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RESEARCH ARTICLE

Neurobehavioral deficits and brain oxidative stress induced by chronic low dose exposure of persistent organic pollutants mixture in adult female rat

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Abstract Persistent organic pollutants (POPs) are long-lived organic compounds that are considered one of the major risks to ecosystem and human health. Recently, great concerns are raised about POPs mixtures and its potential toxicity even in low doses of daily human exposure. The brain is mostly targeted by these lipophilic compounds because of its important contain in lipids. So, it would be quite interesting to study the effects of exposure to these mixtures and evaluate their combined toxicity on brain cells. The present study was designed to characterize the cognitive and locomotors deficits and brain areas redox status in rat model. An orally chronic exposure to a representative mixture of POPs composed of endosulfan (2.6 μg/kg), chlorpyrifos (5.2 μg/kg), naphthalene (0.023 μg/kg) and benzopyrane (0.002 μg/kg); the same mixture with concentration multiplied by 10 and 100 was also tested. Exposed rats have shown a disturbance of memory and a decrease in learning ability concluded by Morris water maze and the open field tests results and anxiolytic behaviour in the test of light/dark box compared to control. Concerning brain redox homeostasis, exposed rats have shown an increased malondialdehyde (MDA) amount and an alteration in glutathione (GSH) levels in both the brain mitochondria and cytosolic fractions of the cerebellum, striatum and hippocampus. These effects were accompanied by a decrease in levels of cytosolic glutathione S-transferase (GST) and a highly significant increase in superoxide dismutase (SOD) and catalase (CAT) activities in both cytosolic and mitochondrial fractions. The current study suggests that environmental exposure to daily even low doses of POPs mixtures through diet induces oxidative stress status in the brain and especially in the mitochondria with important cognitive and locomotor behaviour variations in the rats.

Keywords POPs mixtures · Neurodegeneration · Behavioural alterations · Brain mitochondria · Oxidative stress

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Introduction

What make us special and spatial among all living creatures are our brains; however, lately, we are putting what is making us special in danger. In fact, from 1990 to 2010, mental and behavioural disorders increased by more than 37 %, Parkinson's disease increased by 75 %, Alzheimer's disease doubled, autism increased by 30 % and attention deficit hyperactivity disorder (ADHD) increased by 16 % (Murray et al. 2012). This scary increase in prevalence incidence is mostly linked to pollutions, where persistent organic pollutants (POPs) could play the main role (EIA 2013). Several epidemiological studies have reported the implication of POPs in aetiology of neurodevelopmental (Perera et al. 2009; Perera et al. 2006) and neurodegenerative diseases particularly Parkinson disease (PD) (Fleming et al. 1999; Moretto and Colosio 2011). Pesticides ,for example, are known for their

alteration of metabolism and function of neurotransmitters. In fact, endosulfan, an organochlorine (OC) acts on insects by blocking Cl^- channels linked to the γ-amino-butyric acid (GABA)-receptor (Silva and Beauvais 2010), while organophosphorates (OP) like chlorpyrifos affect cholinergic system through inhibition of acetylcholine esterase (ACHE) and muscarinic receptors (Kobayashi et al. 1986). Disturbance of serotoninenergic and catecholaminergic systems by POPs is also reported in the literature and strongly linked to neurobehavioral effects induced by these compounds (Saunders et al. 2006). Other than neurotransmission, endocrine disruption (Briz et al. 2011) and epigenetic effects (Kodavanti et al. 2011) have been also reported. Moreover, POPs including OC, OP and polycyclic aromatic hydrocarbons (PAH) are reported to disturb Ca^{++} homeostasis (Meijer et al. 2014; Heusinkveld et al. 2012) leading to mitochondrial dysfunction which also could be induced by alterations of the activity of respiratory chain enzymes (Hargreaves 2012; Hatcher et al. 2004); and due to high energetic demands of brain and its poor antioxidant system (Radák et al. 2001; Halliwell 2006), any slight mitochondrial dysfunction could enhance a state of oxidative stress (OS); furthermore, POPs are able to produce reactive oxygen species (ROS) during metabolism process, what aggravates mitochondrial dysfunction which in turn produces more ROS leading to a vicious and detrimental cycle that ends up with apoptosis and neurodegeneration (Federico et al. 2012; Kaur et al. 2007).

Recently, great concerns are raised about neurodevelopmental effects of POPs mixtures in the range of daily exposure as epidemiological studies have been supported by experimental studies. Guillemette et al. (2012)) report that *in uterus* and lactational exposure to a low dose mixture of 16 PAH have induced an increase in anxiety and a neuronal hypometabolism in exposed animals on adulthood; in a related study, prenatal exposure to a representative POPs mixture has induced on adulthood, transcriptional changes in cholinergic system and structural genes, while lactational exposure to a representative mixture of PCB found in contaminated fish matrices has induced oxidative stress and apoptosis in juveniles and an increase in anxiety and transcriptional changes on adulthood (Ardzivian et al. 2012).

Effects of exposure on adult age remains less concerning since the brain has already reached a steady state of developmental processes including neurogenesis, migration, synaptogenesis, gliogenesis and myelination (Garman et al. 2001); however, lately, studies on adult brain revealed its sensitivity towards exposure to environmental relevant mixtures (Moser et al. 2012; 2005; Roszczenko et al. 2013). A preclinical study in adult rats, about exposure to compounds forming golf ware including CPF also, reported pathological changes in morphometry and synaptic integrity in different brain regions (Ojo et al. 2014). In the same context, Du et al. (2014) demonstrated microstructural changes in the central nervous system of agricultural workers with low chronic exposure to pesticide.

In the present study, taking in consideration that oxidative stress and mitochondrial dysfunction are early alterations in neurodegenerative diseases (Manczak et al. 2006) and that POPs are well known by their prooxydant effects and mitochondrial alteration, so we aimed to evaluate the effect of chronic low dose POPs on the neurobehavioral and the oxidative stress of adult female rats. The POPs mixture consisted of two HAP, benzopyrane, a highly prooxydant and carcinogenic compound (Saunders et al. 2006) and naphthalene, a relatively less toxic PAH and two pesticides, endosulfan (END), an OC, which is banned or restricted from almost all the parts of the world but still found in nature due to its high persistence and non-authorized use (ATSDR 2015) and chlorpyrifos (CPF), an OP, still in debate to be classified or not as a POP since the rate of its persistence does not meet the classification criteria of Stockholm convention; however, its toxicity is well established even in doses largely under NOEL without taking in consideration possible interactions with other chemicals present in environment (Giesy et al. 2014).

Materials and methods

Chemicals

The components of the mixture used in this study are consisted of two pesticides on their commercial forms: endosulfan (35 %) and chlorpyrifos (480 %) and two HAPs: α-benzopyrane (95 %) and naphthalene (99.5 %). The majority of chemicals were purchased from Sigma Aldrich, Germany. Assay Kits for enzymes were purchased from Biomerieux, and endosulfan was purchased from Pharmacia, St. Quentin inYvelines, France.

The dose of each compound in the mixture was determined as the estimated daily intake (EDI) calculated according to international guidelines (Iñigo-Nuñez et al. 2010). Residue levels of pesticides used in this study were derived from a reel exploration study on pesticides in vegetables in Algeria (data not published), while residue levels of HAPs were taken from the study of Martorell et al. (2010). Finely, the daily food consumption was determined according to the study of Serra-Majem et al. (2003).

Pesticides and HAPs were dissolved in corn oil and administered to rats as one mixture. The mixture was renewed each 5 days. Three doses were prepared by successive dilution, D × 100, D × 10 and D, where D is consisted of chlorpyrifos (5.2 μg/kg), endosulfan (2, 6 μg/kg), naphthalene (0.023 μg/kg) and α-benzopyrane (0.002 μg/kg).

Animals and exposure protocol

Twenty-eight female Wistar rats, weighing 200–250 g, were obtained from Pasteur institute (Algeria). Upon arrival, the rats were housed, five per cage. Animals were maintained under a daily 12 h light/dark cycle at a constant temperature (22 ± 2 °C), a relative humidity of 55 ± 10 % and a free access to food and water. Rats were adapted for 2 weeks before the indicated treatments. All experimental assays were carried out in conformity with international guidelines for the care and use of laboratory animals. Rats were divided to four groups; control group who received only 0.5 ml of corn oil, group D treated with the lowest dose (D), group D × 10 and D × 100 treated with the dose D folded by 10 and 100, respectively. Each group received the treatment by gavage every day for 90 days between 9:00 and 10:00 pm.

Behavioural testing

From the day 75 of exposure, behavioural tests performed were the Morris water maze test (MWM), the open field (OF) and the light/dark box test (LDB). MWM was performed for five consecutive days, and the probe trial conducted on the 6th day of the test. OF and LDB were performed in one session over the following week; the interval between the two tests was 2 days. All tests were performed between 13:00 and 17:00 p.m. The testing order was randomized between rats from the four groups, to avoid circadian variation. Tests were recorded and all the variables were analyzed by the same experimenter, using the video tracking program Etho-Vision® from Nolduls Information Technologies.

Morris water maze

Spatial learning and retention were tested in a water maze according to a test modified from the procedure of (Morris 1981; Bromley-Brits et al. 2011). The water maze consisted of a circular pool (diameter, 150 cm; height, 50 cm) divided into four equal-sized quadrants. During testing, the pool was filled with water at 22 ± 2 °C. A transparent platform (diameter, 10 cm; height, 25 cm) was set inside the tank being the top submerged 2 cm below the water surface, in the centre of one of the four quadrants of the maze. Water was made opaque by milk powder. Animals were subjected to four trials per day for five consecutive days (training sessions). Each trial started from one of four points assigned on different arbitrary quadrants of the circular tank. The maximum duration of each trial was 60 s, being each trial separated by a 60 s interval. At the beginning of each trial, the rat was placed into the pool with the nose pointing towards the wall from one of four starting positions. If the rat did not locate the platform within 60 s, the animal was then placed on the platform for 20 s. Twenty-four hours after the last training session, retention of the task was assessed by a probe trial which consisted of a 60 s free swim without the escape platform. The swim path length and the latency to find the escape platform during the training sessions, as well as the cumulative time in the quadrant where was the platform and the frequency to pass by the platform zone during probe test were analyzed as the measures of water maze performance.

Open field

This test was performed to assess the general locomotor activity (Riebe and Wotjak 2012). The open-field chamber is a 50 cm × 50 cm × 40 cm rectangular transparent glass box and opened from the top. The floor was divided into equal size cases numbered from 1 to 25 and three squares limiting peripheral, intermediate and centre areas. The chamber was stood in an isolated room. At the beginning of the test, the rat was placed on one of the corners facing the wall of the apparatus and was let free to explore it for 5 min. During this period, the total number of crossed cases, the number of rearing, the number of crossed cases in each of the three areas (peripheral, intermediate and centre) and the total distance moved were recorded. Between each animal, the apparatus was cleaned with a 30 % ethanol solution.

Light dark box test

This test was performed to assess anxiety behaviour (Riebe and Wotjak 2012). The apparatus is a glass box divided into two compartments; one compartment "light" (30 × 30 × 50 cm) is transparent; the other one "dark" is painted black (20 × 20 × 50 cm); a hole in the partition separating the two compartments allows access between compartments. This system is based on the internal conflict between the approach and avoidance of anxiety-provoking areas (here, the light compartment). At the beginning of the experiment, the rat was placed in the light compartment of the box head oriented to the hole and was let free to explore it for 5 min. During this time, latency to enter to the dark compartment, the number of entries to each compartment and the time spent in the illuminated compartment were recorded; the apparatus was cleaned with a 30 % ethanol solution between trials.

Biochemical analysis

After 90 days of exposure, rats were sacrificed by decapitation after deep ether anaesthesia; brain was removed quickly. Right hemisphere was used for the extraction of the whole mitochondrial and cytosolic fractions as described by the method of Clayton and Doda (2001) with slights modifications. Briefly, the brains were washed in cold respiration buffer,

pH 7.4 (50 mM Tris-HCl, 250 mM sucrose, 1 mM methyl diamine acetic acid (EDTA), 0.2 % BSA) and then chopped and homogenized in three volumes of the same buffer and centrifuged at 3500 g for 10 min, than the pellet was recentrifuged in the same conditions. Supernatants from the two centrifugations were mixed and centrifuged at 15,000 g for 20 min. The supernatants were considered as a cytosolic fraction and conserved at −20 °C until ulterior determination of CAT, SOD and GST activities, while the resultant pellet was washed twice with PB buffer (50 mM Ttis-HCl, 250 mM sucrose) pH7.4 in the same conditions; resultants mitochondrial pellets were suspended in 300 μl of PB buffer and frozen at −20 °C until its ulterior use.

Mitochondrial matrix was prepared from mitochondria by freezing and defrosting with repeated homogenization in order to burst mitochondria. After centrifugation at 10,000 g for 10 min, the supernatant was considered as the source of mitochondrial CAT, SOD, MDA and GSH. In other hand, the left hemispheres were dissected immediately after sacrifice to four regions (the striatum, the hippocampus, the cortex and the cerebellum). Then tissues were homogenized in three volumes of phosphate buffer 0.1 M with KCl 1.17 % (ph 7.4) and centrifuged at 2000 g for 15 min. The resultant supernatant was used to determine levels of regional MDA and GSH.

Protein content was determined by the Bradford method (Bradford 1976) using bovine serum albumin as standard. SOD, CAT, and GST activities were determined according to the methods described by Beauchamp and Fridovich (1971), Aebi (1984) and Habig et al. (1974), respectively. Finally, GSH levels were assessed according to Ellman essay (1959), and MDA levels were assessed using Ohkawa method (1979).

Statistical analysis

Data from behavioural and biochemical tests were analyzed using one-way analysis of variance (ANOVA). For the training sessions of MWM, a two-way ANOVA analysis was applied, considering time as a repeated factor. Post hoc comparisons have been performed using the Bonferroni's *t* test when ANOVA was significant. The correlation between GSH and MDA levels was tested by Pearson correlation coefficient. Significance was set at $P < 0.05$. All statistical analyses were carried out using Excel SPC software package.

Results

Behavioural tests

Morris water maze test

In MWM, all groups have shown a decrease in the distance travelled and the latency time across training sessions. In the 5th session before the probe test, statistical analysis revealed a significant increase in distance travelled and latency time in groups treated with D × 10 and D × 100 when compared to control reflecting thus a learning impairment. In contrary, the group treated with D has shown instead a significant decrease in the travelled distance with a non-significant decrease in latency time (Table 1). In the probe trial, exposure to the POPs mixture had a negative impact on spatial memory retention since a significant decrease in frequency to pass by the platform zone was also observed in both groups treated with D and D × 100 (Fig. 1), and a significant decrease in the cumulative time in the probe zone was observed in the group treated with D × 100 (Fig. 2). However, the group treated with D10 did not show any alteration in memory retention unlike its performance during training sessions where an important learning ability impairment was observed.

Open field test

In the OF test (Table 2), a highly significant decrease in rearing was observed in the group treated with the highest dose D × 100 whereas the decrease was not significant in the group treated with dose × 10. However, in the group treated with the lowest dose D, the number of rearing increased instead, although this increase was not significant when compared to control. The treatment had also a significant effect on the total distance moved where Bonferroni's *t* test revealed a highly significant decrease in groups treated with dose × 10 and D × 100 accompanied by a highly significant decrease in the number of crossed cases in peripheral area but only in the group treated with the highest dose D × 100. The number of crossed cases in the intermediate and central area was not affected in all treated groups.

Light/dark box test

In LDB, there is a significant effect of the treatment on the latency time and the time spent in the light compartment. The result of this test showed that only exposure to the dose D × 100 and D has induced a significant increase in these two parameters (Table 3). On the other hand, there was no significant effect of the treatment at any dose on the number of transitions and the number of entries to each compartment (data not shown).

Oxidative stress parameters

MDA levels

MDA levels as an indicator of lipid peroxidation have shown a significant increase in whole brain mitochondria in all treated groups (Fig. 3). In the cerebellum, MDA also showed a highly dose dependent increase in all treated groups; however,

Table 1 Effects of the POPs mixture on memory and learning in the 5th acquisition session in MWM

Variable Group	Control	D	D × 10	D × 100
Latency time (s)	6.36 ± 1.36	4.087 ± 1.14	10.99 ± 2.68	16.44 ± 3.54**
Path length (m)	1627.17 ± 350	790.46 ± 208*	3388.86 ± 1081	5027.67 ± 762***

Results are expressed as mean ± SE (*n* control = 5; *n* treated groups = 7). Bonferroni's *t* test was used for multiple comparisons

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ as compared to control

in the hippocampus, statistical analyzes revealed a significant increase only in the group treated with the highest dose D × 100 while in the striatum the increase was significant in both groups treated with D × 100 and D × 10. In the cortex, the studied mixture seems to have no effect on lipid peroxidation since MDA levels were normal in all treated groups compared to control (Fig. 4).

GSH levels

GSH in whole brain mitochondria and striatum has shown an increase in all treated groups; however, Bonferroni's *t* test revealed that this increase in whole brain mitochondria was significant only in the group treated with the highest dose D × 100 (Fig. 5) while in striatum the significant increase was noticed in the group treated with the intermediate dose D × 10. In the hippocampus GSH has shown a dose dependent increase, and in contrary to striatum, Bonferroni's *t* test revealed a highly statistical significance in both groups treated with the dose D × 100 and D × 10, whereas the increase in the group treated with D was not significant (Fig. 6). In the other hand, GSH and MDA levels seems to be correlated in the hippocampus and striatum, where Pearson test revealed a strong positive correlation ($r = 0.88$, $P \approx 0$). In contrast to the striatum and hippocampus, GSH level in cerebellum has shown instead a significant decrease in all treated groups where the lowest level was observed in the group treated with the intermediate dose D × 10. Moreover, this decrease was significantly correlated to the increase noticed in MDA levels in the same region ($r = 0.53$, $P = 0.020$). In the cortex and similarly to MDA, GSH levels were not affected in all treated groups compared to control (Fig. 6).

Antioxidant enzymes activity

Levels of antioxidant enzymes activity are presented in (Table 4). An increase in whole brain mitochondrial CAT activity was noticed in all treated groups; however, this increase was statistically significant only in the groups treated with the highest and intermediate dose. Whereas in cytosol, Bonferroni's *t* test revealed a highly significant increase in CAT activity in all treated groups where the highest activity was noticed in the group treated with D × 10 and the lowest in the group treated with the D × 100. Furthermore, the noticed decrease in the group treated with D × 100 was statistically significant when compared to the group treated with D × 10.

Mitochondrial SOD activity also increased significantly in all treated groups, except the group treated with the lowest dose D where the increase was not statistically significant as revealed by statistical test. In cytosol, a highly significant increase in SOD activity was noted in groups treated with D and D × 100 compared to control. In contrary, the group treated with D × 10 has shown instead a non-significant decrease in SOD activity as revealed by Bonferroni's *t* test. GST activity

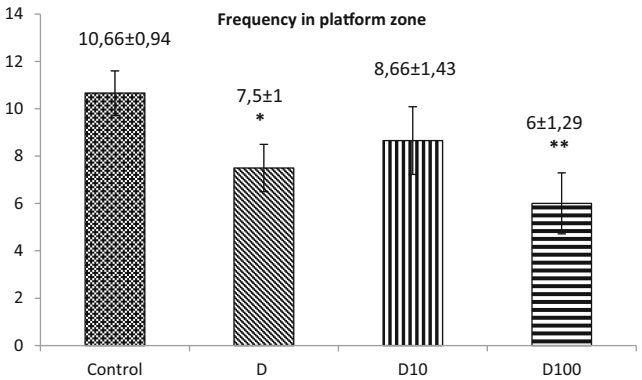


Fig. 1 Effect of the POPs mixture on frequency of passing by the platform zone in the probe test of MWM. Value are mean ± SE (*n* control = 5; *n* treated groups = 7). Benferroni's *t* test was used for multiple comparisons, *$P < 0.05$, statistical significant difference from control, **$P < 0.01$, statistical significant as compared to control

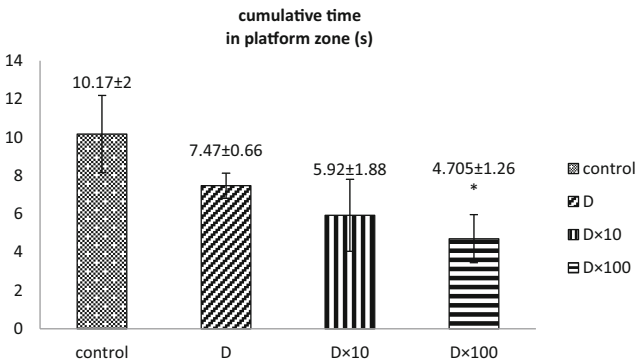


Fig. 2 Effect of the POPs mixture on cumulative time passed in the platform zone in the probe test of MWM. Values are mean ± SE (*n* control = 5; *n* treated groups = 7). Benferroni's *t* test was used for multiple comparisons, *$P < 0.05$, statistical significant as compared to control

Table 2 Effects of POPs mixture on locomotor activity and anxiety assessed in the open field

Variable Group	Control	D	D × 10	D × 100
Number of crossed squares:				
In peripheral area	67 ± 5.6	55.28 ± 17.18	55.5 ± 10	24.14 ± 5.55**
In intermediate area	6.25 ± 1.5	8.5 ± 2.14	7.33 ± 2.38	5.57 ± 3.06
In central area	2.5 ± 0.8	2.28 ± 0.69	1.33 ± 0.38	1.57 ± 0.65
Total distance moved (m)	1303.93 ± 156.61	1202.97 ± 224.43	699.65 ± 117.89**	533.36 ± 83.06**
Total number of rearing	17.25 ± 0.7	19.42 ± 2.08	14.33 ± 3.03	11 ± 2.8**

Results are expressed as mean ± SE (*n* control = 5; *n* treated groups = 7). Bonferroni *t* test was used for multiple comparisons

***P* < 0.001, ***P* < 0.01, **P* < 0.05 as compared to control

decreased significantly only in the group treated with the highest dose D × 100 while in groups treated with D and D × 10 changes were not significant.

Discussion

The components of the mixture used in the present study are two pesticides and two PAHs. The studied pesticides are commonly used by agricultures in the region of Jijel, East of Algeria. Before starting this work, a recent study was conducted by another research team in our lab to explore the level of the contamination of vegetables and fruits with pesticides. Results of this study showed both high amounts of END and CPF, neighboring 4 mg/kg of dry matter (data not published). For the two PAHs of the mixture, it is well referenced that benzopyrene is a very toxic substance, produced by different anthropogenic and natural combustion, contaminating food, biological matrix and consequently animal and human organism. In contrast to naphthalene, which is less toxic but widespread in environment, furthermore, this hydrocarbon is used domestically as an antimite.

To the best of our knowledge, the doses of the environmental mixture used in this study are largely below doses frequently used in the literature. Thus, neurobehavioral and biochemical alterations noticed might be the result of synergism or additivity in the effects of the components of this mixture. Moser et al. (2005; 2012) have also reported additivity and synergism in neurotoxicity after exposure to environmental pesticide mixtures in per weaning and adult rats. On the light of these new facts, it is concerning that the chemical legislation is based only on assessments carried out on individual substances with only an incorporation of safety factors to take account of a range of uncertainties. It becomes a necessity to reconsider methods of toxicity assessments to address risks from exposure to different microdose chemical mixtures widespread in the environment.

In the present study, chronic exposure to the POPs mixture has induced behavioural impairments and oxidative stress. We noticed a decrease in locomotor activity in OF in the groups treated with intermediate and high dose and an impairment in learning ability during acquisition sessions of MWM. In fact, previous studies reported that acute exposure to PAH in adult rats decreases locomotor activity (Saunders et al. 2006). The same thing was observed in juvenile and adult rats after postnatal acute exposure to CPF (Icenogle et al. 2004). Although chronic exposure to the same component, at low doses in adult age, did not induce any effect (Alvin et al. 2007); however, chronic exposure in adult rats to other OP like dichlorovos and malathion decreased locomotor activity (Binukumar et al. 2010; N'Go et al. 2013).

OP such CPF are known by their effect on cholinergic system, inhibiting in particular ACHE (Kobayashi et al. 1986). These effects are proven to disturb motor activity and cognitive ability (Icenogle et al. 2004) which is in accordance with the decrease in locomotor activity and learning ability noticed in groups treated with D × 10 and D × 100. However, in the group treated with the lowest dose D, we noticed a slight but significant improvement in learning ability. Ivens et al., (1998) indicated also that chronic exposure to a low dose of

Table 3 Effects of POPs mixture on anxiety assessed in the Light Dark Box

Variable Group	Control	D	D × 10	D × 100
Latency time (s)	20.25 ± 14.7	193.14 ± 107*	120.33 ± 87.23	210.71 ± 111*
Time spent in (s) the light box	49.35 ± 32.28	202.97 ± 96*	134.3 ± 90.74	231.77 ± 81*

Results are expressed as mean ± SE (*n* control = 5; *n* treated groups = 7). Bonferroni *t* test was used for multiple comparisons

**P* < 0.05 as compared to control

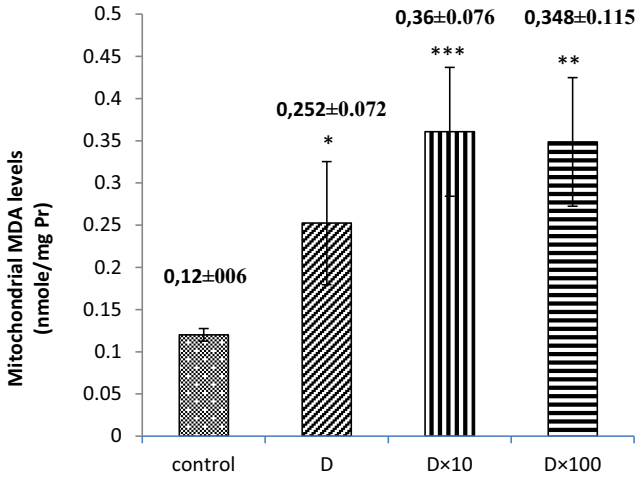


Fig. 3 Effect of the POPs mixture on whole brain mitochondrial MDA levels. Results are expressed as mean ± SE ($n = 5$). Bonferroni t test was used for multiple comparisons. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ statistical significant as compared to control

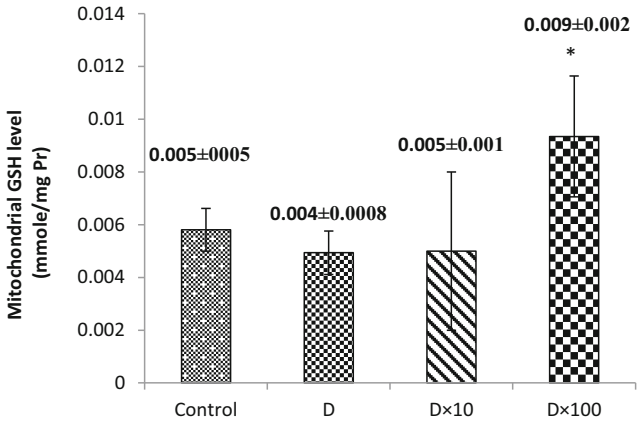
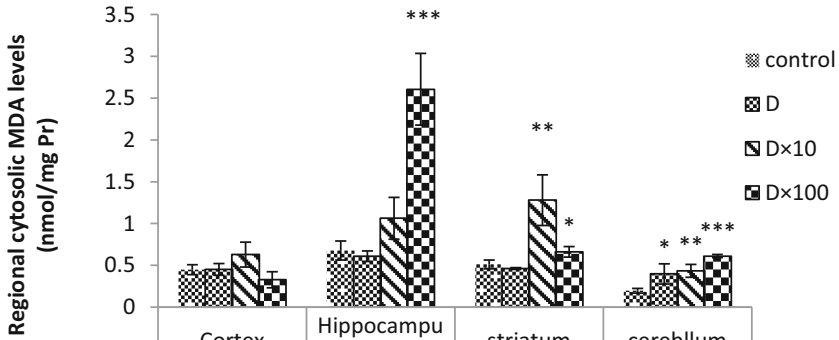


Fig. 5 Effect of the POPs mixture on the whole brain mitochondrial GSH levels. Results are expressed as mean ± SE ($n = 5$) Bonferroni t test was used for multiple comparisons. $^{*}P < 0.05$ as compared to control

parathion, another ACHE OP inhibitor, has improved performance of rats in MWM task. This improvement could be attributed to the role of ACHE in the process of learning and memory. In fact, it might be possible that a slight inhibition of ACHE could modulate motor activity, learning ability and memory (Araujo and Greig 2010; Araujo et al. 2011). Despite the improvement in learning ability, we noticed in the group treated with D impairment in memory retention during the probe test; we noticed the same impairment in the group treated with D × 100 beside the impairment in learning ability. Surprisingly, the group treated with D × 10 did not show any impairment in spatial memory retention despite the impairment in learning ability, which suggests that in the group D × 10 once learned the information is retained. Differences in MWM performance between the three groups also suggest that for the same mixture the dose is a key factor in determining the mechanism of toxicity altering memory and learning ability.

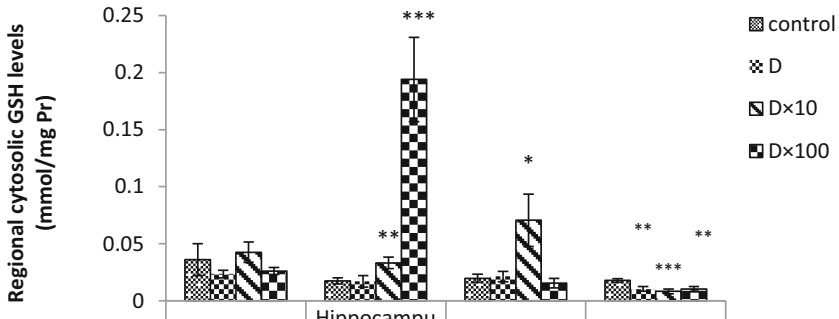
Other than ACHE inhibition, CPF is like END, could alter behaviour by disturbance of catecholamin and serotonin metabolism (Aldridge et al. 2005a; Chen et al. 2011). PAHs ingestion or inhalation was also reported to induce similar effects (Konstandi et al. 2007). Lately, deficiency in serotonin was linked to depression like behaviour induced by postnatal exposure to CPF in adult rats (Aldridge et al. 2005b). In the present study, we noticed in the LDB test an increase in the time spent in the light compartment in groups treated by D × 10 and D × 100. This could be explained by the decrease in locomotor activity noticed in the same groups; however, this possibility is unlikely since the number of transitions and attempts to enter each compartment remained unchanged. We noticed also an increase in latency time to enter the dark compartment suggesting that animals preferred to stay in the light compartment, which indicates that the low and high doses of the studied mixture have an anxiolytic like effect.

Oxidative stress is one of the main common toxicity mechanisms between POPs (Lukaszewicz-Hussain 2008).



	Cortex	Hippocampus	striatum	cerebllum
control	0.449	0.677	0.511	0.197
D	0.452	0.611	0.463	0.398
D×10	0.63	1.063	1.28	0.434
D×100	0.329	2.606	0.663	0.611

Fig. 4 Effect of the POPs mixture on regional cytosolic MDA levels. Results are expressed as mean ± SE ($n = 5$). Bonferroni t test was used for multiple comparisons.$^{***}p < 0.001$, $^{**}P < 0.01$,$^{*}P < 0.05$ statistical significant as compared to control. Only mean values appear in the tab



	Cortex	Hippocampus	striatum	cerebllum
control	0.0361	0.0174	0.0197	0.0178
D	0.0235	0.017	0.0213	0.0104
D×10	0.0425	0.0332	0.0706	0.0083
D×100	0.0261	0.194	0.0155	0.0102

Fig. 6 Effect of the POPs mixture on the cortex, hippocampus and striatum cytosolic GSH levels. Results are expressed as mean ± SE ($n = 5$) Bonferroni t test was used for multiple comparisons.***$P < 0.001$ **$P < 0.01$, *$P < 0.05$ statistical significant as compared to control. Only mean values appear in the tab

Moreover, it is strongly linked to the neurobehavioral effects induced by these compounds (Saunders et al. 2006; Bouayed et al. 2009; Rammal et al. 2010). In this study, chronic exposure to the studied mixture has induced a state of oxidative stress in both the brain cytosol and mitochondria. MDA as an end product of lipid peroxidation was increased significantly in mitochondria even in the group treated with the environmental dose, indicating that mitochondria is a privileged targets to the mixture toxicity.

In fact, it is proved by many authors that POPs could induce oxidative stress in mitochondria in so many ways, mainly by disturbance of calcium uptake (Kodavanti 2005), or by interaction with respiratory chain enzymes (Hargreaves 2012;Hatcher et al. 2004) leading finally to DNA fragmentation and apoptosis (Kaur et al. 2007). Cytosolic MDA was also highly increased in regions of the cerebellum striatum and hippocampus but not in the cortex. Lipid peroxidation in the striatum hippocampus and cerebellum after exposure to OP, OC and PAHs was also reported by Cicchetti et al. (2001); Saunders et al. (2006); Lafuente and Natividad (2013) and others.

Effect on GSH levels has also shown a regional selectivity, GSH in fact is a crucial molecule in neurons antioxidant system (Pathak and Khandelwal 2006). Depletion in its level was noticed in the brains of PD patients (Di Monte et al. 1992; Sechi et al. 1996) and reported to be implicated in the process of neurodegeneration (Franco et al. 2009). It was also noticed after exposure to the POPs (Jia and Misra 2007; Venkataraman et al. 2010; Ojha et al. 2011). In the present study, in the cerebellum, chronic exposure to the POPs mixture induced GSH depletion and MDA increase in a dose dependant manner. This could explain why motor activity impairments were induced only by exposure to D × 10 and D × 100, since this region plays a key role in movement and controls coordination. Acute exposure to the same environmental mixture D folded by 1000 increased instead GSH levels (not published data). This pattern is in agreement with the hormesis effect described in literature, indicating that acute exposure to POPs may induce a response of adaptation by increasing GSH levels, while chronic exposure to low doses fails to induce this adaptation but reduce, on the contrary, its level gradually (Lee and Jacobs 2014).

Table 4 Effect of POPs mixture on antioxidant enzymes activity

Variable Group	Control	D	D × 10	D × 100
Mitochondrial CAT activity (IU/mg Pr.)	0.0642 ± 0.017	0.135 ± 0.004	0.144 ± 0.017	0.145 ± 0.022
Cytosolic CAT activity (IU/mg Pr.)	0.881 ± 0.31	3.559 ± 0.3***	8.359 ± 0.89***	2.245 ± 0.28**
Mitochondrial SOD activity (IU/mg Pr.)	0.107 ± 0.017	1.011 ± 0.52	1.61 ± 0.24***	2.03 ± 0.35***
Cytosolic SOD activity (IU/mg Pr.)	4.83 ± 0.39	19.03 ± 0.89***	2.99 ± 0.92	13.19 ± 0.91***
Cytosolic GST activity (IU/mg Pr.)	6.09 ± 1.02	5.52 ± 0.17	8.19 ± 0.61	2.50 ± 0.81*

Results are expressed as mean ± SE ($n = 5$). Bonferroni t test was used for multiple comparisons
***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ as compared to control

In contrast to this pattern, in the hippocampus and striatum chronic exposure to the mixture has induced an increase in GSH levels; moreover, this increase was tightly correlated with the increase in MDA levels. Thus, if GSH increase was a response of adaptation, it failed to prevent lipid peroxidation noticed in these brain regions. Furthermore, GSH itself might be directly implicated in OS induction. In fact, the ability of GSH to protect against, but in some instances to mediate the toxicity of chemicals is already reported. For example, Monk and Lau (1998) indicated that GSH conjugated could be more toxic than the original xenobiotic. Furthermore, GSH was identified lately as a neuromodulator and neurotransmitter. These additional roles provide a pharmacological basis coupling alterations in GSH homeostasis to the development of certain neurodegenerative processes. Thus, chemical-induced changes in the brain GSH concentrations, like the POPs mixture in this study, may have profound consequences on brain function (Monk et al. 1999).

Besides GSH, antioxidant enzymes, GST, SOD and CAT play a crucial role in cell antioxidant system. GST catalyses the conjugation of GSH to various electrophiles, and it is already described to be a specific target to POPs (Monteiro et al. 2006). Moreover, Bassi et al. (2015) reported that benzopyrane potentiates the inhibitory effect of diazionon, an OP, on GST. In this study, a decrease in GST activity was noticed, but only in the group treated with the highest dose.

SOD dismutates O_2° to H_2O_2 and CAT transforms H_2O_2 to H_2O and O_2. Thus, any imbalance in activity between those two enzymes could alter redox homeostasis. In fact, SOD activity is reported to be much higher than CAT activity in brain, which is another reason why the brain is vulnerable to oxidative stress (Casetta et al. 2005). In the present study, chronic exposure to the POPs mixture has induced an increase in CAT and SOD activity in cytosol and mitochondria of all treated groups. However, this increase was more important in CAT than in SOD. The increase in SOD activity could be the result of an intense production of O_2° in mitochondria (Massicotte et al. 2005) probably by respiratory chain enzymes which are known to be altered by OP and OC (Hatcher et al. 2004; Kaur et al. 2007; Hargreaves 2012). Such increase leads automatically to an increase in H_2O_2 levels that could induce in turn a hyperexpression of CAT (Kale et al. 1999; Rezvanfar et al. 2010). This may explain the increased activity of CAT noticed in the present study. The works of Anupama et al. (2011) reported also an increase in brain CAT activity after exposure to a mixture of OP pesticides, while the study of Lukaszewicz-Hussain (2013) reported that the brain CAT and SOD activity have increased when animals were exposed to chlorfenvinphos, even in a dose two times smaller than LOEl (little observable effect level). On the other hand, it is well established that high levels of H_2O_2 inhibit CAT activity, which may explain the significant decrease in CAT activity noticed in the group treated with the highest dose, when compared to groups treated with D and D × 10, which could suggest that ROS production is more intense in the group treated with D × 100 and follows a dose dependent curve, which is in accordance also with MDA levels found to be higher in the hippocampus and cerebellum in the group treated with D × 100. This oxidative stress increase may explain partially severe impairments in learning abilities, memorization, anxiety and motor activity noticed in the group Dx100, unlike the group D × 10 which expressed only impairment in learning aptitude and motor activity, at the time when the group D which expressed impairment in memory retention and anxiety. In fact, several researchers have suggested that there is a potential relationship between increase of oxidative damage in the brain and disturbance of neurobehavioral abilities, such mild cognitive impairment (Grova et al. 2007; Dominico et al. 2002; Bouayed et al. 2009; Rammal et al. 2010). However, it is not clear whether oxidative stress intensity is the only mechanism responsible for these differences in behaviour or there are other implicated mechanisms. We believe that, studding farther targets, particularly those related to neurotransmission will provide a better image about the mechanisms of the toxic effect of complex environmental mixture of POPs.

Conclusion

Chronic exposure to the POPs mixture, used in this study, was able to induce neurobehavioral abnormalities and the state of oxidative stress in different brain regions of adult female rats. The results of the present study indicate that mature brains could be affected by dietary exposure to environmental POPs mixtures. Mitochondrial dysfunction and regional specific alteration of GSH homeostasis seems to be key factors in oxidative stress induction and neurobehavioral alterations. Actually, the role of alteration GSH homeostasis in oxidative stress induction remains unclear and requires more investigations. In this context, farther researches are required to understand well patterns of the brain response to dietary exposure to POPs mixture and their implications in aetiology of neurodegenerative diseases.

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