



Learning and memory disorders related to hippocampal inflammation following exposure to air pollution

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Received: 18 June 2020 / Accepted: 14 December 2020 / Published online: 22 January 2021
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Abstract

It has been demonstrated that sub-chronic exposure to air pollution containing nanoscale (<100 nm) diesel exhaust particles (DEPs) may lead to excessive oxidative stress and neuro-inflammation in adult male mice. Hereby, we investigated the effects of DEPs on hippocampus-dependent spatial learning and neuro-inflammation and memory-related gene expression in male mice. In this study, we divided 48 adult NMRI male mice into control group VS. three exposure groups. Mice were exposed to 300–350 $\mu\text{g}/\text{m}^3$ DEPs for 2, 5, and 7 h daily for 12 weeks. The Morris Water Maze (MWM) and Elevated Plus Maze device were used to examine anxiety, spatial memory and learning, respectively. The mRNAs expression of pro-inflammatory cytokines, N-methyl-D-aspartate (NMDA) receptor subunits, and glutaminase were studied in hippocampus (HI) by real-time RT-PCR. Besides, malondialdehyde (MDA) tests were used to determine the state of oxidative stress. After 5 and 7 h. of DEPs exposure, mRNA expression of NR2A and NR3B IL1 α , IL1 β , TNF α , NMDA receptor subunits and MDA levels increased significantly ($P < 0.05$). Also, DEPs exposed mice for 2, 5, and 7 h. showed diminished entrance into open arms with short time spent there. Indeed, 5 and 7 h/day exposed mice required a longer time to reach the hidden platform. Sub-chronic exposure to DEPs increased oxidative stress markers, neuroinflammation, anxiety, impaired spatial learning and memory.

Keywords Air pollution · Diesel exhaust particles · Nanotoxicology · Neuroinflammation · Learning and memory

Introduction

Air pollution is a complex combination of solid particles, gases, metals, and organic compounds [1, 2]. Traffic-related air pollution is an important source of environmental pollution. It is estimated that 20 to 70% of environmental pollutions is due to the combustion of traffic which constitute 85% of

particulate matter (PM) in urban air pollution [3]. Global estimates have depicted that daily particulate air pollution is related to an increased risk of various adverse health consequences [4, 5]. Besides, according to information derived from the World Health Organization (WHO), more than 7 million untimely deaths per year are associated with air pollution, making it the greatest environmental risk factor, worldwide. Diesel engine exhaust (DE) is an intricate combination of gases, sulfur, hydrocarbons, particulates, and heavy metals generated from combustion of diesel fuel [6]. DEPs include various combinations with potentially deleterious impacts on immune system and brain growth [7]. In 2013, the International Agency for Research on Cancer (IARC) identified DEPs as a human carcinogenic entity based on evidence originated from exposure to particulate with subsequent development of malignancies of lung and central nervous system (CNS) [8]. The precise composition of DE features depends on the operating conditions, the character of the engine, fuel composition, additives, lubricating oil, and emission control system [9]. Diesel engines are classified typically by their service requirements, and the operating conditions into heavy

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(such as trucks) and light-duty diesel engines (such as automobiles and light trucks) which vary regarding fuel composition, engine emission controls, expected load, and engine speed [10]. Typically, Light-duty vehicles operate at higher speeds than do heavy-duty vehicles. Regarding fuel composition and engine-control technology, light- and heavy-duty diesel engines can respectively emit 50 to 80 and 100 to 200 times as much particulate mass as typical catalyst-equipped gasoline engines [11, 12]. Almost 92% of DEPs diameter are less than 1.0 μm [13]. The elemental and organic carbon account for almost 80% of the total PM mass and the remaining is combined with sulfate (sulfuric acid) [14]. The major inorganic fraction of the DEPs is nuclei carbon particles in addition to some inorganic additives and components of motor oil and fuel [15]. Generally, the organic compounds identified in DE emissions include hydrocarbons and hydrocarbon derivatives, poly-aromatic hydrocarbons (PAHs) and PAH derivatives and PAHs multifunctional derivatives, aldehydes, heterocyclic compounds and heterocyclic derivatives and multifunctional derivatives [6]. The range of organic fraction of DEPs is often 20% to 30%, but it may be as high as 90% based on operating conditions and vehicle type [16]. DEPs could adsorb relatively major amounts of the organic material originated from lubricating oil, unburned fuel, and pyrosynthesis during fuel combustion due to their extensive surface area [17]. A variety of carcinogens and mutagens such as nitro-PAHs, and PAHs are adsorbed by DEPs [18]. Recently, it has been demonstrated that prenatal exposure to DEPs impair neurogenesis in the hippocampus of offspring adult male mice accompanied with learning and memory disorders [19]. Although DEPs have distinct chemical compositions, their sizes are within the nanometer range [20]. Such a small size allows their entry into multiple organs with subsequent development of adverse health effects. Indeed, DEPs can easily translocate into the CNS. Common routes for entrance of nano-sized particles entry into the CNS include their passage across blood-brain barrier (BBB) or retrograde transport along olfactory nerve [21, 22].

The hippocampus is crucial for spatial learning and memory acquisition, and this transmission is mainly mediated through glutamate receptors [23]. It has been reported that hippocampal NMDA-type glutamate receptors are vital for learning and memory and synaptic plasticity [24]. Transgenic studies have shown that missing NR2 subunit in Cornu Ammonis 1 (CA1) of the hippocampal NMDA receptor resulted in disabled performance on the Morris water maze (MWM) test in knockout mice. Therefore, as assessed using the MWM task, it is believed that hippocampal NMDA receptor activity and NMDA receptor-associated synaptic plasticity are critical for learning and memory functions [21].

Glutaminase is an amino-hydrolyzate enzyme which produces glutamate from glutamine [25]. The Glutaminase is a tissue-specific isoenzymes that plays a vital role in glial cells.

One of the most important functions of glutaminase is in the axonal ends of CNS neurons. Glutamate acts as an excitatory neurotransmitter in the CNS. After being released by presynaptic neurons, glutamate is rapidly absorbed by adjacent astrocytes and converted to glutamine. This glutamine is then returned to the presynaptic terminals of neurons, where the glutaminases turn then back to glutamate with re-entrance into synaptic vesicles again [26]. In vitro studies using neuroglial culture also illustrated that DEPs can activate microglia and induce oxidative stress that leads to dopamine neurotoxicity [27]. Many reports indicate that intranasal instillation aerosols of nanoscale carbon black dependence of brain inflammation, and these particles capability to produce reactive oxygen species (ROS) and so have comprehensive toxicity [28].

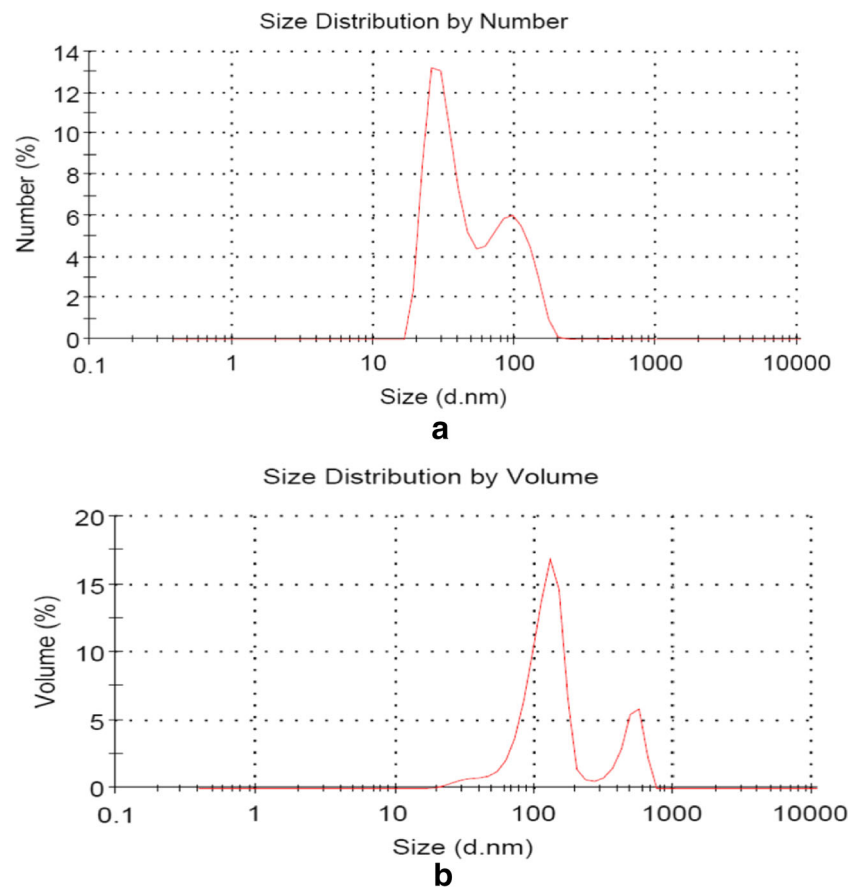
The impacts of DEPs on memory functions and learning has not been throughout explored yet. Formerly, our laboratory has shown that sub-chronic exposure of DEPs affects oxidative stress, neuro-inflammation with subsequent induction of behavioral changes such as depression [21]. This study aimed to evaluate sub-chronic exposure to DEPs. In this work, we assumed that sub-chronic exposure to 300–350 $\mu\text{g}/\text{m}^3$ of nanoscale ($< 100 \text{ nm}$) DEPs would induce neuro-inflammation through the induction of pro-inflammatory cytokines in normal individuals. The neuroimmune system, especially the microglia, may be activated by these inflammatory signals. Activated microglia may release send toxic substances such as ROS, pro-inflammatory cytokines, and excitatory neurotransmitters (e.g., glutamate). Excessive amounts of glutamate may induce abnormal hippocampal NMDA receptor activation; which in turn may lead to impaired spatial memory and learning activity. Thus, in this study, we aimed to evaluate the effect of DEPs exposure for 2 h, 5 h, and 7 h per day during 12 weeks in adult male mice on anxiety and learning and memory function-related hippocampal gene expressions.

Materials and methods

DEPs collection and extraction

The engine used for collection of DEPs was a light-weight pickup truck (4-cylinder diesel engine, 2776 cc) that uses standard diesel fuel with a 10 kg/ m torque at a speed of 1500 rpm. The exhaust gas entered a dilution tunnel made of stainless steel (300 \times 5800 mm). Dynamic light scattering (DLS) measurements to determine the particle size distribution of DEPs in the suspension were performed using a Zetasizer Nano-ZS system (Malvern Instruments Ltd., UK) (Fig. 1a and b). The derived DEPs were suspended at 1 mg/mL in an isotonic sodium chloride solution and then vortexed for 5 min and sonicated for 30 min before application [19, 21].

Fig. 1 Peaks of the size distribution of DEPs were detected by the Zetasizer Nano-ZS system. **a** Size vs. number and **(b)** Size vs. volume



Animal exposure and ethics considerations

48- adult NMRI male mice (between 7 and 8-week-old) were purchased from animal laboratory facilities, Kashan University of medical sciences, Kashan, Iran. Mice were maintained with unlimited access to food and water in a room with 35–40% humidity at 23 °C temperature and a 12 h dark-light cycle (light on at 6:00 a.m.). All animal experiments were performed under the guidance of the National Council for Research and Care of Laboratory Animals, approved by the National Institutes of Health and the Comics Ethics Committee for research purposes. Mice were randomly assigned into case and control groups ($n = 12$ per group in a separate mesh cage). Control groups were just exposed to saline solution. The case mice were maintained in controlled environmental conditions (temperature, 21 ± 0.5 °C; humidity, 55–60%) in a closed systemic room (1 m^3) and exposed to $300\text{--}350 \mu\text{g}/\text{m}^3$ DEPs for 2, 5, and 7 h. (8 AM to 3 PM)/day in a closed system chamber, connected to an ultrasonic nebulizer (NE-UO7; Omron Corporation, Tokyo, Japan) with an output of 1 mL/min.

At the end of the last behavioral tests, mice were sacrificed after induction of sedation using sodium pentobarbital (65 mg/kg body weight, intraperitoneal) and some of their brain regions such HI were dissected immediately, flash-

frozen in liquid nitrogen, and stored at -80 °C for assessment of malondialdehyde, mRNA expression of inflammatory cytokine in HI, and mRNA expression in the HI.

Behavioral experiments

Elevated plus-maze

To investigate anxiety in mice, the elevated plus-maze was used. The elevated plus-maze device contains four $50 \text{ cm} \times 10 \text{ cm}$ arms and a $10 \text{ cm} \times 10 \text{ cm}$ central platform which rises 60 cm from the ground. Two opposing arms are open and the other two arms surrounded by a 40-cm wall. As a rule, an entrance is defined as having four paws on the arm. The tests were conducted in a room with moderate lighting. Each mouse was placed facing the open arm in the central platform and left for 5 min to explore the maze. The measured parameters are the time in open arms (OAT) and the number of entrances into open arms (OAE). The percentages of OAT and OAE are calculated as follow:

$$\text{OAT\%} = \text{duration spent in open arms (s)} / 300 \text{ (s)} \times 100$$

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

Spatial memory and learning performance

Spatial memory and learning were assessed using a MWM setup with a water maze (100 cm) and a platform (10 cm). The platform was placed at a fixed location in the center of one of the four maze quadrants. The starting point was set at the midpoint of the quadrant opposite to the platform quadrant. Milk powder was added to the water to form an opaque solution. The water temperature was set at 22 ± 1 °C. All mice were trained before DEPs administration, which consisted of 3 repeated trials per day (inter-trial interval 30–60 min) starting at 9 am for 4 consecutive days when mice were released at the starting point. Mice were allowed to spend the 90s to find out the hidden escape platform on water and stay there for 10s. If mice could not find the platform within 90s, the experimenter helped them locate this platform and the mice stayed there for 10s. Mice were placed in their cages after each trial. One day after the cessation of training experiments, mice began to receive DEPs administration. Next, another session of navigation tests was performed after DEPs administration (three times per day for 4 consecutive days), starting at 9 am. Mice were released from the starting point and allowed to swim to arrive at the platform or for up to 90s. Distance traveled, swim speed, and escape latency were recorded for each trial. On the fifth day, the probe trial was performed using the same procedure described above after the removal of the platform. Time spent in each quadrant, frequencies of entering the platform area, and swim speed were recorded using a fixed overhead camera and tracking system.

Measurement of MDA in the hippocampus

Frozen brain-region samples were thawed and homogenized in SBB. To obtain a 0.350 ml of tissue, the homogenate was added into 0.550 ml of 5% trichloroacetic acid (TCA) and 0.100 ml of 500 ppm butylated hydroxytoluene (BHT) in methanol. The sample in a boiling water bath was then heated for 30 min. The sample was centrifuged after cooling on ice. The supernatant fraction was mixed 1:1 with saturated thiobarbituric acid (TBA). The samples were again heated for 30 min in a boiling water tub. After cooling it on ice, 0.50 ml of each sample was extracted using 1 ml of n-butanol and centrifuged to facilitate the separation phase. MDA levels were measured using the Duo Sets ELISA test. The excitation and the emission wavelengths were set at 540 nm and 640 nm, respectively. The concentration of the TBA-MDA complex in the mixture was determined using a calibration curve obtained from a malondialdehyde bis (dimethyl acetal) standard solution.

Total RNA isolation

Immediately, after finishing the behavioral tests, the mice were eluted under deep pentobarbital anesthesia and their brain samples were taken and maintained at the temperature of -80 °C before analyzing their mRNA expression patterns. The RNA was extracted from the HI samples using RNX-Plus kits (SinaClon).

Quantification of the hippocampal mRNA expression levels

The purity and quantity of total RNA were assessed and calculated using the NanoDrop RNA Assay protocol (Thermo Scientific NanoDrop One C, USA). Then, the first-strand cDNA was synthesized per sample (one μ g) using Takara Kit reverse transcriptase (Takara Bio USA, Inc.), per manufactured protocol. Afterward, the mRNA expression of IL-1 α , IL-1 β , IL-6, TNF- α , HO1, nNOS, NR2A, and NR2B in the HI was assessed. The expression level of target genes was estimated comparatively after normalization against the housekeeping gene, Hypoxanthine phosphoribosyltransferase (HPRT). The primer sequences used in this study are listed in Table 1. Quantitative real-time RT-PCR was performed using SYBR Green Real-Time PCR Master Mix (Toyobo Co., Ltd., Osaka Japan) in a BioRad Real-Time machine. The amplification of the target gene was monitored by measuring each sample's fluorescence intensity during each cycle.

Nissl staining

Histological examinations after DEPs exposure was performed using an optical microscope (Nikon, Japan) after Nissl staining. For Nissl staining, 5- μ m of coronal sections were first prepared. These sections were deparaffinized in xylene and then hydrated. After rinsing with tap water and then distilled water, sections were stained with 0.1% cresyl violet for 3 min. After dehydration, samples were finally placed under a glass coverslip. The Nissl body was dyed purple-blue.

Statistical analysis

Data are expressed as mean \pm SEM unless indicated otherwise. The significance threshold was set at P-values < 0.05 . The $2^{-\Delta\Delta C_t}$ method was used to analyze the qRT-PCR results. Statistical comparisons were performed using repeated-measurement ANOVA for behavioral tests and one-way ANOVA with Bonferroni correction for multiple comparisons. Statistical analysis was performed in Prism ver.7.3 software.

Table 1 Primer sequences for qRT-PCR assays

Gene		Sequence	T _m
HPRT	Hypoxanthine phosphoribosyltransferase	Forward: 5'GCT GGT GAA AAG GAC CTC T-3' Reverse: CAC AGG ACT AGA ACA CCT GC-3'	60
NR2A	N-methyl-D-aspartate receptor subunit 2A	Forward: 5'-AGCCCCCTTCGTCATCGTAGA-3' Reverse: 5'-ACCCCTTGACGACTTCTTAC-3'	60
NR2B	N-methyl-D-aspartate receptor subunit 2B	Forward: 5'-GCCATGAACGAGACTGACCC-3' Reverse: 5'-GCTTCTGGTCCGTGTCATC-3'	60
NR3B	N-methyl-D-aspartate receptor subunit 3B	Forward: 5'-TCCTACTCCTCCGCTCTCAA-3' Reverse: 5'-TGGATTCCAGACAGCTCCTC-3'	60
Glutaminase	Glutamate metabolism	Forward: 5'-GACTTCTCAGGGCAGTTTGC-3' Reverse: 5'-TCCTTCTCTCCGAGGATCAA-3'	60
TNF- α	tumor necrosis factor alpha	Forward: 5'-GGCCTTCTACCTTCAGACC-3' Reverse: 5'-AGCAAAAGAGGAGGCAACAA-3'	58
IL-1 β	Interleukin 1 β	Forward: 5'-CAG CTC ATA TGG GTC CGA CA-3' Reverse: 5'-CTG TGT CTT TCC CGT GGA CC-3'	60
IL-1 α	Interleukin 1 α	Forward: 5'-GCAACGGGAAGATTCTGAAG-3' Reverse: 5'-TGACAAACTTCTGCCTGACG-3'	60

Results

There was no significant difference between control VS. case adult male mice exposed to 300–350 $\mu\text{g}/\text{m}^3$ DEPs for 12 weeks regarding body length ($P = 0.083$), body weights ($P = 0.071$), vibrissae, and sensorimotor responses ($p < 0.05$).

Behavioral experimental

The impact of DEPs exposure on anxiety

The elevated plus-maze test was used to measure the anxiety of mice exposed to DEP. Anxiety levels are usually calculated based on the two factors of open arm entry and elapsed time in the arms. Our data demonstrated a significantly decreased ability to enter into the open arms accompanied with shorter elapse time in this state in male mice exposed to DEPs at 2, 5, and 7 h. compared to control mice (Fig. 2a, b), $P < 0.05$). Consequently, reduced number of open arm entrance and decrease time spent in this state, means development of more anxiety in this maze.

The impact of DEPs exposure on spatial learning ability

Through two maze navigation strategies, training and probe trial, spatial memory, and learning of mice exposed to DEPs were examined. To investigate hippocampal-dependent spatial memory and learning, we conducted a MWM test. Despite similar swimming speeds across groups (data not shown), four groups of mice introduced into the training phase showed different performance in maze task learning using analysis of variance ($P < 0.0001$). During 4 days of the experiment, all mice improved their performance. Follow-up data showed a significantly diminished behavioral performance in 5- and 7-

h. exposure mice compared to control mice ($P = 0.03$ and $P < 0.0001$, respectively). Meanwhile, the 2-h. exposure group did not demonstrate significantly lower performance VS. control group (Fig. 3a). Probe experiment illustrated significantly altered reference memory in DEPs exposed mice compared to control mice (Fig. 3b). After platform removal, we have measured the time spent in the correct maze quadrant. Results showed that regarding the learning phase, the 5- and 8- h. groups had a smaller area in the correct quadrant compared to the control group ($P = 0.002$ and $P < 0.0001$, respectively). The overall changes were shown by statistical analysis between the exposure groups ($P < 0.0001$) in task. The values for the groups are 17.1 ± 1.47 s and 22.2 ± 1.31 s for group 5 h and group 7 h, respectively. Although the behavior of exposed mice in the 2-h. group was similar to the control group, there was a significant difference between the 2- h. compared to 5-h exposed groups ($P < 0.015$ and $P < 0.0001$, respectively).

The impacts of DEPs exposure on MDA in the hippocampus

MDA levels also increased immediately in all exposure groups ($P < 0.05$). A significant change in the MDA level was observed between the 2- and 7- h exposed groups as well as the 2- and 7- h. exposed groups ($P < 0.001$) (Fig. 4).

The impacts of DEPs exposure on hippocampal NMDA receptor subunits

In addition to the impacts of DEPs exposure on spatial memory and learning, we also examined the impacts of DEPs exposure on hippocampal NMDA receptor subunit mRNA expression in adult mice. We observed significantly enhanced NR2A and NR3B mRNA expression in 5- and

7- h. exposed groups compared to the control group (Fig. 5a, c) ($P < 0.05$). However, the expression level of NR2B mRNA was not significantly different between control VS. exposed groups (Fig. 5b).

The impacts of DEPs exposure on hippocampal mRNA expression of Glutaminase

We have evaluated the **impacts** of DEPs exposure on hippocampal glutamine mRNA expression in mice. There was a significantly increased hippocampal glutaminase mRNA expression in 5- and 7- h. groups than in the control group ($P < 0.05$). However, we did not observe any significant difference between the 5- and 7- h. groups (Fig. 6) ($P < 0.05$).

Effect of DEPs exposure on hippocampal cytokine mRNA expression

We have assessed the expression of pro-inflammatory hippocampal cytokines, such as IL1 α , TNF- α , and IL1 β , in adult male mice that were exposed to DEPs for 12 weeks. The mRNA levels of invasive cytokines such as TNF- α , IL1 β , and IL1 α were significantly higher in the 5- and 7- h. groups ($P < 0.05$). We observed a significant change in IL1 α mRNA expression between 2- and 7- h. groups, as well as 2- and 5- h. groups. There was also a significant change in the expression levels of TNF- α and IL1 β mRNA between the 2- and 7- h. groups (Fig. 7) ($p < 0.001$).

Hippocampal morphological analysis

NISL staining in HI of control and mice exposed to DEPs 2-, 5- and 7- h. per day for 12 weeks indicated altered neuronal morphology in CA1 and CA3 regions of the hippocampus especially with more than two hrs. of exposure per day during long term period (Fig. 8) ($P < 0.05$) (data not shown).

Discussion

The main finding of this study was that 12 weeks of DEPs exposure would affect anxiety, spatial memory, and up-regulate NMDA receptor subunits of NR2A, NR3B, TNF- α , IL1 α , IL1 β , and glutamine Enzyme expression in the HI of adult male mice. Impaired learning and memory in male mice was also associated with enhanced expression of hippocampal NR2A and NR3B following exposure to DEPs. The dose used in this study did not significantly affect mice body weight. However, diminished performance in MWM test suggests impaired memory and learning ability.

Activated microglia secrete inflammatory cytokines and chemokines which contributes to development of chronic inflammatory brain diseases [29]. Upon inflammation, these

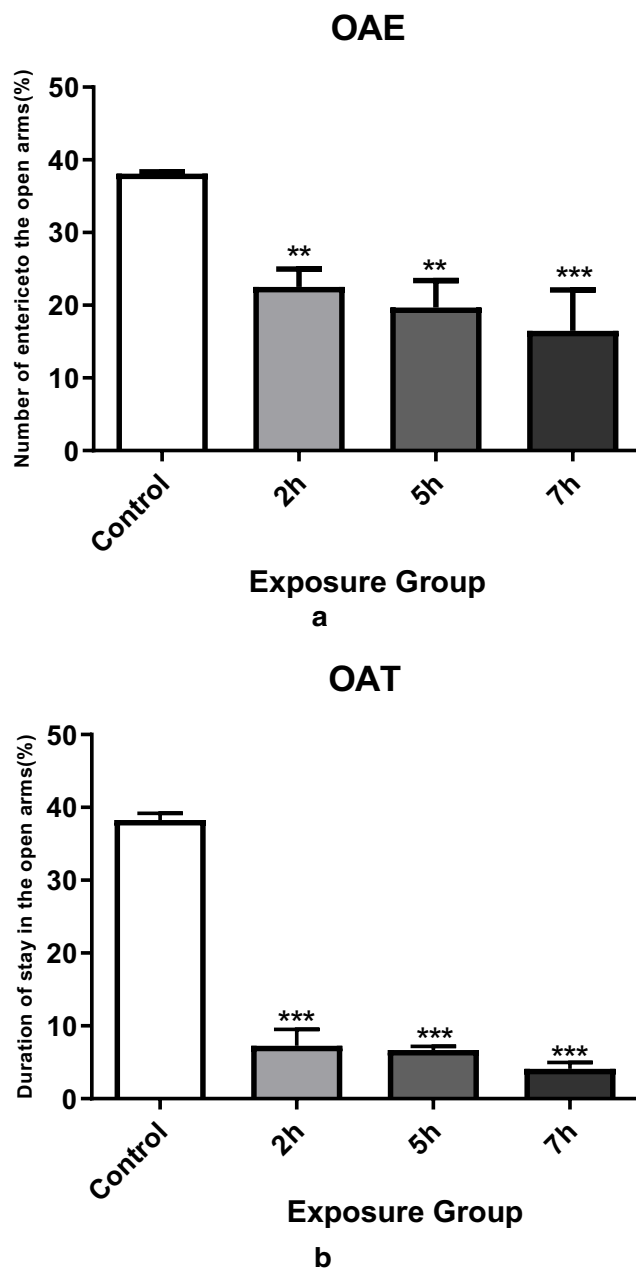
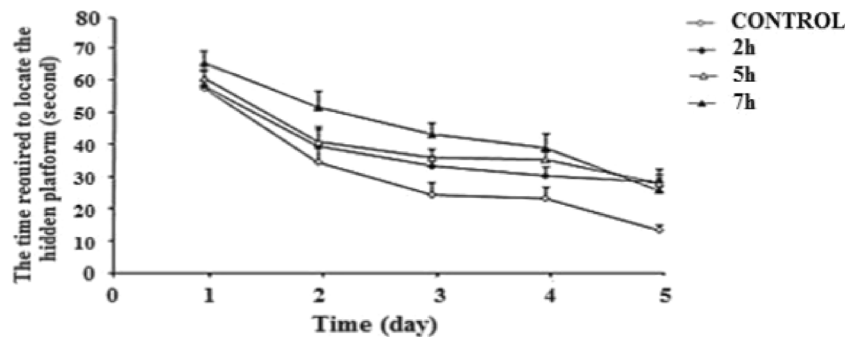


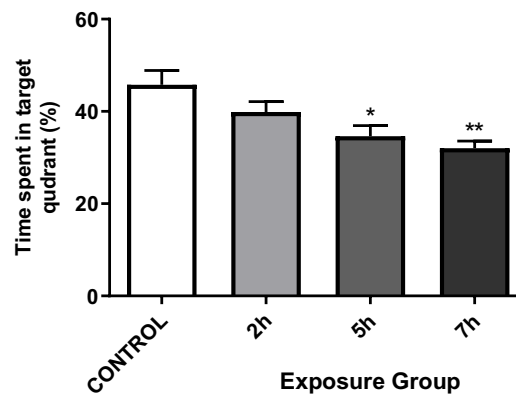
Fig. 2 Elevated plus-maze navigation for different groups of mice. **a** Percentages of open arms entered by different groups of mice when searching for this maze. Exposure to DEPs in all exposure groups significantly reduced arm penetration. **b** Percentage of time spent in open arms while navigating the maze. All exposure groups showed a significant decrease in open arm command compared with control group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are shown as mean \pm SEM

resident CNS immune cells can modify synaptic connectivity essential for learning and memory processes [30]. Another study indicated that rodents exposed to DEPs and lipoteichoic acid (LTA) took longer time to attain the hidden plateau in the MWM test accompanied with upregulated mRNA expression of pro-inflammatory cytokines and NMDA receptor subunits.

Fig. 3 **a** Spatial learning performance in the Morris water maze during the acquisition phase of adult male mice exposed to 2 h/day, 5 h/day, and 7 h/day for 12 weeks when compared with the control group ($P < 0.038$ and $P < 0.001$, respectively). **b** Memory retention during the probe trial of adult male mice exposed to Control, 2 h/day, 5 h/day, and 7 h/day for 12 weeks when compared with the control group. $*P < 0.05$, $**P < 0.01$. Data are shown as mean \pm 10 SEM



a
Prob trial MWM



b

It was also shown that co-exposure of DEPs and LTA caused glutamate-induced neurotoxicity, followed by changes in the

expression of related kinases, transcription factors, hippocampal NMDA receptor subunits and olfactory bulb in the mouse [31].

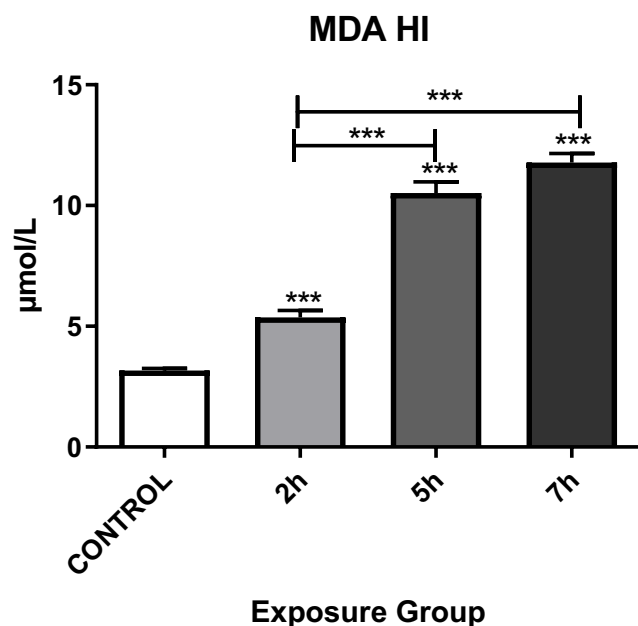
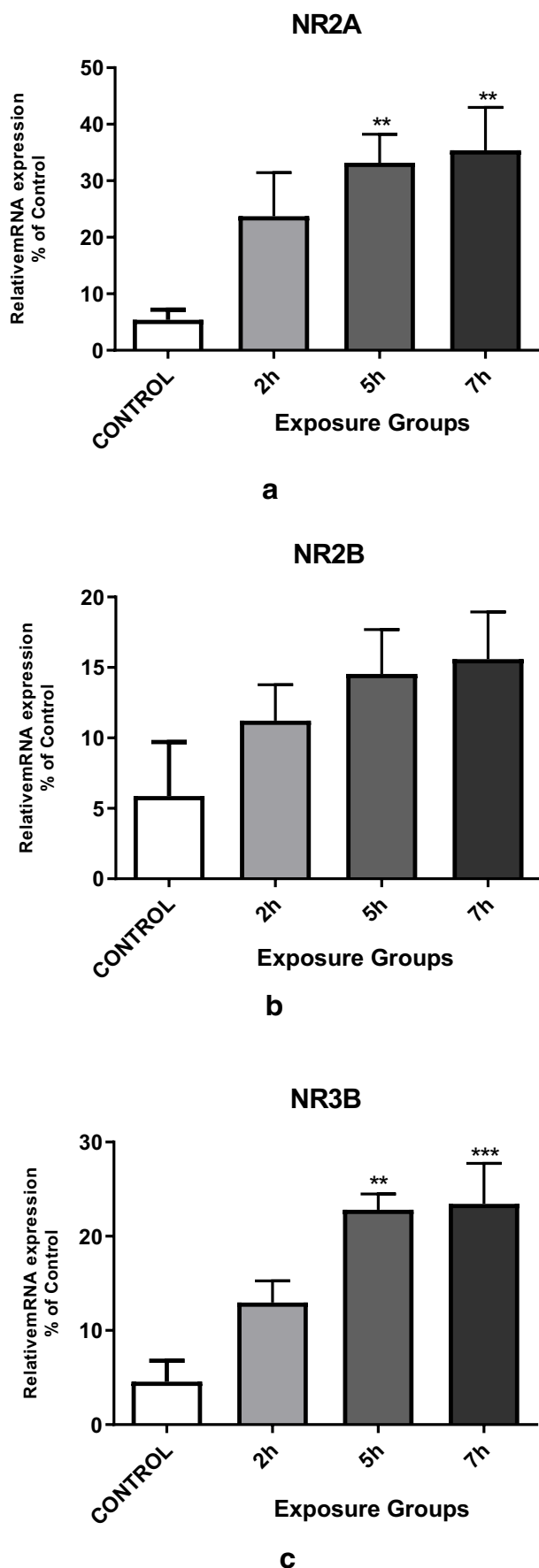


Fig. 4 Alteration of the MDA in the hippocampi of mice exposed to Control, 2, 5, or 7 h. for 12 weeks. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Each bar represents the mean \pm SE ($n = 6$)

In line with these reports, our results have linked diminished learning and memory to increased inflammation, oxidative stress, and altered expression of NMDA receptor subunits in mice hippocampus upon DEPs exposure. In one study, it was demonstrated for the first time that co-exposure with bacterial cell wall and DEPs component LTA for one month correlated with altered expression level of NMDA receptor subunits in the hippocampus with subsequent poor spatial memory and learning in male mice [32]. In female rodents, estrogen acts on the brain to induce reproductive behaviors, including female-like behavioral properties such as lordosis, as well as non-reproductive behaviors, including anxiety and depression-like behaviors, and cognitive behavior [33, 34]. Thus in this study, we limited the experiment to male mice, during DEPs exposure. Morris water maze test indicate that learning and reference memory are impaired in male mice exposed to DEPs, which requires a fully functional HI. Besides, hippocampal NR2A and NR3B expression were significantly higher in adult male mice exposed to DEPs than in control mice. In rodents, the hippocampus has always been considered as a key structure for coding spatial information [35]. Oxidative stress can lead to stress-induced learning and



◀ **Fig. 5** Messenger RNA expression of the hippocampal NMDA receptor subunits (a) NR2A, b NR2B, and c NR3B in the exposure groups (2, 5, or 7 h. per day for 12 weeks) and control mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Each bar represents the mean \pm SE (n = 6)

memory disorders [36]. Oxidative damage induced by oxidative stress was associated with a significant decrease in glucocorticoid receptors in the CA1 region of the HI [37]. It has been suggested that oxidative stressors increase glucocorticoid levels and impair cognitive function due to damaging the HPA axis [38]. As several researchers have demonstrated, chronic stress exposure or glucocorticoids can lead to oxidative damage in the HI with subsequently impaired learning and memory [39]. In rats, the volume of dorsal hippocampal tissue injury is related to the extent of spatial learning deficits. Indeed, dorsal HI lesions cause more severe injury than ventral HI lesions and CA1 neurons are essential for learning [40]. In male mice hippocampus, treated with high-dose of DEPs for one month, the mRNA expression level of the TNF- α was elevated [32]. TNF- α is reported to be a key pro-inflammatory cytokine through autocrine [41]. The way to stimulate the release of a wide range of microglia glutamate up-regulates glutaminase, which results in excitation neurotoxicity [42]. In this study, we found an increase in IL6, IL1 α , and TNF α mRNA levels in exposed mice that maybe due to chronic DEPs exposure related to the activation of different signal transduction pathways in NMDA receptor-associated memory. A significant change in the level of TNF α expression was also observed. Indeed, mRNA expression of cytokines related with DEPs exposure-induced neuro-inflammation was significantly increased in the 6- and 8- h groups. This finding

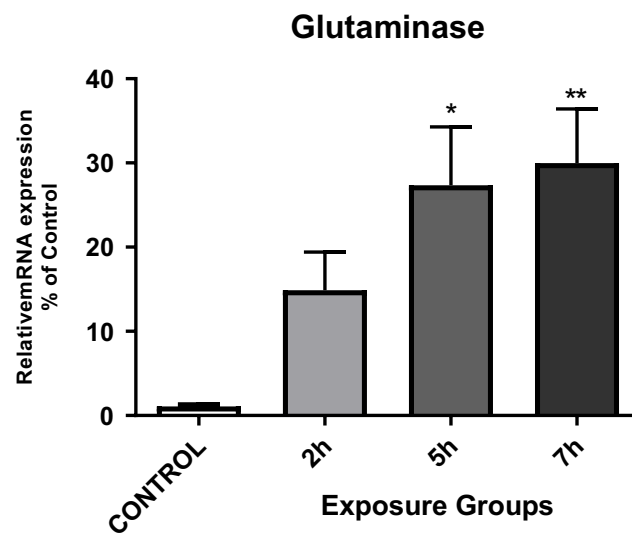
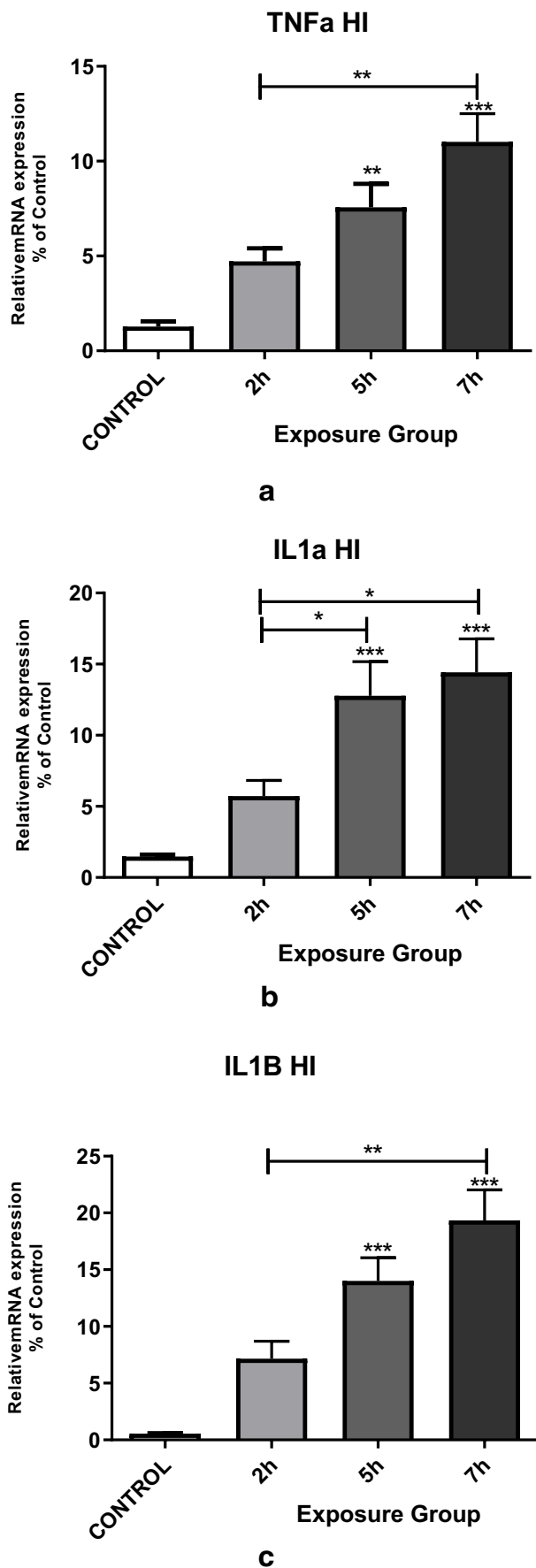


Fig. 6 Messenger RNA expression of glutaminase in the hippocampi of mice exposed to DEPs (2, 5, or 7 h per day for 12 weeks) compared with control mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Each bar represents the mean \pm SE (n = 6)



◀ **Fig. 7** Messenger RNA expression of the pro-inflammatory cytokines (a) TNF α , (b) IL1 α , and (c) IL1 β in the hippocampi of mice exposed to DEPs (2, 5, or 7 h per day for 12 weeks) compared with control mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Each bar represents the mean \pm SE (n = 6)

suggests that mere DEPs exposure for 12 weeks may induce inflammation of the mouse hippocampus. We have reported that morphological alterations of hippocampal neurons in CA1 and CA3 regions were associated with up-regulation of inflammatory cytokines gene expression in the setting of sub-chronic DEPs exposure. Changes in the morphology of CA3 accompanied with disrupted learning and spatial memory have been previously reported in association with low-grade inflammation [43]. These findings are the same to our previous study in which significant pathological lesions in the hippocampal CA1 region of adult offspring male mice were observed due to maternal exposure to inhaled DE [19]. In the HI, NMDA receptors play an important role in spatial learning and memory tasks [44]. Significantly enhanced expression of NR1 subunits in the male mice hippocampus was demonstrated after one month of exposure to high doses of DEPs and bacterial cell wall components compared to the expression of NR2A and NR2B subunits [32]. Also, co-exposure with high-dose DEPs and bacterial cell wall components in the same month affected extracellular glutamate levels and expression of NR1, NR2A, and NR2B mRNA in the olfactory bulb in male mice [31]. In this study, DEPs exposure in adult male mice significantly increased hippocampal NR2A and NR3B expression, which may be related to the spatial memory and learning deficits. The NR2B subunit is required for fetal neuronal patterning and fetal survival. Although DEPs exposure did not significantly affect mRNA NR2B expression [45], NR2A subunit expression and synaptic binding gradually increased throughout development [40, 41]. In fact, synaptic NR plays a key role during brain development, plasticity, and pathology [46]. The insertion of NRs into synaptic sites follows a different mechanism, depending on the receptor subunit composition. With increasing levels of NR2B gene expression, synaptic insertion of receptors containing NR2B does not increase, while synaptic insertion of receptors containing NR2A, that is essential for synaptic activity, is raised by increased expression of the NR2A mRNA. Therefore, exposure to DEP may affect the insertion of NR2A and NR3B into hippocampal synapses since NR2A and NR3B expression is increased in the HI. According to the results derived from epidemiological studies, exposure to high concentration of DEPs might result in Alzheimer’s disease [47]. Our results highlight the need to identify measures to prevent and control the impacts of DEPs exposure on development of cognitive function. The living environment makes sense to prevent the development of DEPs. A rich environment can also prevent

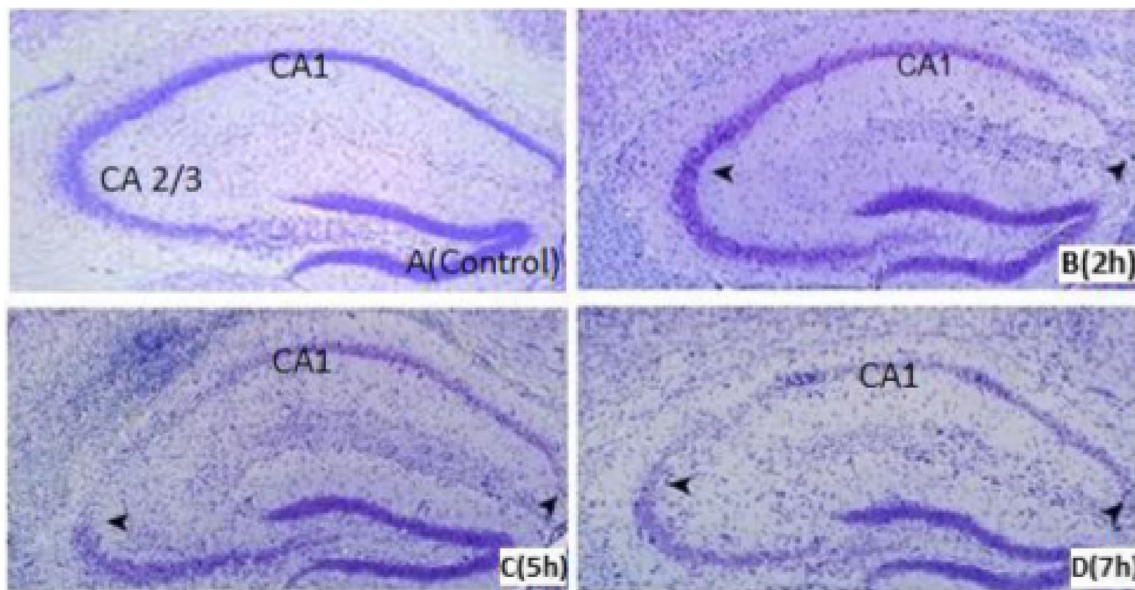


Fig. 8 Nissl staining in the hippocampus of the Control (a) and 2, 5, and 7 h/days exposure to DEPs for 5 days/week within 12 weeks. By 5 h/d exposure, neurons of CA1 and CA3 region are lost in the mouse (c) but minimal neuronal dropout is evident in the 3 h/day exposure mouse (b). By 7 h/d exposure, the majority of CA1 and CA3 neurons are lost in mice

(d). Hippocampal CA1 and CA3 neurons were loose and absent; nucleoli were missing or indistinct (arrows). In the control group, neurons were fairly well preserved and sparse in the CA1 and CA3 sub-region. Sections are representative of at least 4 mice of each group

hippocampal impairment [48, 49]. In this study, it was found that the level of neurotransmitters was not determined. We believe that impairment of learning ability and spatial memory was due to excitatory neurons, especially glutamate, that induce neuronal damage to intercede by abnormal activation of specific NMDA receptor subunits in the HI. Up-regulation of mRNA expression of NMDA receptor subunits NR2A, NR3B, and glutaminase may be caused by a compensatory response to neuronal damage. Further investigations are needed to determine measures to prevent the impacts of DEPs exposure on recognition through early environmental enrichment or interventions.

Conclusions

In conclusion, DEPs exposure induced neuro-inflammation, and anxiety as well as altered NR2A and NR3B expression associated with hippocampus-dependent spatial learning and memory of adult mice. Somehow, we are aware that DEPs exposure time used in this study would jeopardize human cognitive function and further research is needed to elucidate the impacts of chronic DEPs exposure on NMDA receptor, memory, and learning function in rodents.

Acknowledgments We thank Dr. Ehsanifar Lab. for technical assistance. This work was supported by Anatomical Sciences Research Center, Kashan University of Medical Sciences, Kashan, Iran.

Data availability The dataset used in this study is available with the authors and can be made available upon request.

Compliance with ethical standards

Competing interests The authors declared that they have no competing interests.

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