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Exposure to nanoscale diesel exhaust particles: Oxidative stress, neuroinflammation, anxiety and depression on adult male mice

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ABSTRACT

Exposure to nanoscale diesel engines exhausted particles (DEPs) is a well-recognized risk factor for respiratory and cardiovascular diseases. Rodents as commonly used models for urban air pollution in health effect studies demonstrate constant stimulation of inflammatory responses in the main areas of the brain. Nevertheless, the primary effect of diesel exhaust particulate matter on some of the brain regions and relation by behavioral alterations still remains untouched. We evaluated the brain regional inflammatory responses to a nanosized subfraction of diesel engines exhaust particulate matter (DEPs < 200 nm) in an adult male mice brain. Adult male mice were exposed to DEPs for 3, 6, and 8 h per day, 12 weeks and five days per week. Degree of anxiety and the depression by elevated plus maze and Forced Swimming Test respectively (FST) did measurement. After behavior tests, the plasma and some of the brain regions such as olfactory bulb (OB) and hippocampus (HI) were analyzed for oxidative stress and inflammatory responses. The inflammation and oxidative stress changes in OB and HI, markedly coincides with the results of behavioral alterations. These responses corresponded with rapid induction of MDA and nitrite oxide (NO) in brain regions and neuronal nitric oxide synthase (nNOS) mRNA followed by IL6, IL1α, and TNFα in OB and HI. The different times of DEPs exposure, leads to oxidative stress and inflammatory in plasma and brain regions. That this cumulative transport of inhaled nanoscale DEPs into the brain and creating to inflammation responses of brain regions may cause problems of brain function and anxiety and depression.

1. Introduction

Air pollution is an intricate combination of metals, particulate matter, organic compounds, and gases. Pollution caused by particulate matter (PM) is a major modifiable global public health threat. Fine particles are smaller than 2.5 μm in diameter ($PM_{2.5}$) and are released by industrial sources and ignition engine exhaust. Diesel combustion can produce nanoscale PM. It has been estimated that up to 85% of PM in cities is related to traffic. Diesel exhaust (DE) is a compound fusion of gaseous-phase combinations and diesel exhaust particles (DEPs). DEPs are a main component of ambient PM and the majority of particles that are directly released by diesel exhaust have the diameter of below 1 μm (Jonidi Jafari and Arfaeinia, 2016; Kittelson, 2007; Kittelson et al., 2004; Wichmann, 2007). DEPs contain several compounds with lethal impacts on the immune system, pulmonary system, reproductive system (Fujimaki et al., 2006; Li et al., 2009; Takano et al., 2002), and brain development (Suzuki et al., 2010). In DE, PMs' soluble organic fraction has above 1000 mixtures plus different heavy metals and polycyclic aromatic hydrocarbons. In addition, great quantities of toxic substances (e.g., metals and hydrocarbons) can be carried on the surface of nanoscale PM, suggesting that nanoscale PM may cause direct neurotoxic effects (Hesterberg et al., 2010; Wichmann, 2007).

With regard to the abundant documents on elevated risk of lung cancer by DE exposure, DE was introduced as carcinogenic to humans (Group1) by a section of the World Health Organization recognized as the International Agency for Research on Cancer. Developmental

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toxicity following DE exposure has been also reported in this regard (Auten et al., 2012; Claxton, 2015). Now, all over the world, we see the exposure to particulate air pollution in all stages of life of individuals. Therefore, the real health problem caused by exposure to PM might be mostly unrecognized. Impaired cognitive function occurs due to PM exposure in the atmosphere. Moreover, there is an increase in amyloid-β deposition in brain tissue, blood-brain barrier (BBB) damage, and oxidative stress response, proposing a causal association between increased pace of formation of neurodegenerative diseases (e.g., Alzheimer's disease) and exposure to PM. It has been reported that BBB can be passed through by these particles, specifically nanoscale PMs (< 100 nm in aerodynamic diameter), which leads to their penetration into the cerebral tissue (Block and Calderón-Garcidueñas, 2009; Calderón-Garcidueñas et al., 2008).

Air pollution generally targets the brain. Studies based on population show the elevation in cognitive disorders proportionally to the levels of PM2.5 and ozone, which amounts to accelerated cognitive loss up to three-five years (Ailshire and Crimmins, 2014; Chen and Schwartz, 2009). Consistently, in a Magnetic Resonance Imaging (MRI) of old female individuals in the Women's Health Initiative Memory Study cohort, there was an increase in the white matter loss (1% per 3 μg/m3 $PM_{2.5}$). In a Mexican city with a high pollution rate, inflammation and changes in the volume of cortical white matter were observed in a small number of postmortem neonates (Calderón-Garcidueñas et al., 2011; Cheng et al., 2016). Rodent models provide valuable data on midbrain, cortex, and OB's inflammatory responses to fairly temporary exposure to air particulate matter produced by automotive. Notably, neurite outgrowth impairment might be caused by TNFα induced by nanoscale particulate matter (nPM) (Block et al., 2012; Morgan et al., 2011). Extensive research has been conducted on the long- and short-term exposure and the impact of PM2.5 on the peripheral systems and showed major adverse cardiovascular events (Brook et al., 2010; Mills et al., 2009). Nevertheless, little research has been conducted on the impact of PM exposure for a long time on the central nervous system (CNS). Stimulations of reactive oxygen species and proinflammatory pathways by $PM_{2.5}$ are thought to initiate dysfunctional responses, which might have adverse impacts on the function of organs in return. Lack of immunity of the CNS to the effects of $PM_{2.5}$ is probable (Campbell et al., 2005; Win-Shwe et al., 2008), suggesting the need for investigating the impact of $PM_{2.5}$ on the brain. Given the presence of several pathways that can be passed by inflammatory signals from the periphery to the brain, neuroinflammation might be caused by peripheral immune activation in individuals who were exposed to $PM_{2.5}$. Chemokines, oxidative stress, and cytokines facilitate inflammatory reactions in the brain, which leads to neurodegeneration, neurotrophin signaling, aberrant protein aggregation, compromised neurotransmitter and neuronal remodeling (Brook et al., 2010; Teeling and Perry, 2009).

Excessively susceptible to inflammation and injury, the brain area of the hippocampus (HI) has numerous receptors for pro-inflammatory cytokines (e.g., Interleukin [IL] 1α, IL6, and tumor necrosis factor α [TNFα]). Therefore, it might be targeted by exposure to $PM_{2.5}$ for a prolonged period (Maier and Watkins, 1998). The pre-existing neurodegenerative disorders (e.g., Alzheimer's disease) can be intensified by the restorative upregulation of inflammatory markers caused by PM exposure for a long time (Cunningham et al., 2009; Godbout et al., 2005). Moreover, data developed in this area confirm the inflammation's involvement in the CNS in the pathogenesis of affective disorders and impaired cognition (Clark et al., 2010; Raison et al., 2006). Reported in inhalation studies, axons of olfactory sensory neurons in the olfactory epithelium (OE) can change the physical location of nanoscale particles to the brain and OB, which project directly to synapses in the OB glomerulus. Nasal instillation evaluation of ultrafine particles that demonstrate the change of location to the OB and TNFα and MIP1α induction in OB confirmed these results (Elder et al., 2006; Yamamoto et al., 2006). In vivo and in vitro, nPM induces IL-1α, IL-6, and TNFα responses. Correspondingly, diesel exhaust at nanoscale prompts TNFα, IL-6, and MIP1α in the OB and post-olfactory brain regions. Thus, the OE may be an important gateway for the impact on CNS by the ultrafine PM (Cheng et al., 2016; Levesque et al., 2011a).

In this work, it was assumed that PM exposure for a long time stimulates oxidative stress, brain inflammation, and depression. There is increasing evidence that systemic inflammation may contribute to neurodegenerative diseases (Perry et al., 2010). However, there are not sufficient studies exploring the effect of long-term exposure to particulate matter in the context of the CNS. The CNS is likely not immune to the effects of $PM_{2.5}$, suggesting an investigation of the impact of $PM_{2.5}$ on the brain is warranted (Campbell et al., 2005; Win-Shwe et al., 2008).

We hypothesized that sub-chronic exposure to DEPs would induce brain inflammation and behavior alteration; we used for long-term of DEPs exposure to evaluate how extended DEPs exposure may impact individuals activates immune measures and induced brain inflammation. 48 sevem-week-old male NMRI mice were ordered from Kashan University of Medical Sciences Laboratories (Kaums) and were exposed to either DEPs for 3, 6 and 8 h per day, 5 days per week for 12 weeks in a locked exposure system. Following 12-weeks of DEPs exposure, mice underwent a battery of behavioral tests assessing physical abilities, anxiety and depression. After the conclusion of behavioral testing, blood for biochemistry biomarkers, and brains were collected processed or flash frozen for PCR analyses. Thus, this study was conducted to assess biological probability of the association between anxiety, depression and neurological effects and exposure to inhaled Nanoscale DEPs.

2. Materials and methods

2.1. Animals and ethics considerations

Animal studies were conducted on seven-week-old adult NMRI male mice (body weight average: 23 g; n = 12/treatment/time, 48 total) purchased from Kashan University of Medical Sciences (Kaums) Laboratories of animal facilities (Kashan, Iran). During the tests, the mice had access to food and water at all times and were maintained in a room with the temperature of 23 °C, air of humidity 35–40% and a 12:12 h light–dark cycle (light on at 6:00 a.m.). The researcher dedicated extensive efforts to minimizing the suffering of a limited number of mice. In addition, pain and distress of the animals were reduced by taking suitable measures. It is notable that the ethics committee of Kashan University of Medical Sciences approved the research stages, performed while adhering to the directive 2010/63/EU on animals' protection applied for scientific objective.

2.2. DEPs collection and extraction

The DEPs used in this study were collected via the following method. The engine used for the preparation of DEPs was a pickup (Iran Khodro Diesel Co., Tehran, Iran), light-duty (2776 -cc), 4-cylinder, diesel engine. At the speed of 1500 rpm and under 10 load torque (kg/m), a standard diesel fuel was exploited to connect the engine to the dynamometer and operate it. The exhaust was introduced into a stainless steel dilution tunnel (300 × 5800 mm) in a constant-volume sampler system installed at the dilution tunnel's end. The sampling point had a temperature of below 50 °C. DEPs were gathered on samples of filter to be subsequently resuspended for exposure to animals. In addition, subjecting of the DEPs to dynamic light scattering (DLS) measurements was carried out by a Zetasizer Nano-ZS system (Malvern Instruments Ltd., UK) so that the level of distribution of DEPs in the suspension could be determined (Fig. 1) (Organization and UNAIDS, 2006; Yoshizaki et al., 2010).

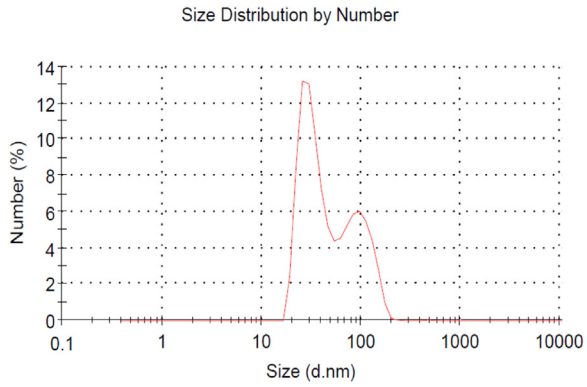


Fig. 1. Size distribution of diesel exhaust particles (DEPs) in suspension as determined by dynamic light scattering.

2.3. Animals and exposure conditions

In this step, 48 NMRI seven-week-old male mice were randomly assigned to groups (n = 12 in $^{\text{each group) and}}$ used in the experiments. Under controlled conditions (humidity, 50 ± 5%; temperature, 22 ± 0.5 °C), the mice were maintained in chambers that expose the whole body (1 m^3) in separate wire mesh cages (n = 12). Being administration, suspension of diesel exhaust particle in saline was carried out for 30 min, followed by 5 min of vortexing and 30 min sonication. The mice were sub-chronically exposed to DEPs inhalation to 350–400 μg DEPs/m3 for 3, 6, and 8 h (7:30 a.m. to 3:30 p.m.)/day, 5d/week for 12 weeks in a locked system compartment fastened to an ultrasonic nebulizer (NE-UO7; Omron Corporation, Tokyo, Japan) with an output of 1 ml/min. The control mice were administered and exposed to saline solution alone. Killing of the mice was carried out by intraperitoneal administration of 65 mg/kg body weight of pentobarbital sodium, which was an overdose (Morgan et al., 2011; Wang et al., 2013).

2.4. Behavioral studies

2.4.1. Forced swimming test

Forced swimming test in mice, established previously (Porsolt et al., 1977), is a behavioral despair test. The mice were separately positioned in glass cylinders (10 cm diameter and 20 cm height), which encompassed 10 cm depth of 25 °C water. Removing, drying, and returning the mice back to their cages was performed five minutes later. After 24 h, the animals were replaced in the cylinder to measure the immobility duration for five minutes after the primary minute of the duration of adjustment. Mice were considered to be immobile when they were floating motionless. The duration of swimming was recorded for 5 min from side-view using small fire-wire cameras and the ANY-maze software (Stoelting Co., Wood Dale, IL).

2.4.2. Elevated plus maze

In mice, spatial stress was assessed using the elevated plus maze. (Carobrez and Bertoglio, 2005; Haller and Alicki, 2012). In short, the apparatus, which had four 50 × 10 cm arms and a 10 × 10 cm center platform, was raised up to 60 cm above the ground. Two arms were remained open, whereas two opposite arms were circled by 40-cm walls. In addition, moderate brightening of the test room was carried out.

Facing an open arm, each animal was situated in the platform at the center. The mice were allowed to walk in the maze for five minutes, during which we observed the animals' behavior. It was agreed to define an entry as having all four paws in the arm. The time spent in open arms (OAT) and the numbers of entries into open arms (OAE) were the measured factors for the plus maze navigation.

We calculated the OAE and OAT percentages, as presented below:

$$\text{OAE\%} = \text{number of entries into the open arms/total number of entries} \times 100$$

$$\text{OAT\%} = \text{durationspentinopenarms(s)/300(s)} \times 100$$

2.5. Collection of blood samples

Immediate centrifuge of the blood samples was carried out at 4 °C and 1500 RCF for 10 min. In this process, we separated the plasma and kept it at −70 °C in Eppendorf tubes for further applications. Moreover, samples of plasma were applied to measure the levels of NO, MDA and TAC.

2.6. Measurement of Nitric oxide (NO)

After the nitrates were converted to nitrites using vanadium chloride, the modified Griess reaction was applied to measure the NO in the plasma as nitrites, followed by the preparation of standard curves for sodium nitrite. In addition, standard calibration plots of $NaNO_3$ and $NaNO_2$ were applied to estimate the values (Miranda et al., 2001).

2.7. Measurement of malondialdehyde (MDA) in the plasma

As designed by Kurtel et al., measurement of the development of the thiobarbituric acid reactive compounds was carried out to quantify the MDA. Moreover, the centrifuge of the aliquots (0.5 ml) was performed, followed by adding the supernatants to the solution (1 ml), which had 0.375% (wt/vol) thiobarbituric acid, 0.25 N HCl, and 15% (wt/vol) tricarboxylic acid. More lipid peroxidation during the following stages was prevented by removing the protein precipitate through centrifugation and transferring the supernatants to glass test tubes encompassing 0.02% (wt/vol) butylated hydroxytoluene. In the next stage, a boiling -water bath (96 °C) It was used to heat the samples for 15 min, temperature of which was lowered after that to be centrifuged to eliminate the precipitant. Each sample's absorbance was estimated at 540 nm.

2.8. Measurement of malondialdehyde (MDA) in the OB and HI

The description by Draper et al., was applied to determine the MDA content, followed by the brief homogenization of the tissue in SBB. Following that, 0.100 ml of 500 ppm butylated hydroxytoluene (BHT) in methanol and 0.550 ml of 5% trichloroacetic acid (TCA) was added to 0.350 ml of the tissue homogenate. In the next stage, boiling water was used to heat the samples for 30 min, which were then cooled on ice and centrifuged. A ratio of 1:1 mixture of saturated thiobarbituric acid (TBA) and supernatant fractions was prepared, followed by reheating the samples for 30 min in a bath filled with boiling water. Extraction of 0.50 ml of each sample after being cooled on ice was carried out using one ml of *n*-butanol. After that, the centrifugation of the samples was performed so that the separation stages could be facilitated. MDA levels were measured using ELISA DuoSets (R&D System). We set the emission and excitation wavelengths at 640 nm and 540 nm, respectively. Furthermore, we determined the TBA-MDA complex's concentrations in the combination applying the calibration curve, which was attained from a standard solution of malondialdehyde bis(dimethyl acetal).

2.9. Measurement of total antioxidant capacity (TAC)

Total antioxidant capacity of plasma or brain tissue homogenate was measured according to the method of Benzie and Strain (1996). Briefly, a working solution of FRAP (ferric reducing antioxidant power) was provided by mixing 10 volumes of buffer acetate (300 μmol/L, pH = 3.6) with 1 vol TPTZ solution in HCl (40 mM/L). After that, 1 ml

solution of $FeCl_3$ (20 mM/L) was added and mixed. For measurement, 1.5 Ml of FRAP working solution was put in the cuvette and incubated for 10 min at room temperature, then the optical density of the blank was measured spectrophotometrically at 532 nm. For test samples, 50 μL of plasma or brain tissue homogenate replaced by the working solution, and change in absorbance was measured.

2.10. Quantitative real-time RT-PCR

TThe mice received pentobarbital anesthesia 24 hours after completing the social behavioral tests, followed by collecting OB and HI to analyze mRNA. Immediately after that, liquid nitrogen was used to freeze the OB and HI samples, then were maintained at the temperature of –80 °C until total RNA extraction. Applying the RNX- Plus kits (SinaClon), the RNA was totally extracted from the HI and OB samples. Afterwards, the quantity and purity of the total RNA were calculated and assessed, respectively applying the NanoDrop RNA Assay protocol (Thermo scientific NanoDrop One C, USA). Following that, first-strand cDNA synthesis was carried out for total RNA (one μg). Applying the Takara Kit reverse transcriptase (Takara Bio USA, Inc.), a template was exploited per sample for synthesizing cDNA based on the company directions of the mentioned kit. After that, the mRNA expressions of nNOS, IL6, IL1α, and TNFα in the OB and HI were assessed. One research tool was quantitative real-time RT-PCR with SYBR Green Real-Time PCR Master Mix (Toyobo Co., Ltd., Osaka Japan) in a BioRad Real-Time. Amplification of the target gene was monitored by measuring each sample's fluorescence intensity during each cycle. After normalization against the housekeeping gene, Hypoxanthine phosphoribosyl transferase (HPRT), comparative levels of expression of target genes were estimated for each sample. According to the results, no significant difference was observed between the groups in terms of HPRT expression (unavailable data). It is notable that the target primers were custom-made. The obtained sequences are shown in Table 1.

2.11. Western blots

Supplemented with 1 mM Na_3VO_2, phosphatase inhibitor cocktails (Sigma), 1 mM PMSF, 10 mM NaF, and Roche Complete Mini EDTA-free Protease Inhibitor Cocktail Tablet (Roche), a Millipore buffer (1x RIPA) was occupied by a motor-driven pestle on ice, which homogenized the OB and HI brain tissues. This process was followed by the centrifugation of the samples at the temperature of 4 °C and at 10,000 × *g* for 10 min. After that, we transferred the supernatant to a tube that was filled by one-third size of the combination of 500 mM dithiothreitol, 0.1% bromophenol blue, 200 mM Tris, 40% glycerol, and 5% sodium dodecyl sulfate. The product was destroyed by five minutes of boiling and then kept at the temperature of − 20 °C until further applications. Protein separation was performed by loading 45 μg of the protein sample onto each gel lane and subsequent electrophoresis at 200 V for 90 min. After electroblotting the proteins onto the nitrocellulose membranes, they were washed with phosphate-buffered saline with 0.05% Tween-20 (PBST). Afterwards, they were blocked with 5% milk solution in 0.05% Tween for one night 20 in 10 mM Tris and 150 mM NaCl. Finally, they were incubated with 1 μg/ml anti-nNOS (neuronal) antibody (ab1376) directed to the carboxy terminus (ab) for 1 h followed by a secondary antibody (mouse anti-goat secondary antibody, coupled to horseradish peroxidase from Santa Cruz). It should be noted that the Tween/Tris /NaCl solution was applied to wash the immunoblots between each step. Chemiluminescent detection reagents for western blotting (NEN, Dupont) were applied to visualize the immune complexes, which were then placed in front of X-ray film. The density of bands was assessed with imageJ. Through background subtraction, the concentrations of 161 kDa immunoreactive protein bands, which showed correspondence to nNOS, were measured.

2.12. Data analysis

Data were reported in the form of mean ± standard deviation. Data analysis was carried out via Prism ver.7.3 software using One-way analysis of variance (ANOVA) followed by Turkey test. One-way ANOVA with depression and the assessment of stress among the variables of the subjects and DEPs exposure as a variable in individual subjects was performed on the behavioral processes. Moreover, ANOVA with oxidative stress markers and level of inflammatory cytokine mRNA expression as variable between the subjects and DEPs exposure as a variable in the subjects was carried out on the inflammation and oxidative stress level of plasma and brain tissue. The basis of statistical decision was a significance Alpha level of $P < 0.05$.

3. Results

Body weights ($P = 0.078$), body length ($P = 0.093$), eye appearance, vibrissae, gross olfactory abilities, and sensorimotor responses did not differ between DEPs exposure and Control mice ($p < 0.05$).

3.1. Behavioral experiments

3.1.1. The effect of DEPs exposure on depression

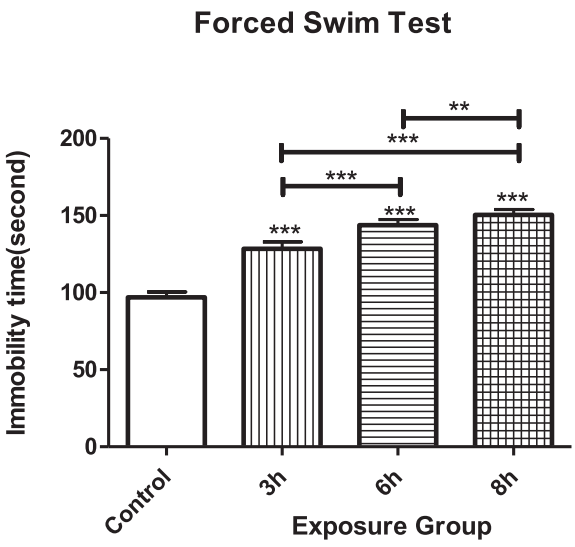
Since our sub-chronic DEPs exposure did sufficiently depressive-like responses induce immobility in the forced swim test. That evaluated using a modified version of the Porsolt FST (Porsolt et al., 1977) revealing increased behavioral despair among DEPs exposure mice. DEPs exposure mice elevated depressive-like behaviors with elevated floating frequency and duration in the forced swim test. We studied the effect on immobility duration in forced to swim models. Our observations indicate that chronic DEPs exposure for 3, 6, and 8 h/day caused significant enhancement of immobility duration in the forced swim test (Fig. 2A).

3.1.2. The effect of DEPs exposure on anxiety

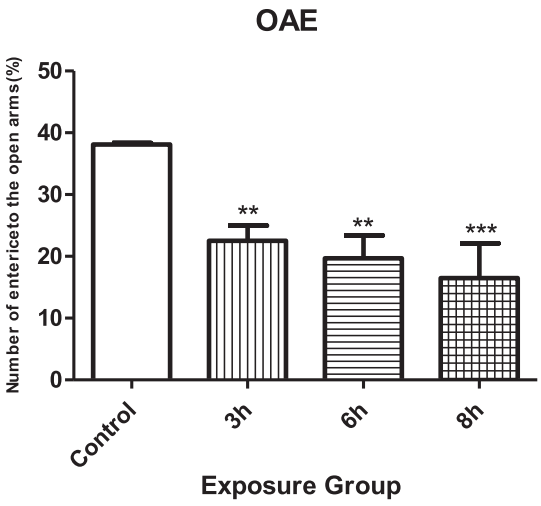
The plus maze test was presented to assess the stress level of the mice exposed to DEPs. In general, the stress level was calculated based

Table 1
Primer design for quantitative RT-PCR.

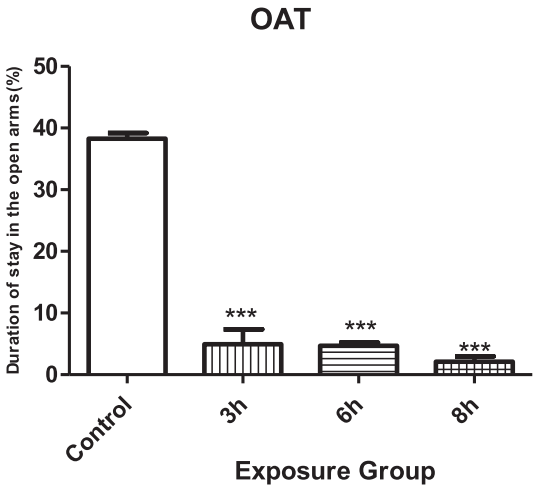
Gene		Sequence	T_m
HPRT	Hypoxanthine phospho ribosyl transferase	*Forward:*5′- *GCT GGT GAA AAG GAC CTC T-3′* *Reverse:*5′- *CAC AGG ACT AGA ACA CCT GC-3′*	60
nNOS	Neuronal Nitric oxide synthase	*Forward:* 5′- *CTTGGCTTGGAGGTCTTCTG-3′* *Reverse:* 5′- *AACTTCCAGAGCGCTGTCAT-3′*	57
TNF-α	Tumor necrosis factor alpha	*Forward:* 5′- *GGCCTTCCTACCTTCAGACC-3′* *Reverse:* 5′- *AGCAAAAGAGGAGGCAACAA-3′*	58
IL-6	Interleukin 6	*Forward:* 5′- *TTGCCTTCTTGGGACTGATG-3′* *Reverse:* 5′- *AGGTCTGTTGGGAGTGGTAT-3′*	60
IL-1 α	Interleukin 1α	*Forward:* 5′- *GCAACGGGAAGATTCTGAAG -3′* *Reverse:* 5′- *TGACAAACTTCTGCCTGACG -3′*	60



A



B



C

Fig. 2. Effects of sub-chronic DEPs exposure on enhanced immobility in the (A) forced swim task. Bar diagram representing the day-dependent study on the effects of sub-chronic DEPs exposure, increased duration of time spent floating and immobility (in secs) of mice in forced swim test which is consistent with a depressive-like state. The elevated plus maze navigation by the different groups of animals.(B) Percentage of the entered open arms by the different groups of mice during the plus maze searching. While the DEPs exposure led a marked decrease in entering the open arms in all exposure groups. (C) Percentage of the duration of stay in the entered open arms during the plus maze navigation. The all exposure animals displayed a considerable decrease in the time of the open arm steering. Results are represented as mean ± SEM with n = 12 in each group. *P < 0.05, **P < 0.01, ***P < 0.001 when compared with control group.

on two factors of entering the open arms and time spent in the arms. According to results of ANOVA, there was a difference between the research groups in terms of performance. The mice exposed to 3, 6 and 8 h DEPs displayed a significant decrease in the ability to enter the open arms, compared to the samples in the control group (Fig. 2B). Furthermore, the mice in the 3, 6 and 8 h groups passed a shorter time in the open arms (Fig. 2C) ($p < 0.05$).

3.2. The effect of DEPs exposure on plasma biochemistry biomarkers

To investigate the plasma responses to DEPs, adult male mice were exposed to the nebulizer and inhaled DEPs. As it can be seen in Fig. 4a and b, after 3, 6, and 8 h exposure in 5 days a week for 12 weeks, the plasma showed rapid increases in the MDA, NO (Fig. 3A, B) and decreased TAC were significantly at all exposure groups (Fig. 4A) ($p < 0.05$).

3.3. Extended induction of biochemistry biomarkers in OB and HI caused by exposure to DEPs in vivo

To investigate the earliest OB and HI responses to DEPs exposure, the MDA levels were significantly increased in all groups (Figs. 3C and 3D) and TAC levels were significantly decreased in all exposure groups (Fig. 4B, C) ($p < 0.05$).

3.4. Extended induction of expression of pro-inflammatory cytokine in OB and HI caused by exposure to DEPs in vivo

To discern whether DEPs caused neuroinflammation at all, we measured proinflammatory mRNA expression in the OB in mice exposed for 12 weeks by inhalation. DEPs caused a significant increase in IL6, IL1α, and TNFα in all exposure groups in OB (Figs. 5A, 5B, and 5C) and were significantly increased after 6 and 8 h/day exposure in HI (Figs. 5D, 5E, and 5F). OB and HI demonstrated equally moderate oxidative stress and inflammatory responses to exposure to DEPs in an extended manner.

TNFα responded more slowly than IL6 and IL1α mRNA fluctuated with a possible transient increase at 3 h/day. During DEPs exposure, nitrite in the plasma increased progressively over 3 h/day (Fig. 3 A), suggesting that nNOS significantly contributes to the induction of nitrite. Moreover, nNOS mRNA showed a slower induction, significantly increasing in IL6, IL1α, and TNFα after 6 and 8 h/day (Fig. 4 A). TNFα mRNA in HI showed an increase at 6 and 8 h/day of exposure, while nNOS mRNA in HI was slowly increased at 6 and 8 h/day exposures. We compared the HI with the OB with over 180 h total exposure in 12 weeks and noticed that TNFα mRNA increased in HI and in OB at 6 and 8 h/day (Fig. 5 A, 5Bb, and 5 D). OB IL6 and IL1α mRNA was increased at 6 h and 8 h/day, but HI responded slowly (Fig. 5 A and 5 B). Data show that DEPs exposure increased expression of nNOS protein in HI as measured by Western blot in parallel to mRNA at all (Fig. 6 A and 6 B). These data suggest that DEPs exposure caused generalized oxidative stress and neuroinflammation that extended throughout the entire

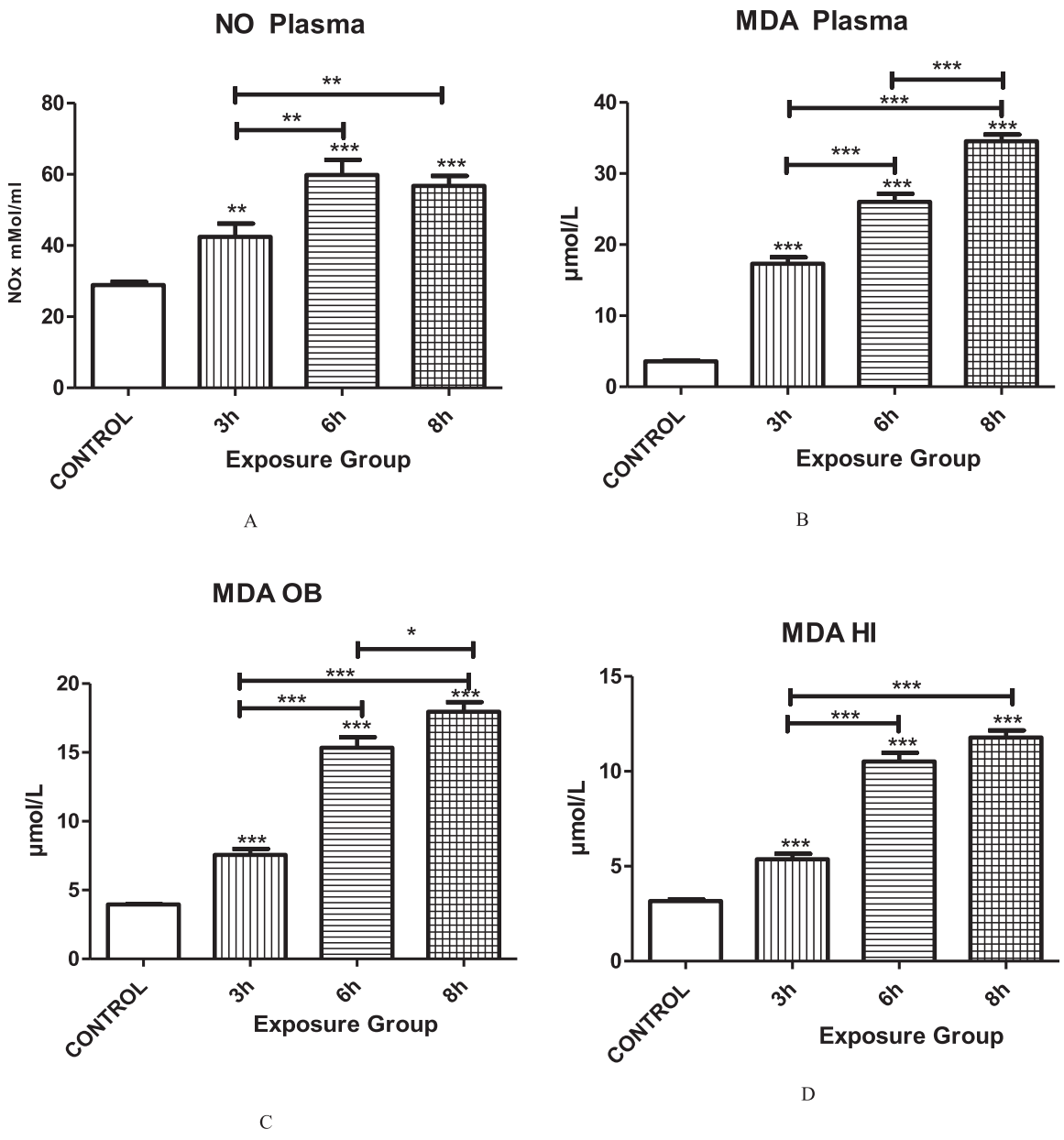


Fig. 3. DEPs in vivo exposure induced MDA and NO in the plasma, OB and HI. NO and MDA were significantly increased at all exposure groups (A,B), and MDA were significantly increased in OB and HI at all exposure groups (C,D). Results are represented as mean ± SEM with n = 5 in each group. *P < 0.05, **P < 0.01, ***P < 0.001 when compared with control group.

brain.

4. Discussion

Copious reports have indicated an altered behavioral performance in sub-chronic to DEPs inhalation exposures. The cognitive changes are proved to be associated with oxidative stress and neuroinflammation changed in some brain regions. The present research assessed the impact of DEPs exposure in adult male mice on anxiety, depression, oxidative stress and neuroinflammation. We show that DEPs caused effectively weaken the behavioral performances on adult male mice After 12 weeks of inhalation exposure. The negative effect of the DEPs exposure was pronounced in the groups. The animals in the 3, 6 and 8 h/day groups had lower performance efficiency, compared to the control group, whether in plus maze test and forced swimming test. Fewer abilities to enter the open arms in the plus maze test and staying in the arms for a shorter duration were interpreted as stress in mice. In addition, it was concluded that the anxious mice did sufficiently induce immobility duration in forced to swim test (Fig. 2 A). The major finding of this study is that sub-chronic exposure to DEPs in male NMARY mice leads to DEPs inhalation and its response transmission from blood and OB to HI and various regions of the brain. These experiments exposed rodent brain tissues to DEPs, a nano-sized subfraction of $PM_{2.5}$, which is enriched in saline. A quick elevation in the biochemistry biomarkers was indicated by the plasma models in vivo by 3, 6, and 8 h/day of DEPs exposure (Fig. 3 A and 3 B). The biological probability of the association between depression, being exposed to DEP inhalation, and neurological impacts was evaluated in the current article. We anticipated that the OB would also show increased tissue levels of IL6, IL1β, and TNFα because nanoscale gold PM was axonally transmitted by olfactory neurons of the olfactory epithelium (OE) into the OB and due to the quick accumulation of inhaled PM (nano size) of intranasal Mn and of ^{14}C-graphite in the OB (De Lorenzo, 2008; Elder et al., 2006). The HI had a smaller changes, level and slower response to biochemistry

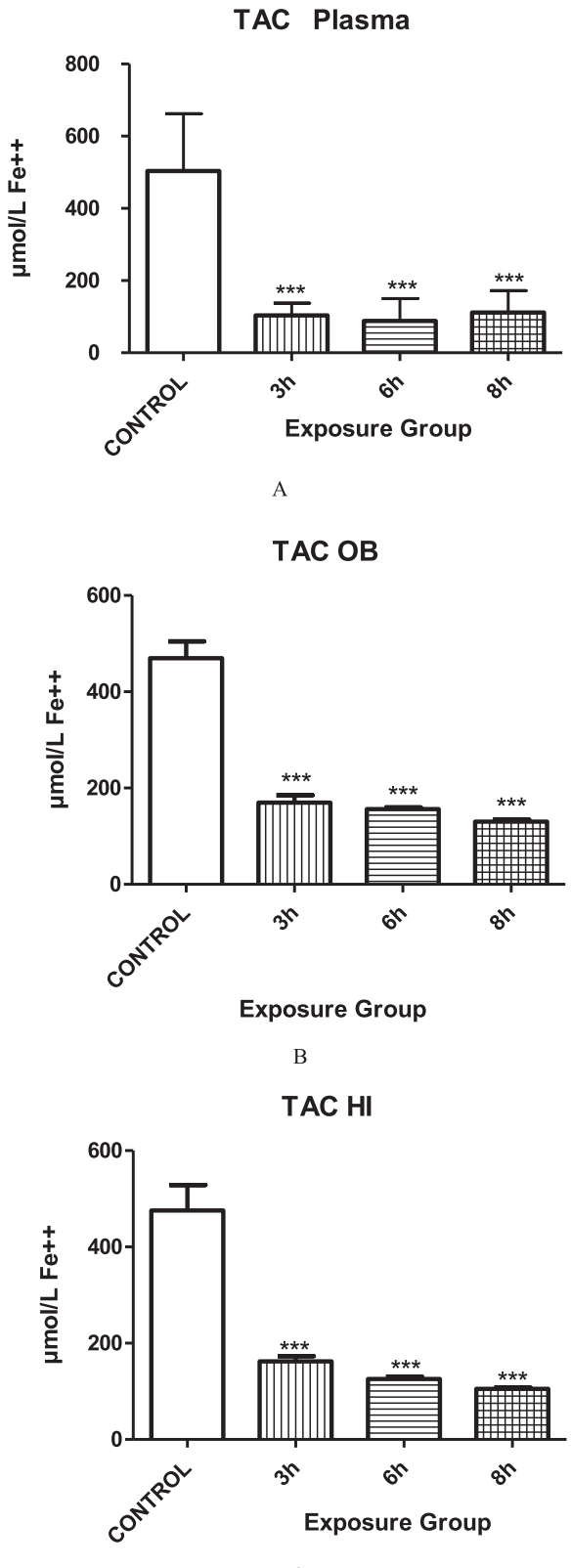


Fig. 4. DEPs in vivo exposure induced total antioxidant capacity of plasma (TAC, as µmol per ml) in the plasma (A), OB (B) and HI (C). TAC were significantly decreased at all exposure groups. Results are represented as mean ± SEM with n = 5 in each group *P < 0.05, **P < 0.01, ***P < 0.001 when compared with control group.

biomarkers. However, the OB show changes in biochemistry biomarkers and response to IL6, IL1α, and TNFα mRNA (Fig. 3 and Fig. 4). The TNFα mRNA in the cerebral cortex reached the levels from 150 h of exposure to nPM during 10 weeks just at 45 h of total nPM exposure within the interval of three weeks (Morgan et al., 2011). The direct olfactory nerve pathway from nose to brain might be interacted by systematic responses' contributions, as suggested by the smaller reactions. In addition, a neuronal degenerative OE response, which was different from the downstream areas of the brain, was regarded in the research. The only neurons, which have direct exposure to the external environment in the respiratory tract, are the olfactory sensory neurons (OSN), which express the OE. Therefore, they inhale the contaminants in air as the first neuronal responder. Initial elevation in oxidative stress and IL6, IL1α, and TNFα mRNA after exposures were histochemically localized to the OB, concurrent with increased oxidative stress in the plasma and OB. However, TNFα increases were slow in the OB (Fig. 3). OB biochemistry biomarkers responses were more modest and smaller than in the plasma while IL6, IL1α, and TNFα mRNA were increased by exposure (Fig. 5). Furthermore, HI showed smaller increases in IL6, IL1α, and TNFα mRNA at any time (Fig. 5). We anticipate that longer exposure to DEPs would increase inflammation in the brain, as observed for cumulative exposure to diesel exhaust (Levesque et al., 2011b). It is worth mentioning that TNFα induction expressed in the previous study was similar to the elevation of TNFα in the HI and OB in terms of size. The comparatively greater size of OB can be attributed to the systemic mechanisms involved, or it would be expected that the transferred DEPs be weakened to the brain mass from the OB to HI. However, the increase in Oxidative stress ratio and inflammation factors in OB and HI proves the latter. Various studies have confirmed the role of particulate material's systemic import or of pro-inflammatory factors. In the highly respected study of Oberdörster et al. (2004), after five days, ^{4}C brain levels became higher in the cerebellum as in the OB due to being exposed to inhaled nanoscale ^{14}C-graphite. The mentioned researchers assessed the possibility of "displacement in the barrier of blood and brain in specific areas". Presence of a source of blood born continuing ^{14}C boosts in cerebrum and cerebellum would be in line with the huge renal residual ^{14}C pool. Another approval for the axis of lung to brain in pollution of air is related to the responses of the brain to a PM10 air pollutant fraction's intratracheal instillation, which induces the HO-1 gene that is oxidatively sensitive by > 100% in both the lung and brain. In addition, systematic transportation of inflammatory variables and particles is in congruence with the impacts on the intratracheal DEP in the brain of the fetus (Bolton et al., 2014; Farina et al., 2013). Furthermore, vehicular emissions that were inhaled elevated the absorbency of the BBB in the animals, while the exposure of the mice to pollution's serum elevated the BBB absorbency in a model in vitro and altered vasorelaxation with CD36 dependence (Oppenheim et al., 2013; Robertson et al., 2013). Finally, it was pointed out that elevated mixing of cytokines from inflammation of the respiratory tract can pass the BBB and induce neuroinflammatory responses. Therefore, it is suggested that more studies be conducted on the air pollution's systematic impacts together with the recognized direct pathway of the nose to the brain. During exposures for a long time, it is expected to observe compound conversions in specific pathway mechanisms. During exposure in vivo, induced IL6, IL1α, and TNFα showed inflammatory and oxidative mechanisms. The quick elevation in IL6, IL1α, and TNFα mRNA in OB led to nitrosative stress. In addition, incubation of intact OB with DEPs in vivo and condition according to previous research on pieces of the hippocampus was carried out, where there was an elevation in nitric oxide (NO) and S-nitrosylation by nPM (Davis et al., 2013; Erickson et al., 2012). The in vivo HI responded to DEPs with increased nNOS mRNA. The in vivo OB also showed induction of cytokines (IL6, IL1α, and TNFα). These changes parallel are in line with quick inflammatory responses to inhalation of DEPs in vivo, such as increased IL6, IL1α, and TNFα in the HI (Fig. 4). The inflammatory alternations seen in the brain and OB might be proliferated

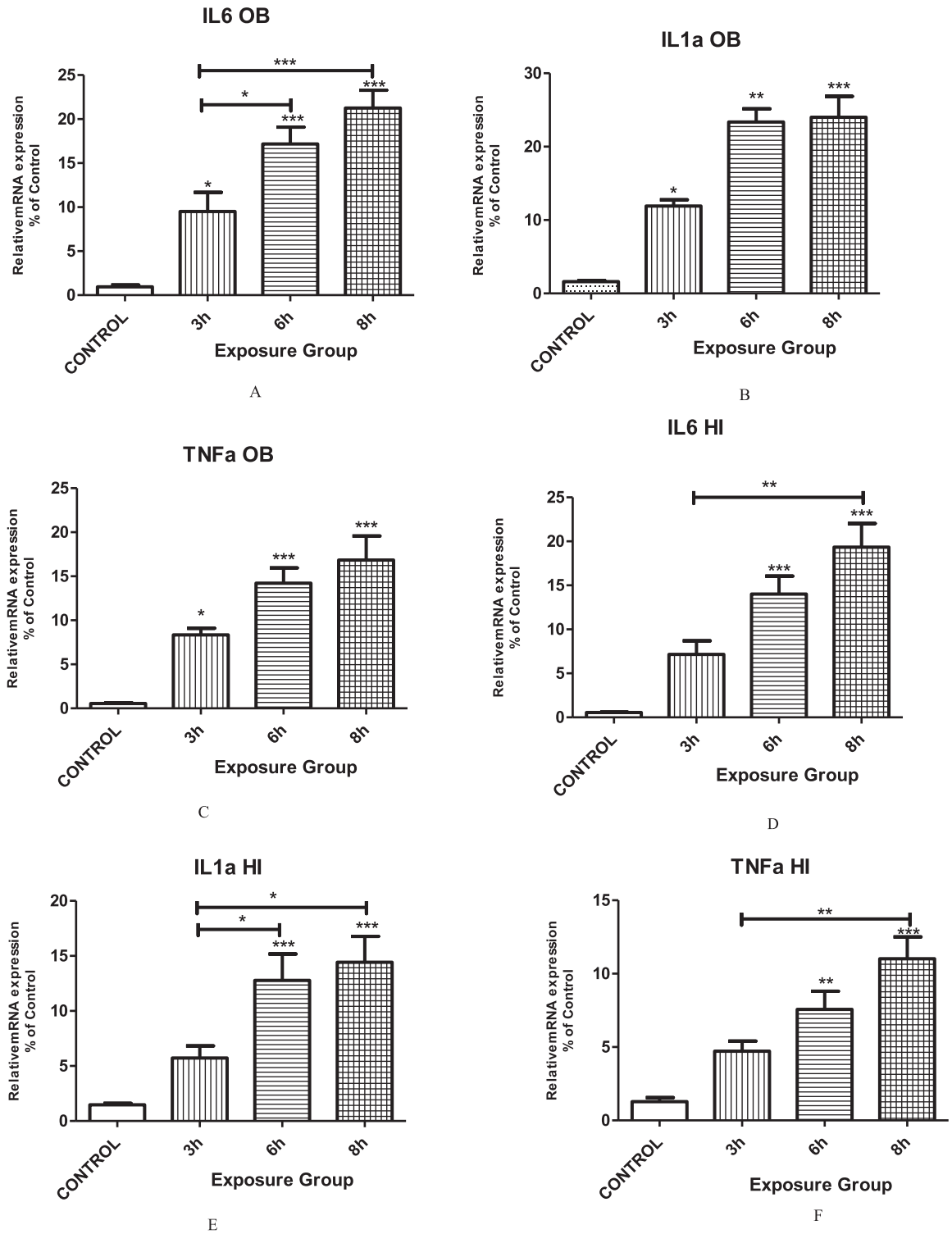


Fig. 5. Extended DEPs in vivo exposure induced IL6, IL1α and TNFα in the OB (A, B, C) and HI (D, E, F,). IL6, IL1α and TNFα mRNA level were significantly increased at all groups in OB and were significantly increased after 6 and 8 h/d exposure in HI. Exposure vs. controls (n = 5 mice/group/time). *P < 0.05, **P < 0.01, ***P < 0.001 when compared with control group.

by responding to the induced oxidative stress by DEPs via macrophage/microglial activation. Oxidative markers are involved in the pathogenesis of neurodegenerative disorders, including the Alzheimer's disease (Butterfield et al., 2007; Dalleau et al., 2013). Changes in mood responses like as Depressive was also evaluated using a modified version of the Porsolt FST (Porsolt et al., 1977), revealing increased behavioral despair among DEPs mice. Depression and Cognitive impairment are often related to changes in the hippocampus in both rodent models and humans (Bremner et al., 2000). Recent findings that demonstrate an interconnected in humans relationship between changes in mood, asthma risk and air pollution exposure. DEPs exposure is implicated in potentiating cardiopulmonary conditions and asthma, and negative mood are associated with asthma and high airway inflammation (de Miguel Díez et al., 2011; Kullowatz et al., 2008). The

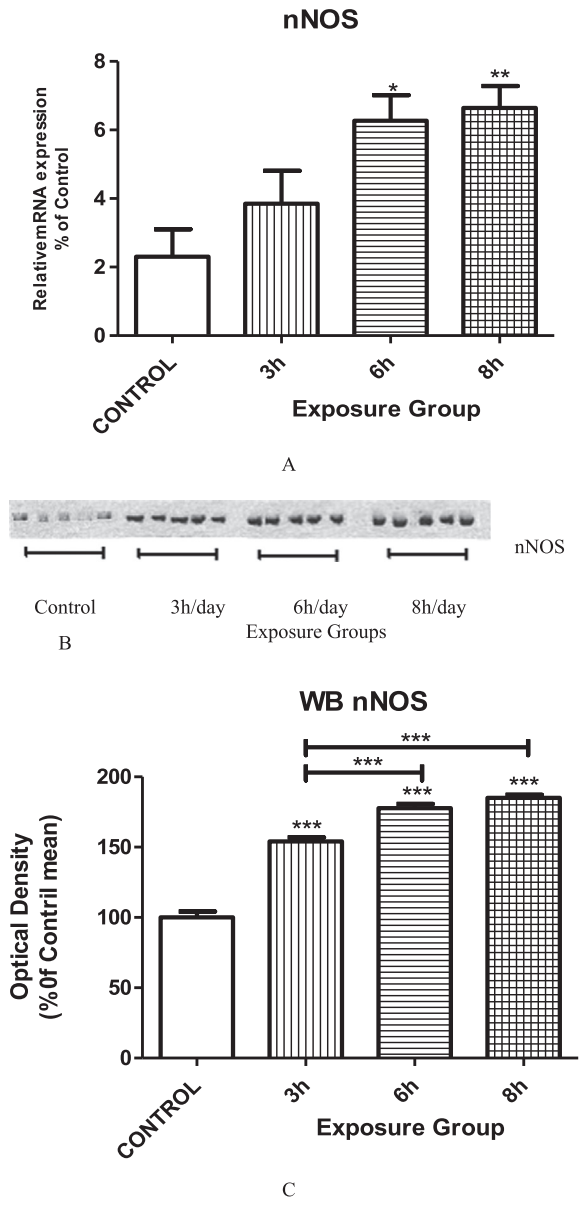


Fig. 6. Extended DEPs in vivo exposure induced nNOS in the HI (A). nNOS mRNA increased in HI after 3, 6 and 8 h/d exposure. nNOS protein increased after 3, 6 and 8 h/d exposure. Quantitative assessment of nNOS levels reflected as optical density, in control and exposure groups(B). Each lane contains 45 μg of protein used for gel electrophoresis and western blotting. Asterisks show values considered to be statistically significant on one-way analysis of variance (ANOVA) when compared to control values(C). Each histogram bar reflects mean + S.E. for brain homogenates taken from HI of each brain (n = 5 for each group). *P < 0.05, **P < 0.01, ***P < 0.001 when compared with control group.

western blotting technique shows that DEPs exposure in the evaluated animals resulted in significant changes in nNOS expression in HI and during this period. The primary increase in the expression of nNOS happens in the OB with decrease in the levels of nNOS in brain residues. Moreover, nNOS levels significantly increase in the middle brain (including HI), by 6 and 8 h/d exposure. Accordingly, the final outcome of DEPs exposure in mice leads to a higher of nNOS protein levels throughout the brain encompassing the whole brain, i.e. OB and HI. Furthermore, increased expression of nNOS was accompanied by destruction of cells and axons, increased s-nitrosylation, glutamate excitotoxicity and release of glutamate (Nicotera et al., 1997). Several studies have indicated the upregulation of nNOS in the pathological processes, including secondary-to-axotomy peripheral nerve regeneration (González-Hernández and Rustioni, 1999); followed by lipopolysaccharide administration, methylmercury exposure and methamphetamine administration (Deng and Cadet, 1999). Alternatively, increased nNOS expression is also known to be co-local in the neurons of the striatum and hippocampus carrying NMDAR1 glutamate receptors (Weiss et al., 1998). Among the exposure model, we detected considerable differences in responses of OB. Weakened or absent responses of OB in exposures over a longer period with baseline return of TNFα mRNA by 6 and 8 h/day exposure in our data suggests compensatory OB mechanisms. In this regard, Ong et al. (2016) showed that exposing the mice for 4 h for more than a day to ozone of 0.5 ppm (a gasiform pollutant without nPM) induced temporarily the TNFα mRNA in the nasal mucosa of the mice and returned to controlling levels by four days of exposure to pollution-free air (Guerra et al., 2013; Ong et al., 2016). In our model, the slow responses in TNFα, IL1α, and IL6 mRNA to OB and HI by sub-chronic exposure of DEPs inhalation are described by different mechanisms. DEPs exposure also resulted in increased TNFα, IL1α, and IL6 mRNA expression in OB and HI, further supporting the hypothesis that nasal entry through the olfactory bulb may be necessary for DEPs to cause neuroinflammation.

5. Conclusions

Taken together, exposure to DEPs, endorses oxidative stress and neuroinflammation, and deeply undergoes the behavioral alteration linked Anxiety and depression. Our findings confirmed the hypothesis on quick formation of oxidative stress in the plasma, olfactory bulb (OB), and hippocampus (HI), with inflammatory responses in the brain by DEP inhalation. Despite receiving direct input from olfactory neurons, there was a delay in the oxidative stress and inflammatory responses of OB. The HI also responded to a smaller increase of oxidative stress and showed smaller increases at IL6, IL1α, and TNFα for all time periods. These smaller reactions of the brain demonstrated the contribution of DEP inhalation to neurodegenerative impacts of particulates in the polluted air. In addition, systematic variables have values for more contemplation over the responses of the brain to the polluted air. Using a quantitative western blotting technique, we demonstrated that sub-chronic exposure by diesel exhaust particles in male mice results in significant changes in nNOS expression in HI in the long run. In the present study, it was confirmed the relevance of targeting of the brain by the impacts of inhaled DEPs, proposing prolonged exposure to this universal combination of air pollution may lead to increase of oxidative stress markers, TNFα, and other cytokines and development of behavioral alteration. It is recommended that more research be conducted in this area to clarify the impact of chronic exposure to DEPs on behavioral alteration and brain performance both in female and male mice.

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Competing financial interests

None.

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