International Journal of Biomedical Science & Bioinformatics– IJBSB
Volume 2 : Issue 1 [ISSN 2475-2290]
Publication Date : 30 April, 2015

Chronic exposure of female rats to a low dose POPs mixture induced oxidative stress in brain cytosol and mitochondria.

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***Abstract*— Persistent organic pollutants (POPs) are long-lived toxic organic compounds and are of major threat for human and ecosystem health. Recently, great concerns are raised about POPs mixtures and its potential toxicity even in doses of daily human exposure. Taking in consideration that current scientific consensus states that deficits in energetic metabolism and oxidative stress are common characteristics between neurodegenerative diseases and a large range of POPs is incriminated in the pathogenesis of these diseases, it would be quite interesting to study the effects of exposure to these mixtures on brain. For that, an orally chronic exposure to a representative mixture of POPs composed of Endosulfan (2.6µg), Chlorpyrifos(5.2µg),Naphthalene(0.023µg)andBenzopyrane(0.002 µg)/kg, or the same mixture folded by 10 or 100 were tested on the oxidative stress state in different brain regions of adult female rats. Exposed rats have shown an increase in malondialdehyde (MDA) levels and an alteration in glutathione (GSH) homeostasis in both mitochondrial and regional cytosolic fractions. These effects were accompanied by a decrease in levels of cytosolic Glutathione S-Transferase (GST) and a very significant increase in levels of Superoxide Dismutase (SOD) and Catalase (CAT) in both cytosolic and mitochondrial fractions. The current study suggests that environmental exposure to low doses of POPs mixtures through diet induces oxidative stress in adult brain where mitochondria could be a privileged target. More studies are required to understand more responses patterns of brain to chronic exposure to POPs mixtures and its implication in neurodegenerative diseases' aetiology.**

***Keywords*— POPsmixtures,neurodegeneration, mitochondria, oxidative Stress, chronic exposure, adult age.**

I. *Introduction*

What make us spatial among all living creatures are our brains, however lately we are putting what making us special in danger. In fact, From 1990 to 2010, mental and behavioral 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% (1),(2). This scary increase in prevalence incidence is mostly linked to pollutions, where persistent organic pollutants (POPs) could play the main role (3),(4). Several epidemiological studies have reported the implication of POPs in aetiology of neurodevlopmantal (5), (6) and neurodegenerative diseases particularly Parkinson Disease (PD) (7),(8). *In vitro* and experimental studies support these reports. Pesticides for example are known by the 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(9), while organophosphorates (OP) like Chlorpyrifos affect cholinergic system through inhibition of acetylcholine esterase (ACHE) and muscarinic receptors (10),(11). Disturbance of serotonergic and catecholaminergic systems by POPs is also reported in literature and strongly linked to neurobehavioral effects induced by these compounds (12),(13),(14). Other than neurotransmission, endocrine disruption (15), (12) and epigenetic effects (16), (17) are also reported. Moreover, POPs including OC, OP, and Polycyclic aromatic hydrocarbons (PAHs) are reported to disturb Ca^+ homeostasis (18),(19) leading to mitochondrial dysfunction which also could be induced by alterations of the activity of respiratory chain enzymes (20),(21),(22), and due to high energetic demands of brain and it poor antioxidant system (23), any slight mitochondrial dysfunction could enhance a state of oxidative stress, furthermore, POPs are able to modulate production of 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 (24),(25),(26).

Although experimental studies have proved many mechanisms of neurotoxicity, most of its used individual compounds at high doses which represent only accidental or professional exposure. However and due to its high bioaccumulation and persistence in ecosystem and organism, we are in constant exposure POPs mixtures at low levels, mainly via food or air. And even these levels are largely under (No Observable effect level) NOEl; it seems that it could produce harmful effects that's for the most part, are unknown and unpredictable (27).

Recently great concerns are raised about neurodevlopemantal effects of mixtures of POPs in the range of daily exposure as epidemiological studies have been supported by experimental studies. (28) reported that *In Uterus* and lactational exposure to a low dose mixture of 16 PAHs has induced an increase in anxiety and a neuronal hypometabolism in exposed animals on

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adulthood, in a related study prenatal exposure to a representative POPs mixture has induced on adulthood, transcriptional changes in cholinergic system and structural genes (29), 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 (30).

Effects of exposure on adult age remains less concerning since the brain is already reached a steady state of developmental process including neurogenesis, migration, synaptogenesis, gliogenesis, and myelination (31),(32). however lately, studies on adult brain reveled it sensitivity toward exposure to environmental relevant mixtures (33),(34),(35).a preclinical study in adult rats on exposure to chemical agents from the golf ware including CPF also reported pathological changes in morphometry and synaptic integrity in different brain regions (36). In the same context (37) reported microstructural changes in the central nervous system of agricultural workers with low chronic exposure to pesticides.

In the present study, taking in consideration that oxidative stress (OS) and mitochondrial dysfunction are early alterations in neurodegenerative diseases (38),(39), and that POPs are well-known by its prooxydant effects and mitochondrial alteration we aim to evaluate the oxidative stress state might be induced after a chronic exposure in the adult age to a low dose POPs mixture consisted of tow PAHs; benzo[a]pyrene, a highly prooxydant and carcinogenic compound (14) and naphthalene, a relatively less toxic HAP and tow pesticides ;endosulfan , an OC, which is banned or restricted from almost all the parts of the world but still found in nature due to it high persistence (40),(41), and chlorpyrifos ,an OP, still in debate to be classified or not as a POP since the rate of it persistence does not meet the classification criteria of Stockholm convention, however it toxicity is well established even in doses largely under NOEL without taking in consideration the possible interactions with other chemicals present in environment (42).

II. Materials and Methods

A. Chemicals

The mixture used in this study is consisted of two pesticides (Chlorpyrifos, Endosulfan) and two PHAs (Naphthalene and α- Benzopyrane), Endosulfan (35%) and Chlorpyrifos (480%) were commercial forms. α -Benzopyrane (95%) was a gift from Dr Lahouel and Naphthalene (99.5%) was obtained from the department of chemistry.

The dose of each compound in the mixture was determined as the Estimated Daily Intake (EDI) calculated according to international guidelines (43).

Residue levels of pesticides were derived from a study on pesticides in vegetables in the region of Jijel, Algeria (data not published) while residue levels of HAPs were taken from the study of (44). Finely, the daily food consumption was taken from the study of (45**).**

Pesticides and HAPs were dissolved in corn oil and administered to rats as a one mixture. The mixture was renewed each five days. Three doses were prepared by the method of successive dilution; D×100, D×10 and D where D is consisted of Chlorpyrifos (5.2µg/Kg), Endosulfan (2, 6µg/Kg), Naphthalene (0,023µg/kg) and benzo[a]pyrene (0,002µg/kg)

B. Animals and Protocol of Exposure

20 female *Wistar rats*, weighing 200–250g, were obtained from Pasteur institute (Algeria). Upon arrival, the rats were housed, 4 per cage. Animals were maintained under a daily 12h 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 two 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 4 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 three months between 9:00 and 10:00 Pm.

C. Tissue Simples

On the 90 day 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 (46) with slights modifications. Briefly, the hemisphere was washed in cold PBS, PH 7.4 (50 mM Ttis-HCl, 250 mM sucrose, 1 mM Methyl diamine tetra-acetic acid (EDTA), 0.2% BSA) than chopped and homogenised in 3 volumes of the same buffer and centrifuged at 3500g for 10 min, than the pellet was recentrefuged in same conditions. Supernatants from the tow centrifugations were mixed and centrifuged at 15000 g for 20 min. The supernatants were considered as cytosolic fraction and conserved at -20C° 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) PH, 7.4 at the same conditions, resultants mitochondrial pellets were suspended in 300 µl of PB buffer and frozen at -20 C° until it 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 Left hemispheres were dissected immediately after sacrifice to the four regions (striatum, hippocampus, cortex and cerebellum).Then tissues were homogenised in 3 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.

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D. *Biochemical Analysis*

Protein content was determined by the Bradford method (47) using bovine serum albumin as standard. SOD, CAT, and GST activities were determined according to methods described by (48), (49), (50) respectively. Finally GSH levels were assessed according to (51) and MDA levels, according to (52).

E. *Statistical Analysis*

Data were analysed using a one-way analysis of variance (one-way ANOVA).Post hoc comparisons have been performed using the Bonferroni's t test when ANOVA was significant. And 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.

III. Results

A. *MDA Levels*

MDA levels as an indicator on lipid peroxidation have shown a significant increase in whole brain mitochondria of all treated groups **(Figure 1)**. in Cerebellum, MDA also showed a highly dose dependant increase in all treated groups, however in hippocampus bonferroni t test revealed a significant increase only in the group treated with the highest dose D×100 while in striatum the increase was significant in both groups treated with D×100 and D×10. In cortex the studied mixture seams has no effect on lipid peroxydation since MDA levels were normal in all treated groups compared to control as revealed by one way ANOVA **(Figure 2)**.

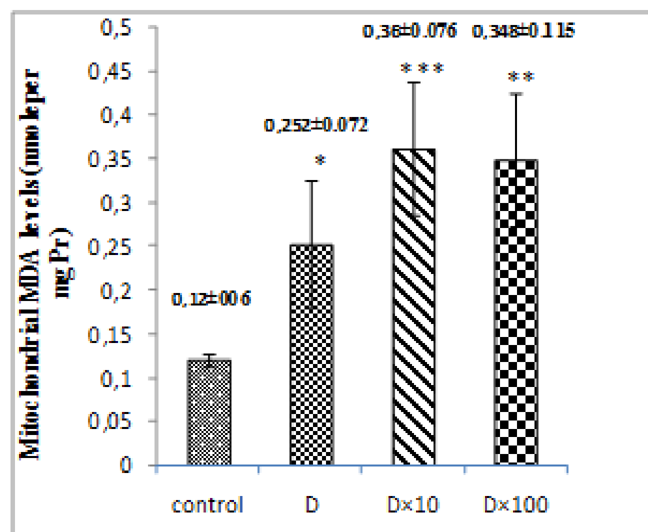

Figure 1. Effect of the POPs mixture on whole brain mitochondrial MDA levels.
*Results are expressed as mean±SE (n=5). Bonferroni t-test was used for multiple comparisons. ***p<0.001, **P <0.01,*P<0.05 statistical significant as compared to control.*

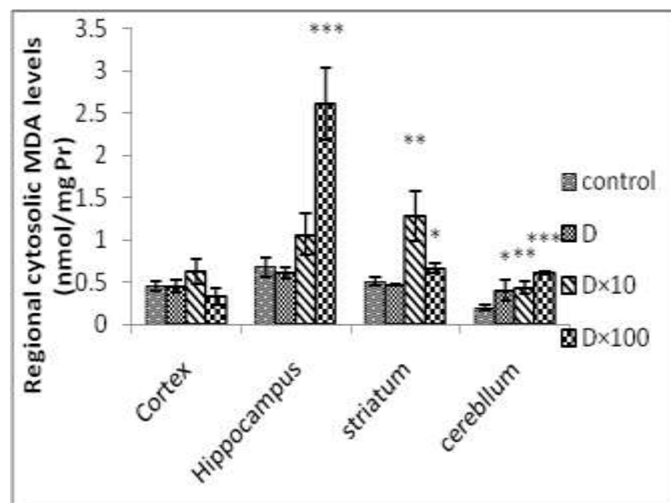

Figure 2. Effect of the POPs mixture on regional cytosolic MDAlevels.
*Results are expressed as mean±SE (n=5). Bonferroni t-test was used for multiple comparisons. ***p<0.001, **P <0.01,*P<0.05 statistical significant as compared to control.*

B. *GSH Levels*

GSH in whole brain Mitochondria and striatum has shown an increase in all treated groups however bonferroni t test revealed that this increase in whole brain mitochondria was significant only in the group treated with the highest dose D×100 **(Figure3)** while in striatum the significant increase was noticed in the group treated with the intermediate dose D×10. In hippocampus GSH has shown a dose dependant increase, and in contrary to striatum, bonferroni 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 **(Figure4)**.
In other hand GSH and MDA levels seems to be correlated in hippocampus and striatum, where Pearson test revealed a strong positive correlation ($r=0.88$, $P \approx 0$).

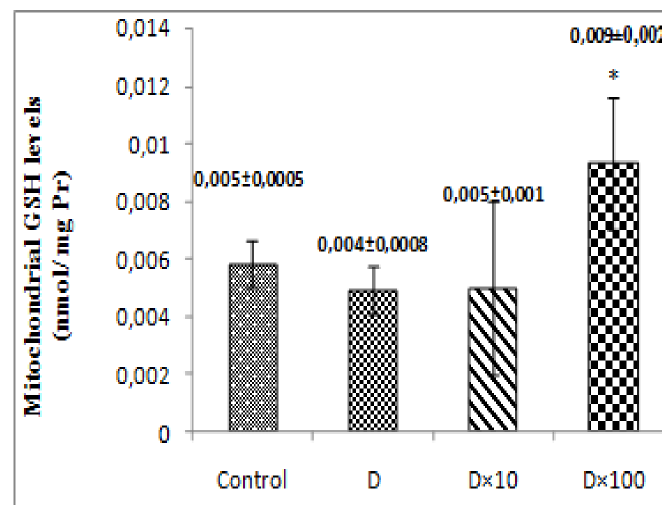

Figure 3. Effect of the POPs mixture on whole brain mitochondrial GSH levels.
*Results are expressed as mean±SE. (n=5) Bonferroni t-test was used for multiple comparisons. *P<0.05 as compared to control*

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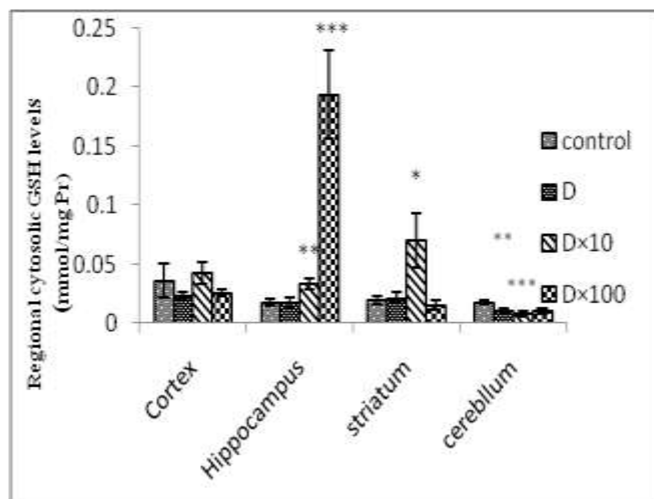

Figure 4. Effect of the POPs mixture on cortex, hippocampus and striatum cytosolic GSH levels.

*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.*

In contrast to 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).

C. *Antioxidant Enzymes Activity*

Levels of antioxidant enzymes activity are presented in **(table 1)**. An increase in whole brain mitochondrial CAT activity was noticed in all treated groups however this increase was statistically significant only in groups treated with the highest and intermediate dose. Whereas in cytosol Bonferroni 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 for the group treated with the lowest dose D where the increase was not statistically significant as revealed by bonferroni t 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 t test.

GST activity 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.

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TABLE 1. EFFECT OF POPs MIXTRE ON ANTIOXIDANT ENZYMES ACTIVITY.

Variables	Groups			
	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.*

IV. Discussion

Oxidative stress is one of the main common toxicity mechanisms between POPs (53); moreover it is strongly linked to the neurobehavioral effects induced by these compounds (14),(54),(55). In this study chronic exposure to the studied mixture has induced a state of oxidative stress in mitochondria and cytosol of different brain regions. MDA as an end product of lipid peroxidation was increased in brain mitochondria of all treated groups, what indicates that mitochondria was a privileged target to the effect of the studied mixture since even the environmental dose was able to induce lipid peroxidation. In fact it is proved by many that POPs could induce oxidative stress in mitochondria in so many ways, mainly by disturbance of calcium up take (56), or by the interaction with respiratory chain enzymes (20),(21),(22). (57) reported that chronic exposure to a low dose of dichlorovos, an OP, has induced an increase in mitochondrial Ca^{++} uptake and a decrease in cytochrome oxydase activity along with altered mitochondrial complex I, and complex II activity, what led to an increase in lipid peroxydation and protein and ADNmt oxidation, a release of cytochrome C from mitochondria to cytosol and activation of caspase cascade leading finally to DNA fragmentation and apoptosis. (28) also reported an alteration in cytochrome oxidase activity in different brain regions in adult male rats after prenatal and postnatal exposure to an environmental mixture of PHAs. These findings correlate with findings in the present study and epidemiological studies indicating that mitochondrial dysfunction is an early event in neurotoxicity of low POPs exposure that leads to PD.

Cytosolic MDA was also highly increased in regions of cerebellum, striatum and hippocampus but not in cortex. Lipid peroxidation in striatum, hippocampus and cerebellum after exposure to OP ,OC and HAP was also reported by (58),(59),

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(14), and others. In contrast, it was also reported that acute exposure to Malathion, an OP in adult rats induced lipid peroxydation selectively in cortex (60),(61),(62). This Regional selectivity could be explained by the difference of neuronal population and neurotransmission circuits in deferent brain regions.

In this study regional GSH levels have also shown a regional selectivity, GSH in fact is a crucial molecule in neurons antioxidant system. Depletion in it levels was noticed in brains of PD patients (63),(64) and reported by many to be implicated in the process of neurodegeneration (65). It was also noticed after exposure to POPs (66), (67),(53). In cerebellum in the present study chronic exposure to the POPs mixture induced GSH depletion and MDA increase. 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 fail to induce such an adaptation and reduce it levels gradually (68).

In contrast to this pattern, in hippocampus and striatum chronic exposure to the POPs mixture in this study has induced an increase in GSH levels, moreover this increase was tightly correlated with the increase in MDA levels noticed in those regions. Thus, if GSH increase was a response of adaptation, it failed to prevent lipid peroxydation noticed in those regions. Furthermore GSH might be directly implicated in OS induction. In fact the ability of GSH to protect against, and in some instances to mediate, the toxicity of chemicals is well established. (69) and others indicated that GSH conjugates could be more toxic than the original xenobiotic. Lately other roles are identified for GSH like neuromodulation and neurotransmission, moreover, it is reported to interact with metabolism of neurotransmitters like serotonin and dopamine. These additional roles of GSH provide a pharmacological basis coupling alterations in GSH homeostasis to the development of certain neurodegenerative processes. Thus, chemical-induced changes in brain GSH concentrations like the POPs mixture in this study may have profound consequences. The challenge will be to distinguish between the direct effects caused by chemical exposure and the secondary effects arising from changes in GSH concentrations. (70).

Besides GSH, antioxidant enzymes, SOD and CAT play a crucial role in cell antioxidant system. Regarding the SOD activity in dismutation of O° to H_2O_2 and CAT activity that transform H_2O_2 to H_2O and O_2 , any imbalance in activity of those enzymes could alter redox homeostasis. In fact SOD activity is reported to be much higher than CAT activity in brain, which is another reason of it vulnerability to OS (71).

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° in mitochondria (72) probably via respiratory chain enzymes known to be altered by OP and OC (57), (20). Such increase leads automatically to an increase in H_2O_2 levels that induce **in** it turn an hyperexpression of CAT (73), (74). This could explain the increased activity of Cat noticed in this study.(75) also reported an increase in brain CAT activity after exposure to a mixture of OP while (76) reported that brain CAT and SOD activity could increase due to exposure to chlorfenvinphos even in a dose two times smaller than the LOEl (little observable effect level)

In other hand, it is well established that high levels of H_2O_2 inhibit CAT activity, this may explain the significant decrease in CAT activity noticed in the group treated with the highest dose D×100 compared to groups treated with D and D×10 where ROS production may be less intense.

GST catalyses the conjugation of GSH to various electrophiles, and it is already described to be a specific target to OP (77), moreover (78) reported in his study that benzo[a]pyrene could potentiate the inhibitory effect of diazionon, an OP on GST activity which could explain in a part the decrease in it activity noticed in this study. However, this decrease was significant only in the group treated with the highest dose, unlike activities of CAT and SOD that were more sensible and affected even in the group treated with the environmental dose mixture.

V. Conclusion

Chronic exposure of female rats in adult age to the POP mixture used in this study was able to induce an of oxidative stress state in different brain regions. What indicates that not only brain in development but also mature brain could be affected by dietary exposure to environmental POPs mixtures. Mitochondrial dysfunction and regional specific alteration of GSH homeostasis seem to be key factors in the OS induction. However the role of GSH homeostasis alteration in OS induction remains unclear and requires more investigations. In this context, farther researches are required, to understand well patterns of brain response to dietary exposure to POPs mixtures and it implication in neurodegenerative diseases.

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