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Impact of persistent organic pollutants on the brain: implication in the aetiology of neurodegenerative diseases.

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بِسْمِ اللهِ الرَّحْمنِ الرَّحِيمِ

(إِنَّا كُلَّ شَيْءٍ خَلَقْنَاهُ بِقَدَرٍ) [القمر:49]

"Verily, all things have We created in proportion and measure."

(Surah Al-Qamar, Ayah 49)

(وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا) [الإسراء: 85].

"And of knowledge, you (mankind) have been given only a little."

(Surah Al-Isra , Ayah 85)

Dedication

In memory of my grandfather, the exemplar man in our family.

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Abbreviation list

- (AD) Alzheimer's disease
- (ADHD) Attention deficit hyperactivity disorder
- (APOE) Alipoliprotein gene
- (APP) Amyloid precursor protein
- (Aβ) βAmyloid
- (CAT) Catalase
- (CDK5) Cyclin-dependent kinase 5
- (CP) Chlorpyrifos
- (DEE) Dichlorodiphenyldichloroethylene
- (END) Endosufan
- (GABA) γ-amino-butyric acid
- (GSH) Glutathione
- (GSK3) Glycogen synthase kinase 3
- (hAPP) Human amyloid precursor protein
-(Hfd) high fat diet
-(Hfdp) high fat diet + PFOS
- (IDE) Insulin degrading enzyme
- (LDB) light dark box test
-(Lfd) low fat diet
-(Lfdp) low fat diet + PFOS
- (LTP) Long term potentiation
- (MAPT) Microtubule-associated protein tau
- (MWM) Morris water maze test
- (OCs) Organochlorine pesticides
- (OF) Open field test

- (OP) Organophosphorous pesticides
- (OS) Oxidative stress
- (PCB Polychlorinated biphenyls
- (PD) Parkinson's disease
- (PD) Post natal day
- (PD) Postnatalnatal day
- (PFCs) Perfluorinated chemicals
- (PFOS) Perfluorooctane sulfonic acid
-(PKC) Protein kinase C
- (POPs) Persistent organic pollutants
- (PPARγ) Preoxisom prolifirator activated receptor
- (ROS) Reactive oxygen species
- (SOD) Superoxide dismutase
- (TCDD) Tetrachlorodibenzodioxin
- (VGCCs) Voltage gated calcium channels

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Abstract

Persistent organic pollutants (POPs) are long-lived organic compounds that are considered one of the major risks on ecosystem and human health. Mainly, POPs are known by induction of oxidative stress and mitochondrial dysfunction as well as disturbance of metabolism and endocrine disruption.

Recently, great concerns have been raised about environmental exposure to POPs mixtures and its potential toxicity even that doses of exposure are largely below the estimated acceptable daily intake (ADI). Neurotoxicity of POPs is one of the major concerns, since brain is mostly targeted by these lipophilic compounds because of its important contain in lipids and iron and its deficient antioxidant system. Neurodevelopmental toxicity is particularly considered, because POPs are able to cross placenta and breast milk, thus might interact with processes of neurodevelopment and maturation.

Starting from these concepts, the present work aims to evaluate the neurotoxic effect of POPs at environmental doses from the perspective of three scenarios of exposure in rodents: exposure to an environmental POPs mixture on adulthood, neurodevelopmental exposure to an environmental POPs mixture and the possible exposure to neurotoxic effects of POPs through the axis of a peripheral organ like the liver.

Chronic exposure to a representative environmental mixture of POPs composed of endosulfan (2.6 µg/kg), chlorpyrifos (5.2 µg/kg), naphthalene (0.023 µg/kg) and benzopyrene (0.002 µg/kg), induced in adult female rats memory and locomotor deficits as shown by behavioural tests like Morris water maze and the open field tests. Same exposure induced oxidative stress in mitochondria and cytosol as shown by an increase in lipid peroxidation and a perturbation in GSH levels and antioxidant enzymes activity. Gestational and lactational exposure to the same mixture induced neurodevelopment deficits as shown by a decrease in body weight and the delay in the maturation of some primitive reflexes. In juvenile rats we noticed also locomotor and behavioural deficits as noted by the novel object recognition tests. We noticed as well a state of oxidative stress in both cytosol and mitochondria.

In another context, perfluoroctane sulfonate (PFOS) is a neurotoxic and hepatotoxic POPs. Recent reports revealed its potential modulation of Alzheimer disease (AD) biomarkers; however molecular mechanisms are still poorly understood. In the actual study chronic exposure to this compound (0, 0063 %) in adult mice induced a perturbation in Glycogen synthase kinase (GSK3B) and insulin degrading enzyme (IDE) levels in the brain as noticed by Western blot analysis. We noticed also a perturbation in other AD biomarkers like B Amyloid (AB) and Amyloid precursor protein (APP), total tau protein and it phosphorylated form. These effects are related to perturbation in lipid and glucose metabolism as shown by lipid and glucose blood concentration registered in the same cohort study.

As a conclusion we have shown that environmental exposure to POPs during development or in adult age could alter brain integrity. Such alterations might pave the way to the development of neurodegenerative diseases in the aging brain. In this context, further researches are required, to understand well patterns of brain response to environmental exposure to POPs and its implications in the aetiology of neurodegenerative diseases.

Keywords: *POPs, Neurotoxicity, Oxidative stress, Neurodevelopment, Behaviour, Liver axis.*

Resumé

Les polluants organiques persistants (POP) sont des composés organiques à vie longue qui sont considérés comme l'un des principaux risques sur la santé de l'écosystème et de l'être humain. Principalement, les POPs sont connus par l'induction du stress oxydatif et le dysfonctionnement mitochondrial ainsi que la perturbation métabolique et endocrinienne.

Récemment, de grandes préoccupations ont été soulevées à la toxicité potentielle de l'exposition environnementale aux mélanges de POPs, même si les doses d'exposition sont largement inférieures aux estimations des doses journalières admissibles (DJA). La neurotoxicité des POPs est une des principales préoccupations, puisque le cerveau est une cible privilégiée de ces composés lipophiles en raison de sa richesse en lipides et fer et son système antioxydant déficient. La toxicité neurodéveloppementale est particulièrement considérée, car les POPs sont capables de traverser le placenta et le lait maternel, ce qui pourrait interagir avec les processus du neurodéveloppement et de maturation.

A partir de ces concepts, la présente étude a pour but d'évaluer l'effet neurotoxique des POPs à des doses environnementales dans la perspective de trois scénarios d'exposition chez les rongeurs: L'exposition à un mélange de POPs à l'âge adulte, l'exposition neuro- développementale à un mélange de POPs et l'exposition possible aux effets neurotoxiques des POPs via l'axe d'un organe périphérique comme le foie.

L'exposition chronique à un mélange représentatif de POPs composé d'endosulfan (2,6 µg / kg), de chlorpyrifos (5,2 µg / kg), de naphtalène (0,023 µg / kg) et de benzopyrene (0,002 µg / kg) a induit des déficits de locomotion et mémorisation chez les rats femelles adultes selon les résultats obtenus par les tests comportementaux comme le labyrinthe d'eau de Morris et l'essai de champ libre. La même exposition a induit un état de stress oxydatif dans les mitochondries et le cytosol, indiqué par une augmentation de la peroxydation lipidique et une perturbation des taux de GSH et de l'activité des enzymes antioxydantes. L'exposition gestationnelle et lactationnelle au même mélange a induit des déficits neurodéveloppementaux, indiqués par la diminution du poids corporel et le retard dans la maturation de certains réflexes primitifs. Chez les rats juvéniles, nous avons également noté des déficits de comportement et de locomotion, selon les résultats obtenus par les tests des compartiments successifs et le test de reconnaissance de nouveaux objets. Nous avons aussi remarqué un état de stress oxydatif dans le cytosol et la mitochondrie.

Dans un autre contexte, le perfluoroctane sulfonate (PFOS) est un POP neurotoxique et également hépatotoxique. Des rapports récents ont révélé sa modulation potentielle des biomarqueurs de la maladie d'Alzheimer (AD); Cependant les mécanismes moléculaires sont encore mal compris. Dans l'étude actuelle, une exposition chronique à ce composé (0, 0063%) chez des souris adultes a induit une perturbation dans le métabolisme des lipides et du glucose, comme le montrent la concentration sanguine en lipides et en glucose. Ces effets étaient liés à une perturbation de la glycogène synthase kinase3 (GSK3β) et des niveaux d'enzyme dégradant l'insuline (IDE) dans le cerveau, selon l'essai de western blot. Nous avons également remarqué une perturbation dans d'autres boimarqueurs d'AD comme la protéine amyloïde B et la protéine précurseur d'amyloïde (APP), la protéine tau et ses isoformes phosphorylées.

En conclusion, nous avons montré que l'exposition environnementale aux POPs pendant le développement ou à l'âge adulte pouvait altérer l'intégrité du cerveau. De telles altérations pourraient ouvrir la voie au développement de maladies neurodégénératives dans le cerveau au cours du vieillissement. Dans ce contexte, des recherches plus approfondies sont nécessaires pour comprendre les modèles de réponse cérébrale à l'exposition environnementale aux POPs et leurs implications dans l'étiologie des maladies neurodégénératives.

***Mots clés**: POP, Neurotoxicité, Stress oxydatif, Neurodéveloppement, Comportement, Axe hépatique.*

ملخص

الملوثات العضوية الثابتة هي مركبات عضوية طويلة الأجل ,تعتبر واحدة من أهم المخاطر على النظام البيئي وصحة الإنسان. أساسا تعرف الملوثات العضوية الثابتة بالتسبب بالاجهاد التأكسدي واضعاف وظيفة الميتوكوندريا، وكذلك الاخلال بالأيض الغذائي والاختلالات الهرمونية.

في الآونة الأخيرة أثيرت مخاوف كبيرة حول التعرض لخليط هذه المكونات واثارها المحتملة علي صحة الانسان علي الرغم من أن جرعات التعرض منخفضة تماما عن جرعات التعرض اليومي المسموح بها. أخذت السمية العصبية بعين خاصة من الاعتبار لأن الدماغ مستهدف خاصة من هذه المكونات كونه يحتوي علي مستويات عالية من الدهون و الحديد, كما أن نظامه ضد التأكسدي يعتبر ضعيفا نسبيا. الملوثات العضوية الثابتة قادرة ايضا على عبور المشيمة و حليب الأم مما يجعلها قادرة على التأثير على مراحل نمو و نضج الجهاز العصبي.

انطلاقا من هذه المفاهيم, تهدف الدراسة التالية الى تقييم اثار التعرض لجرعات بيئية من الملوثات العضوية على الجهاز العصبي من منظور ثلاثة سيناريوهات للتعرض عند القوارض: التعرض لخليط بيئي من الملوثات العضوية الثابتة بعد مرحلة البلوغ و نضج الدماغ. التعرض لخليط بيئي من الملوثات العضوية الثابتة خلال مرحلة نمو و تطور الجهاز العصبي, اضافة الى ذالك, تقييم احتمال تعرض الدماغ لأثار الملوثات العضوية الثابتة من خلال التأثير على أجهزة محيطية, مثل الكبد.

التعرض المستمرلخليط بيئي من الملوثات العضوية الثابتة يتكون الإندوسلفان (2.6 ميكروجرام/ كغ)، الكلوربيريفوس (5.2 ميكروجرام/ كغ)، النفثالين (0.023ميكروجرام/ كلغ وبنزوبيران (0.002ميكروجرام / كغ)اثر في الحركة والذاكرة عند الفئران الإناث البالغات وفقا لنتائج الاختبارات السلوكية مثل متاهة موريس المائية و الحقل المفتوح. لاحظنا أيضا وجود حالة من الإجهاد التأكسدي في العصارة الخلوية والميتوكوندريا كما أشارت إليه الزيادة في أكسدة الدهون و تأثير على معدل تركيز ونشاط الأنزيمات المضادة للأكسدة. التعرض لنفس لخليط اثناء الحمل والرضاعة اثر على النمو العصبي كما اشار اليه انخفاض وزن الجسم وتأخير نضج بعض ردود الفعل. عند الفئران الأحداث، لاحظنا أيضا عجزا في الحركة والتعلم والذاكرة وفقا لنتائج التي تم الحصول عليها من طريق اختبار الحقل المفتوح واختبار القدرة على التعرف على الاشياء الجديدة. لاحظنا ايضا وجود حالة من الإجهاد التأكسدي في العصارة الخلوية و المتكوندريا.

في سياق آخر، سلفونات فلورو أوكتان المشبع هو من الملوثات العضوية الثابتة ذات سمية عالية عل الأعصاب و الكبد. كشفت دراسات حديثة عن امكانية تأثير هذا المركب تحديدا على اليات اساسية في مرض الزهايمر الا أن الاليات الجزئية لهذه التأثيرات لا تزال مبهمة. في الدراسة الحالية, التعرض المزمن لهذا المركب (0,0063%) عند الفئران أدى الى حدوث اضطراب في مستويات انزيمات تعتبر مؤشرات حيوية هامة لمرض الزهايمر مثل الانزيم المحلل الانسلين و بروتينات Tau/pTau, GSK3β, CDK5, APP

ويبدو أن هذه الاثار متعلقة باضطراب في ايض الدهون و الجلوكوز كما اتضح في تغير تراكيز هذه الأخيرة في دم فئران نفس مجموعة الدراسة الحالية

في الختام، لقد أظهرنا أن التعرض لجرعات منخفضة من الملوثات العضوية الثابتة سواءا عند مرحلة النمو العصبي او بعد البلوغ قادر على احداث تغيرات في الدماغ التي قد تكون أصل المبكرة لحدوث تنكس عصبي في مراحل لاحقة من الحياة .في هذا السياق هنالك حاجة إلى مزيد من البحوث لفهم أفضل لأنماط استجابة الدماغ للتعرض للملوثات العضوية الثابتة على جرعات منخفضة والية في مسببات أمراض التلفه و مدى مساهمة هذه الاخيرة العصبي .

الكلمات المفتاحية: الملوثات العضوية الثابتة, السمية العصبية, الاجهاد التأكسدي, النمو العصبي, محور الكبد,

Introduction

In modern life, we are no longer struggling to achieve the simple daily tasks. But on the other side, we are struggling to protect our health. Diseases like diabetes, cancer, cardiovascular and neurodegenerative diseases are becoming almost epidemic.

The prevalence of neurodevelopmental and neurodegenerative diseases has increased dramatically. 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). Genetic factor alone could not explain these increases, suggesting involvement of environmental factors. Among these factors, pollution is strongly incriminated and persistent organic pollutants (POPs) are likely to play a critical role.

POPs are compounds that are not only toxic, but also resist to degradation and accumulate in ecosystem and the living organism, what makes us daily exposed to mixtures of these compounds mainly through air and food (WHO 2010). And even if the doses of exposure are largely below estimated toxic levels, it is suspected that this kind of exposure might be implicated in aetiologies of neurodegenerative diseases (Zeliger 2011). However, neither the mechanisms by which POPs mixtures induce neurotoxicity nor mechanisms of interaction between different compounds of mixtures are well understood. Exposure could start at an early stage of life; from embryonic life since these compounds could pass by placenta and also by breast milk, what suggests an interaction with neurodevelopmental processes like neurogenesis, synaptogenesis, gliogenesis, and myelination (Garman 2001). Furthermore, many experimental studies suggest that effects of prenatal and postnatal exposure often do not appear until on adulthood (Gill et al. 2013). Recent studies indicate that exposure in adult age independently on prenatal or postnatal exposure to mixtures of POPs at low doses could also alter brain integrity, however and like in developmental exposure, mechanisms of toxicity are not well understood. In general, induction of oxidative stress, disturbance of mitochondrial function, calcium homeostasis and cell cycle (apoptosis and cell proliferation) are all common mechanisms of toxicity between POPs (Meijer et al. 2014; Westernik et al. 2014; Federico et al. 2012). Interestingly, oxidative stress and mitochondrial dysfunction are also alterations that appear early in pathogenesis of neurodegenerative diseases like Alzheimer’s Disease (AD) and Parkinson Disease (PD) thus, supporting studies linking environmental exposure to POPs mixtures to the aetiology of these diseases.

POPs are also known to disturb glucose and lipid metabolism and could be implicated as well in insulin resistance by affecting mainly insulin signalling pathways, and enzymes involved in glycolysis and TCA cycle. In fact, epidemiological studies have already established an association between POPs exposure and diabetes (Lee et al. 2015). Interestingly, deficiency in glucose metabolism and insulin signalling became common hallmarks of neurodegenerative diseases (Steen et al. 2005; Jordan et al. 2013). Furthermore, epidemiological studies revealed that diabetes is a major risk factor for AD (Li et al. 2015).

Epidemiological studies also have succeeded to establish an association between neurodegenerative diseases and POPs blood levels (Singh et al. 2013), as well as association between developmental exposure to POPs and behavioural disturbances in children (Berghuis et al. 2015). In fact, some of these studies have revealed neurological effects such as decreased IQ, and delayed reading ability at levels about a thousand times lower than those that were considered "levels of concern" in the near past (Solomon et al. 2002). Today, the question of "*Is there any safe threshold for POPs exposure?*" is raising strong concerns in the scientific community. Yet, on the experimental level, studies that evaluate neurotoxicity of POPs at environmental (background) levels are few, and the involved mechanisms are still poorly understood.

In the present study, we aimed to evaluate the potential effects of low dose POPs exposure on the brain. Taking in consideration that both adult brain and developing brain are exposed to POPs and more, POPs might alter brain integrity through the axis of peripheral organs like liver, than taking in consideration that oxidative stress and metabolism disturbance are principal mechanisms of POPs toxicity and deeply implicated in the pathogenesis of neurodegenerative diseases, we have chosen to evaluate:

- Neurobehavioral deficits and brain oxidative stress induced by chronic low dose exposure to persistent organic pollutants mixture in adult female rats.

- Neurobehavioral deficits and brain oxidative stress induced by developmental exposure to a low dose mixture of persistent organic pollutants.

- Neurotoxicity induced by Perfloroctane sulfonate (POFS); a disruptor of metabolism and liver function.

Chapter I

Neurotoxicity of persistent organic pollutants (POPs): Implication of oxidative stress as a major mechanism of toxicity

1. Introduction to persistent organic pollutants (POPs)

Persistent organic pollutants (POPs) are organic compounds characterised mainly by high resistance to degradation. Beside the resistance to degradation, POPs share the characteristic of bioaccumulation due to their high lipophilicity. They have also, a large range transport due to their high volatility. But most of all, POPs are extremely toxic to the environment and living organism (WHO 2010). Following these characteristics many substances are classified as POPs. The most credited classification is that of Stockholm convention. It was adopted in May 2001 with the aim of protecting human health and the environment from POPs, targeting at first an initial of 12 POPs. Most of these substances were organochlorine pesticides (OCs); the others were industrial chemicals like polychlorinated biphenyls (PCB), or unintended by-products such as Hexachlorobenzene. In May 2009, governments agreed to list 9 additional substances including the pesticide endosufan as POP under the Convention. Stockholm convention is under continuous updating. Today, four substances are under review to be added to the list, including Dicofol, an OC (Stockholm Convention website 2016).

Several other regional and global conventions established different classifications for POPs. For example some references consider bisphenol A and all the group of PAH as POPs (WHO 2008; Mai et al. 2003). There is also a debate to include or not some organophosphorous pesticides such as chlorpyrifos under the list of POPs (Giesy et al. 2014a,b). The actual difference in the classification of POPs is due to the non-harmonised criteria for evaluating persistence, bioaccumulation, toxicity and long range transport characteristics of substances. However, the overall and most important goal of organisations and governments is to take a global action to protect human health and environment (WHO 2010).

Several POPs were banned from use decades ago due to their high toxicity. For example PCBs were banned from use in USA in 1979 and in Europe in 1989. However, some substances are still in use in a wide variety of industrial and consumer products, like perfluorinated chemicals (PFCs), used in textile industry and food packing (NIH 2016). There is also the non- authorised use of some pesticides, for example endosulfan is still in use in Jijel even that it was banned from commercialisation in Algeria since 2006 (CNTPP 2011).

The high persistence and the non-authorised use of POPs make us constantly exposed to these substances, via air and water, but in a much higher degree via food (WHO 2010). Children are more exposed to these compounds from playgrounds, soil and dust, also, due to their high consumption of vegetables and fruits. Breast milk remains the major dietary route of infants' exposure. POPs could pass to the breast milk; in fact maternal milk is an important route of the excretion of POPs from the body. POPs levels in breast milk are much higher than the usual levels in food due the effect of bioaccumulation, making infants exposed to high concentrations of POPs during a critical period of their development. According to WHO (2006), the daily intake of POPs for breast fed infants is between 1 and 2 orders of magnitude higher than adults. A critical window of exposure can also occur in *uterus* during pregnancy when maternal fat stores are mobilized resulting in transfer of toxicants to the embryo and to the foetus through the placenta (Zeliger 2011).

WHO has estimated the acceptable daily intake for each compound of POPs according to it toxicity. In their estimations, official organisations like WHO take in consideration the potential developmental toxicity and special susceptibility of children as a factor of risk. The limited daily intake of PCBs and endosulfan for example is 6µg/Kg (ATSDR 2000; 2016). The effects of POPs are usually studied for one chemical, or a group of chemicals, at a time and still ignore possible effects of chemical interactions. Toxicology of mixtures has attracted attention in the past few years with the new findings that exposure to multiple contaminants found in the environment at micro-doses could produce increased adverse effects by synergism or even induce different or totally adverse effects (Zeliger 2011).

POPs are toxic at high levels as illustrated by mass poisoning incidents over the time; exposure to high levels of POPS may cause adverse health effects including death, disease, and birth defects among humans and animals. Specific effects can include cancer, allergies and hypersensitivity, damage to the central and peripheral nervous systems, reproductive disorders and disruption of the immune system (WHO 2010).

What remains less clear is whether significant adverse health effects can occur from background levels. In fact, there is a public concern about the possible effect of daily chronic low exposure to POPs mixtures basing on the new insights of epidemiological and experimental studies, linking low exposure to POPs to some disease outcomes like diabetes, obesity (Lee et al. 2006; De Tata et al. 2014) and neurodegenerative diseases, particularly, Alzheimer's (AD) and Parkinson diseases (PD).

There is also a big concern about possible effects of low levels POPs exposure on the developing *foetus*, infants and children and particularly possible effects on the developing nervous system and it implication in developmental basis of neurodegenerative diseases. In the next paragraph we will discuss epidemiological studies linking POPs exposure to the aetiology of neurodegenerative diseases.

2. Epidemiological studies

Many epidemiological studies have contributed to reinforce the positive relation between exposure to POPs and the aetiology of neurodegenerative diseases. In the most recent study plasma concentrations of OCs pesticides was linked with a threefold increase in dementia in a general population (Lee et al. 2016). In a previous study, OCs serum concentration was found to be associated with the increase risk of low cognition in an elderly US population (Lee et al. 2014). In a study conducted on 89 AD patients, Richardson and collaborators (2014) reported a high serum concentration of dichlorodiphenyldichloroethylene (DEE) in AD patients compared to control participants. In the same context a case control study reported high serum concentration of DEE in 70 patients of AD from north Indian population (Singh et al. 2013). Another study on concentrations of 15 OCs in the serum of 40 PD patients reported a high level of β-hexachlorocyclohexane (β-HCH) compared to control (Richardson et al.2009; 2014). Previous studies have also reported high concentrations of OCs in the brain tissue of PD patients (Fleming et al. 1994; Corrigan et al. 2000). Other than OCs, PCB are associated with PD aetiology, in the study of Martin and collaborators (2012), PCB congeners 153 and 180 were found to be significantly elevated in post-mortem brain tissue of PD patients relative to controls. In the same context, Steenland et al. (2006), an increased risk of PD in a cohort of highly PCB-exposed female workers.

Even that a direct relation between exposure to other POPs and the neurodegenerative diseases is not established yet, epidemiological studies indicate that exposure to these compounds induce many neurological disorders. For example, high blood concentrations of perfluoroalkyl acids (PFC) were found to be related to memory impairments (Gallo et al. 2013). Occupational exposure to Benzopyrene has been reported to induce neurobehavioral function and neurotransmitter alterations in coke oven workers according to the study of Qiu and collaborators (2012).

More interestingly, there is a strong positive link between exposure to POPs and neurodevelopmental deficits according to several epidemiological studies from all around the

world (Berghuis et al. 2015). For example, exposure of pregnant mothers to OCs has resulted in an impaired neurodevelopment and postnatal neuropsychological defects, including poor cognitive development, impaired motor functions, inattention and altered activity, and autism, as well as an increased risk of major chronic diseases such as cancers and endocrine system dysfunction later in life (Sagiv et al. 2009; Stillerman et al. 2008). Roberts and collaborators (2007) reported an increased risk of autism among children whose mothers lived near sites where OCs, particularly endosulfan had been applied to fields in California during gestation. In a cohort birth study included children of farm working mothers, high blood levels of OCs in the mother was inversely associated with psychomotor and mental development of the children between the age of 6 and 24 months (Eskenazi et al. 2006). Same effects were induced by perinatal exposure to DEE (Bahena-Medina et al. 2011). Perinatal exposure to PCB impaired primitive reflexes of newborns between the age of 1 and 5 days (Engel et al. 2007). Between the age of 3 and 16 months several studies reported that perinatal exposure to PCB induces mental and motor alterations (Berghuis et al. 2013). During school age, high perinatal exposure to PCB was reported to induce attention deficit/ hyperactivity (ADHD) according to the study of Sagiv and collaborators (2010). Inverse associations between perinatal PCB levels and full-scale IQ and verbal IQ at the age of 9 years were observed in an American cohort (Stewart et al. 2008). Neurodevelopmental effects of PAH were also reported in several studies (Peterson et al. 2015). First of all, in 1998, Perera and colleagues reported some birth outcomes like a decrease in length, weight and head circumference in a new-born cohort from Poland with high levels of PAH DNA adducts in umbilical cord blood. At age of three years perinatal exposure to PAH via air induced a decrease in mental development in a cohort study from New York (Perera et al. 2006). While at age of five the same cohort of study expressed a significant decrease in IQ measures (Perera et al. 2009). These findings are similar to those reported by the study of Edwards and collaborators (2010) on cohort children from Poland also at age of five.

Results of these studies give a strong reason to suggest that developmental exposure to POPs could be an early environmental origin of the late onset of neurodegenerative diseases. The theory of the early environmental origins of neurodegenerative diseases is strongly supported by scientific community (Landrigan et al. 2005). The concept of the developmental origin of diseases was firstly proposed by Barker and colleagues in 1990, known as Barker hypothesis (Osmond and Barker 2000). Barker found that infants with low birth weight, small head circumference, and low ponderal index at birth are at increased risk of developing coronary

heart disease, hypertension, stroke, insulin resistance and diabetes. He concluded from his observations that parameters of foetal infant and childhood growth may be predictors of disease in later life. This hypothesis was extended to encompass the effect of toxic chemicals on brain development (Landrigan et al. 2005), based on the link in epidemiological studies between exposure to some insecticides like paraquat, and PD (Thiruchelvam et al. 2000) and exposure to lead and methylmercury and AD (Grandjean et al. 1997; Jacobson et al. 1990). On molecular level today, epigenetic deregulation and oxidative stress are the most mechanisms proposed to explain the early environmental basis of neurodegenerative diseases (Ho et al. 2012). Interestingly, for the first time, in 2008 an inverse correlation has been proposed between plasma DNA methylation and serum POPs levels in a population highly exposed to POPs (Rusiecki et al.2008). This study was followed by three other studies on populations with background exposure to POPs; they have reported also that DNA hypomethylation is correlated to blood POPs levels, particularly OCs (Kim et al. 2010). On the other side, a recent epidemiological study has presented evidences of the putative role of OCs and PCBs in the disruption of the oxidative microenvironment in adipose tissue. Results obtained suggest that increased adipose tissue POP levels may modulate SOD activity and increase lipoperoxidation and marginally affect glutathione cycle (Artacho-Cordón et al. 2016). In the next paragraph we will discuss the most emergent POPs neurotoxicity mechanisms proposed in literature.

3. Mechanisms of toxicity

In order to understand molecular mechanisms of POPs neurotoxicity, several *in vivo* and *in vitro* experimental studies have been established. Studies mimicking environmental exposure are few in the literature. Crépeaux et al. 2012 reported that, *in Uterus* and lactational exposure to a low dose environmental mixture of 16 HAP has 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 (Gill et al. 2013), while lactational exposure to a representative mixture of PCB found in contaminated fish matrices has induced an increase in anxiety and transcriptional changes in mice on adulthood (Elnar et al. 2012).

Most of experiments in the literature were designed to study the toxicity of individual compounds and mostly in high doses, very far from the range of daily exposure. Even so, these experiments have succeeded to identify the big axises of POPs neurotoxicity

mechanisms. Some of the studies were able to establish a direct relation between the mechanism of toxicity and the molecular structure of the substance (Coats 1990).

According to these studies, principal mechanisms of neurotoxicity are; disruption of signalling pathways and neurotransmission, oxidative stress, mitochondrial dysfunction and disruption of energy metabolism and epigenetic effects (Meijer et al. 2014. Wisternik 2014 Federico et al. 2012). In fact, OCs pesticides kill insects through disturbance of their neurotransmission pathways. Endosulfan for example blocks the Cl^- channels linked to the γ-amino-butyric acid (GABA)-receptor while DTT exerts its toxicity by binding to lipoproteins in the nerve cell membrane,disturbing the ionic homeostasis, especially the sodium/potassium balance across this membrane (coman et al. 2013). By the same mechanisms, these molecules are neurotoxic to animals and human. Furthermore, developmental exposure to endosulfan has been found to alter gabaergic, cholinergic and dopaminergic circuits, by altering the expression of the receptors of these neurotransmitters (Wilson et al. 2014). Cabaleiro et al. 2008 reported that developmental exposure of mice to endosulfan induced an increase in amino acid concentration in prefrontal cortex in critical periods of neurodevelopment post natal day (PD15 and PD30) and increased specifically serotonin concentration in juveniles (PD30 and PD60). However, in striatum according to the study of Lafuente and collaborators (2013), developmental exposure to endosulfan decreased the concentration of both serotonin and norepinephrine and decreased metabolism of serotonin and dopamine in adult male rats. While in female rats endosulfan induced only a decrease of norepinephrine concentration and an increase of dopamine and serotonin concentration after exposure to the highest dose used in this study (6,2mg/kg).

Exposure to PCBs is also known to induce changes to the neurotransmitter system. *In vitro,* PCBs altered release, uptake and metabolism of serotonin (Boix et al. 2012). *In vivo*, perinatal exposure to PCB congener 153 (20mg/Kg) reduced serotonin and 5-HIAA concentration in cerebral cortex and it metabolite, 5-HIAA in the striatum of mice pups (Castoldi et al. 2006). Postnatal exposure to the same molecule significantly increased serotonin and 5-HIAA content in the whole brain at 1 week of age; however, these effects were reversible at one year of age (Honma et al. 2009). Subchronic exposure to benzopyrene (6.25 mg/kg) induced memory and learning impairments and altered the expression of neurotransmitter receptors (Qiu et al. 2011). Beside the alteration of neurotransmission, disturbance of signal transduction is a proposed mechanism of POPs neurotoxicity and particularly during development when, cellular signalling play a key role in nervous system development

processes (Murphy et al. 1987; Girard& Kuo. 1990) and any interference with these processes would have potential profound effects on the function of neurons as well as their development. POPs could alter signalling pathways by the disturbance of Ca^{+} homeostasis. In fact inhibition of voltage-gated calcium channels (VGCCs) is a common mode of action of OCs (Meijer et al. 2014). For PCB, exposure *in vivo* and *in vitro* was reported to increase Ca^{+} intercellular levels by the inhibition of its uptake by mitochondria and microsomes (Kodavanti et al. 2005). One of the down-stream effects of perturbation of Ca^{2+}-homeostasis by POPs is translocation of protein kinase C (PKC) from the cytosol to the membrane where it is activated leading to troubles in learning and memory, particularly trough interfering with the process of long term potentiation (LTP) (Kodavanti et al. 2005). Oxidative stress is another mechanism by which POPs could alter cell signalling and disturb brain development and function. In the next paragraph we will discuss in details mechanisms of induction of oxidative stress through POPs exposure and its implication in the aetiology of neurodegenerative diseases.

4. Oxidative stress

4.1 Oxidative stress is deeply implicated in the pathogenesis of neurodegenerative diseases

Oxidative stress (OS) is a common mechanism of toxicity between all the classes of POPs, not only in the brain, but also in the peripheral organs.

By definition, OS is a disturbance in the balance between the production of reactive oxygen species (free radicals) and the antioxidant defence system in the cell. Reactive oxygen species (ROS) are oxygen derivative molecules, highly reactive due the presence of a single unpaired electron in their outermost shell of electrons. ROS include, superoxide (O^{-}_{2}), hydroxyl radical ($OH^{\bullet}$), or non-radicals (hydrogen peroxide, H2O2) (Kim et al.2015). O^{-}_{2} is suggested to play a gateway role in ROS production; it may be transformed into the more stable form of H_2O_2 or protonated to form H_2O. H_2O_2 may have potential to generate highly reactive hydroxyl radicals $OH^{\bullet}$ (Halliwell et al. 2006). $OH^{\bullet}$ is known to be one of the most reactive ROS that are mainly responsible for the cytotoxic effects of ROS (Bolisetty et al. 2013).

ROS are produced during the process of metabolism of xenobiotics, where the secondary metabolites and conjugates could be high reactive free radicals. O_2^{-} could leak also, from the heme-iron (Fe) centre of the enzyme cytochrome P450 (CYP450), an important detoxifying

enzyme. O_2^- , H_2O_2 and iron ions Fe^{2+} could enter into the Fenton reaction and modulate production of more dangerous ROS specifically, $OH^{\bullet}$ (Bolisetty et al. 2013).

However, the main source of ROS is endogenous, more precisely, mitochondria. During the process of energy production in mitochondria, Oxygen plays the role of the last receptor of electrons in the respiratory chain and by accepting 2 electrons with 2 molecules of H^+, the water (H_2O) will be produced. However, it is estimated that 2% from total oxygen consumption are leaking from mitochondria as O_2^- leading to the production of other ROS and the attack of cell components (Cadenas& Devis. 2000).

To protect the macromolecules (DNA, lipids and proteins) from the reactivity of ROS, the cell has its own antioxidant system, composed of enzymes and molecules. The main enzymes are superoxide dismutase (SOD) and catalase (CAT). SOD catalyses the breakdown of highly reactive O^-_2 to less reactive H_2O_2 and oxygen and CAT convert H_2O_2 into the water and oxygen (Kim et al. 2015).

Glutathione (GSH) is a nonenzymatic antioxidant, composed of three aminoacids and plays a crucial role in reducing free radicals and conjugates secondary metabolites of xenobiotics; also it is a cofactor for antioxidant enzymes. Vitamins and natural products like flavonoids are also strong reducing agents and play an important role in the antioxidant system of the cell (Gandhi et al. 2012; Kim et al. 2015).

The strength of the antioxidant system is different from an organ to another. Liver for example owns a very strong antioxidant system with high concentrations of GSH, SOD and Catalase. On the other side, Catalase activity and GSH amounts in β cells of the pancreas are extremely low (Robertson& Hasman 2007). Catalase expression in the brain is also very low (Rhoads et al. 2012), and the brain is highly exposed to ROS due to its high energy consumption (more than 20% of total oxygen consumption) and its high iron and lipid concentrations (Bouayed et al. 2009). Other than the weak antioxidant system, both, βcells and neurons are post mitotic cells, what makes the damages accruing due to ROS exposure cumulative damages leading eventually to cell degeneration.

In fact experimental and clinical studies suggest that oxidative stress is deeply implicated in the pathogenesis of diabetes and neurodegenerative diseases; however there is confusion in literature between classifying oxidative stress as a cause or consequence of these degenerative diseases (Andersen 2004; Zhao et al. 2013; Kim et al. 2015).

In the case of neurodegenerative diseases, post-mortem brain tissues from patients with PD and AD, clearly display increased indices of ROS in affected brain regions. However, it is impossible to discern from this observation weather oxidative stress is a major cause or a consequence of associated neuronal cell loss (Andersen 2004). Many evidences support the consideration of oxidative stress as an origin cause of neurodegeneration. First of all, oxidative stress is strongly related to the cognitive impairments in both, age-related dementia and neurodegenerative diseases (Berr et al. 2000).

In individuals affected with neurodegenerative diseases such PD and AD, markers of lipid and protein oxidation have been identified in cortex and hippocampus. Surprisingly, targets of protein oxidation seem to be specific to some key proteins in the pathophysiology of neurodegenerative diseases like αsynuclein in PD (Giasson et al. 2000) and tau in AD (Horiguchi et al. 2003). In PD, GSH depletion is the earliest known biochemical indicator of nigral degeneration, furthermore, the magnitude of depletion parallels the severity of the disease (Pearce et al. 1997). In AD, it has been reported that oxidative stress decreases the activity of α-secretase while promoting the expression and activation of β- and γ-secretase; critical enzymes for the generation of the βAmyloid (Aβ) peptides from amyloid precursor protein (APP) (Zhao et al. 2013).

Many studies reported a reduction in antioxidant enzymes activity in the affected regions of the brain. Studies on transgenic mice support these observations, where a reduction in SOD activity to 50% (SOD $^{+/-}$) in human amyloid precursor protein (hAPP) transgenic mice accelerated the onset of hAPP/Aβ-dependent behavioural abnormalities and increased vascular amyloidosis among other molecular and pathological alterations (Esposito et al. 2006).While in the study of Li and collaborators (2004) SOD reduction in hAPP transgenic mice increased instead the overall amyloid burden in the brain.

Evidences supporting the consideration of oxidative stress as a consequence of earlier processes of neurodegeneration are also several. The main hallmark of neurodegenerative diseases is the fibrillar protein aggregates like lewy bodies consisting of synuclein in PD and β amyloid and tau tangles consisting of Aβ and tau in AD (Serrano-Pozoet al. 2011). It is well known that the deposition of proteins aggregates in the brain induces oxidative stress and mitochondrial dysfunction leading to apoptosis (Mattson et al. 1997; Zhao et al. 2013). Moreover, OS induced by the proteins aggregates itself favourites the proteins aggregation, thus, forming a vicious cycle that promotes neurodegeneration (Zhao et al. 2013).

Inflammation and mitochondrial dysfunction are important mechanisms by which proteins aggregates induce oxidative stress in the brain (Manczak et al. 2006). In fact, protein aggregation in brain mitochondria of AD individuals was already reported (Caspersen et al. 2005; Manczak et al. 2006). In isolated mitochondria, Aβ treatment could cause oxidative injury to mitochondrial membrane, disrupt lipid polarity and protein mobility and inhibit key enzymes of the mitochondria respiratory chain, leading to increased mitochondrial membrane permeability and cytochrome c release (Casley et al. 2001; Zhao et al. 2013). Another mechanism by which Aβ peptides could induce mitochondrial dysfunction is alteration of glutamate signalling (Cassano et al. 2016). Aβ deposition is linked to the over stimulation of NMDA glutamate receptors. NMDA receptors are Ca^{2+}favouring glutamate-gated ion channels and their continuous stimulation induces an increase in the Ca^{+} internal load leading to mitochondrial membrane depolarisation and mitochondrial dysfunction, induction of OS and eventually apoptosis (Danysz& Parsons 2012).

On the other side, Eteghad and collaborators (2014) reported that Aβ at physiological concentrations protects neurons from oxidative stress in brain and that these proteins induce toxic effects only in the advanced stages of the disease with extremely high concentrations. Thus, early onset of metabolism failure and mitochondrial dysfunction are independent toxic effects of protein aggregates, what will lead us again to suggest that oxidative stress is one of the earliest alterations that occur during the initiation and development of neurodegenerative diseases.

In overall, we have an idea on the different molecular mechanisms implicated in neurodegeneration, yet, the exact molecular cascade and its chronology is still unknown. However, it is clearly obvious that OS plays a crucial role in pathological cascade during all the phases of neurodegenration.

4.2 Oxidative stress as a major mechanism of POPs neurotoxicity

It is interesting to note that OS seems to play the same pivotal role in the neurotoxicity of environmental pollutants, particularly in the neurotoxicity of POPs.

Experimental studies indicate that all POPs are prooxydants. Chronic or acute exposure in low or high doses alters antioxidant parameters and damage cell components (DNA, proteins and lipids). Although, the intracellular source of ROS is not fully understood and the patterns of

the alteration change according to the scenario of exposure dependently on age and sometimes sex of animals as well as the dose and type of exposure.

Somme POPs could directly induce OS by producing ROS and reactive metabolites during their metabolism process. A wellknown example is benzopyrene. Benzopyrene is metabolised by cytochromes P450 (CYP1 and CYP2), basically in the liver and in a lesser degree in other organs like the brain, resulting on the production of reactive quinones, leakage of $O^{\bullet -}$ and production of $OH^{\bullet}$. Saunders and collaborators (2006) reported that acute exposure to benzopyrene induced behavioural deficits and increased in the brain lipid peroxidation and ROS levels and simultaneously repressed the antioxidant enzymes activity. Moreover, behavioural effects were positively correlated with levels of ROS and benzopyrene metabolites in the brain, indicating that benzopyrene is able to alter behaviour by inducing OS in the brain (Saunder et al. 2006). POPs also could directly produce ROS by altering mitochondrial function. OCs like methoxychlor interferes with complexe I of the respiratory chain of mitochondria and stimulates ROS production with a selective destruction of dopaminergic neurons (Kodavanti. 2005; Schuh et al.2009). Rotenone was the first insecticide reported to induce a selective destruction of dopaminergic neurons by the alteration of complex I activity. Today, rotenone is used in experimental models of PD (Duty& Jenner. 2011).

Prooxidant and degenerative effects of rotenone as well as OCs and other POPs like PCBs and HAP were also linked to the activation of microglia, a hallmark of neuroinflammation (Kodavanti. 2005; Taetzsch& Black. 2013). In fact, in neurodegenerative diseases including AD and PD, microglia is reported to be chronically activated to produce neurotoxic cytokines and ROS. However, origins of the inflammation are not well understood, it could be induced by an instigating endogenous or exogenous pro-inflammatory trigger or in response to neuronal death (reactive microgliosis) (Glass et al. 2010). Interestingly, OCs and PCB are reported to produce ROS in microglia specifically by the activation of NADPH oxidase (NOX2) leading to a selective degeneration of dopaminergic neurons (Taetzsch& Black 2013; Fonnum& Mariussen 2009).

POPs are able to alter mitochondrial function and stimulate ROS production by disturbance of calcium homeostasis (Kodavanti. 2005). OCs for example inhibit voltage gated calcium channels (VGCCs) (Heusinkveld et al. 2011) while PCBs inhibit mitochondrial Ca^{+} uptake (Fonnum et al. 2009), leading to mitochondrial depolarisation, ROS production and

eventually apoptosis and neurodegeneration. POPs also could disturb glutamate signalling and induce exitotoxicity by alteration of Ca^+ homeostasis and ROS production. For example, repeated exposure of PC12 cells to a very low dose of deildrin (60nM) induced inhibition of GABA receptors and disturbed glutamate signalling leading to an increase in internal Ca^+ levels which could lead to mitochondrial dysfunction and exitotoxicity (Briz et al.2010).

Antioxidant system reacts in response to the ROS production and mitochondrial dysfunction induced by POPs exposure. GSH is a key biomolecule in maintenance of redox status of the cell, also, it is the conjugator of the majority of POPs (Sipes et al. 1987). Acute and subchronic exposure to high concentrations of POPs commonly, decreases GSH levels in the brain. Exposure to PCB126 (5mg/kg/day) for 28 days induced a decrease in brain GSH levels and decreased activity of SOD and CAT in mice (Majumdar et al. 2014). Exposure to endosulfan at concentration of 4mg/Kg/day for 30 days induced mitochondrial swelling and both mitochondrial and cytosolic reduction of GSH levels as well as SOD and CAT activity (Lakroun et al. 2015). Yet, effect of exposer to environmental POPs mixtures on GSH levels has not been studied until lately (lee et al. 2014). In the study of Slezak and collaborators (2000), exposure to tetrachlorodibenzodioxin (TCDD) (0.15 ng/kg/day) for 13 weeks, which was estimated to be similar to current human background exposure induced GSH depletion. Interestingly, according to the same study, GSH levels increased with the increase of the dose of exposure (150 ng/Kg/day). This effect is known by hormetic effect indicating that there is a range of nontoxic exposure levels able to stimulate compensatory pathways to increase GSH levels. However, exposure to low doses of POPs mimicking human daily exposure is not sufficient to induce hormetic responses. Thus, human background exposure to POPs induces continuous consumption of GSH through POPs metabolism without compensation which can lead consequently to chronic GSH depletion and OS (Lee et al. 2014).

Exposure to POPs induces OS during development, leading to the induction of alterations that could pave the way to the late onset of neurodegenerative diseases. In literature, epigenetic dysregulation is one of the major mechanisms proposed to explain the early environmental basis of neurodegenerative diseases (Zawia et al. 2009). Epigenetics refers to modifications in gene expression that are influenced by DNA methylation and/or chromatin structure, RNA editing, and RNA interference without any changes in DNA sequences. DNA methylation and histone deacetylation are known to occur shortly after DNA synthesis and could be modified by diverse physiological or pathological factors, altering gene expression for the lifetime of the organism (Zawia et al. 2009). DNA methylation patterns are mainly established *in uteru*s,

and it has been established that the foetal environment may alter such patterns, leading to sustainable changes in gene expression that endure for a lifetime (Wu et al. 2004). Cytosine is the favourite base for DNA methylation in CpG islands, whereas guanine is the favourite base for oxidative damage. Interestingly, it has been reported that guanine oxidation interacts with the methylation of the adjacent cytosine and induces its inhibition. According to these findings OS induced by developmental exposure to POPs is susceptible to alter DNA methylation in the brain and induce permanent modifications in gene expression (Weitzman et al. 1994; Turk et al. 1995). Furthermore, those modifications could give the basis of the late onset of neurodegenerative diseases (Zawia et al. 2009). These results are in accordance with epidemiological studies linking exposure of POPs to oxidative stress, epigenetic effects and development of neurodegenerative diseases.

As a conclusion we can say, that all evidences from epidemiological and experimental studies indicate that POPs are deeply implicated in the aetiology of neurodegenerative diseases. Exposure to POPs alters brain integrity in so many ways, mainly by disturbance of neurotransmission, alteration of cell signalling and oxidative stress. In fact, oxidative stress is deeply implicated in the mechanisms of neurtoxicity. Many studies report that it is directly responsible on initiation and progression of neurodegenerative diseases; yet, oxidative stress is considered as major mechanism of POPs neurotoxic effects. Thus, we can conclude that exposure to POPs could be directly implicated in the aetiology of neurodegenerative diseases through induction of oxidative stress. This effect should not be neglected in order to elucidate mechanisms implicated in neurodegenerative diseases.

Chapter II

Neurotoxicity of perflurooctane sulfonate (PFOS); links between hepatotoxicity and neurotoxicity and its implication on the aetiology of Alzheimer's disease.

1. Introduction to Alzheimer's disease

Alzheimer disease (AD) is one of the most emergent neurodegenerative diseases in the world. There are 47.5 million people suffering from dementia over the world and AD may contribute to 60–70% of cases (WHO 2016). In 1901, *Auguste D* was admitted to the *Frankfurt hospital* where she was examined by Dr. *Alois Alzheimer*. He reported in his notes that she was suffering from a reduced comprehension and memory, paranoia, auditory hallucinations and pronounced psychosocial impairments. When he asked her to write letters she showed big troubles for the task, once, she announced *"I have lost my self"*. On 1906, *Auguste D* died, Dr. Alzheimer performed a histological study on her brain and described the presence of plaques, neurofibrillary tangles, and arteriosclerotic changes (Maurer et al. 1997). Dr. Alzheimer and his team published their works describing the disease for the first time in a series of articles between 1906 and 1909 (Alzheimer A. 1906; Perusini et al. 1909). From that time and until today, people are still losing their selves for AD, there is no radical cure to the disease, mechanisms of pathogenesis and aetiologies are still mysterious or under debate and the only definitive way of diagnostic is still the detection of senile plaques and neurofibrillary tangles in the postmortem brain of the patient.

Yet, over all these years, a lot of progress has been made in determining molecular mechanisms implicated in the pathogenesis of AD. Today, we know that senile plaques are composed of βamyloid aggregates (Aβ). Aβ is the derivative peptide of proteolysis of amyloid precursor protein (APP) by β and γ secretase instead of α secretase. Aβ aggregation leads either to its deposition in form of amyloid plaques or to the formation of Aβ soluble oligomers (Fig.1). After the findings that correlation between cognitive alterations and deposition of amyloid plaques is not linear, neither in humans or mice, it has been proposed that Aβ toxicity could be instead mediated by the form of Aβ soluble oligomers, however mechanisms of toxicity are still poorly understood (Hardy et al. 2002; Serrano-Pozo et al. 2011).

On the other side, tau tangles consisted of hyperphosphorylated tau protein. Tau is a dominant microtubule-associated protein (MAP) attached to the surface of axons to support its structure. Hyperphosphorylation of tau induces its detachment from axons leading to the collapse of neuronal structure and the formation of fibril tangles (Fig.2). Tau contains more than 30 site of phosphorylation, several kinases have been found to be capable of phosphorylating tau *in vitro*, however, it is not yet clear whether all of them participate in tau phosphorylation under pathological conditions, glycogen synthase kinase 3 (GSK3) and cyclin-dependent kinase 5 (CDK5) are the most studied kinases and received a particular attention as potential therapeutic targets (Mi& Johnson. 2006).

The mechanistic link between Aβ aggregates and tau tangles in the pathogenesis of AD is not clear. For years, it has been proposed that the formation of Aβ plaques is responsible on synaptic loss and cell death and that formation of tau tangles is only a consequence of Aβ toxicity. However, later observations in animal models and in elderly individuals, suggested that Aβ-associated clinical decline might occur only in the presence of elevated tau pathology, furthermore, it has been proposed that tau hyperphosphorylation precedes Aβ aggregation (Bloom 2014).

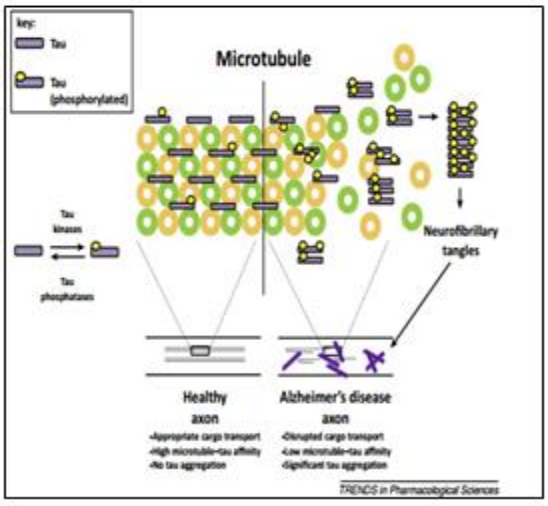

Fig. 1 Tau metabolic pathway. *The microtubule-associated tau protein maintains phosphorylation status through the combined actions of tau-associated kinases and tau-associated phosphatases. When appropriate physiological tau phosphorylation is in place, tau affinity to microtubules is maintained and microtubule structure, axon integrity, and cellular function are preserved. When tau is hyperphosphorylated (as found in Alzheimer's disease), tau is thought to lose affinity for microtubules and form insoluble aggregates, which eventually lead to impaired axonal transport, neuronal ultrastructure damage, and cell death.* ***(Joshi et al. 2015).***

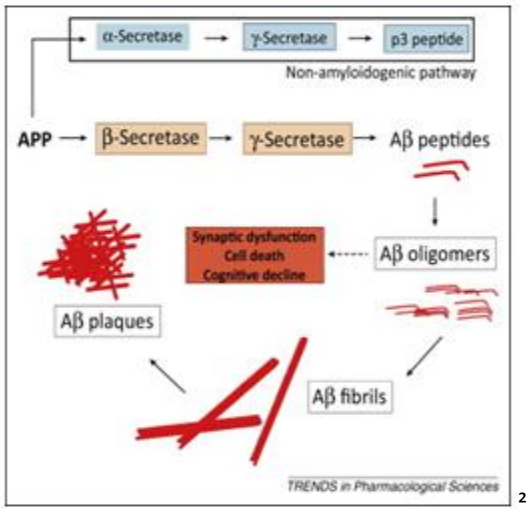

Fig. 2 Aβ metabolic pathway. *The Aβ precursor protein (APP) is processed in one of two main pathways that yield either Aβ peptides or non-amyloidogenic products. If APP is sequentially cleaved by a-secretase and then g secretase, nonamyloidogenic products form. However, if APP is cleaved by b-secretase and then g-secretase, Aβ is produced. As Aβ peptides continue to be produced, they form low-n oligomers, fibrils, and eventually plaques. It is believed that soluble low-n oligomers produce the neuronal and cytotoxic injury in Alzheimer's disease.* ***(Joshi et al. 2015).***

Other than the big hallmarks of AD, Aβ aggregates and tau tangles, experimental and clinical studies suggest that oxidative stress plays a pivotal role in the pathogenesis of AD. In fact, oxidative stress is strongly related to the cognitive impairments in both, age-related dementingdiseases and neurodegenerative diseases (Berr et al. 2000). Post-mortem brain tissues from patients with AD, clearly display increased indices of ROS in affected brain regions. However, it is impossible to discern from this observation weather oxidative stress is a major cause or a consequence of associated neuronal cell loss (Andersen et al. 2004). In fact, toxicity of Aβ aggregates is attributed in the first place to induction of mitochondrial dysfunction and oxidative stress. Oxidative stress in turn favourites deposition of Aβ aggregates and tau tangles, thus, forming a vicious cycle that promotes neurodegeneration (Zhao et al. 2013).

Mitochondrial dysfunction and oxidative stress have been linked also to the metabolic failure noticed in AD. In fact, disturbance of glucose and lipid metabolism as well as disturbance of insulin signalling has been noticed in preclinical stages of AD (Cai et al. 2012). Interestingly, there is a crossing link between these metabolic pathways and pathways implicated in formation of Aβ and tau aggregates. However, the exact mechanism by which these pathways are contributing together in the pathogenesis of AD is not defined yet (Llorens-Marítin et al. 2014).

Aetiologies of AD are like its pathogenesis, still controversial. The constant is that there are familial /early onset AD and sporadic/late onset AD. Familial AD is related to mutations mostly in genes of APP, perensiline1 and perensiline2 which are implicated in APP/Aβ metabolic processing with an autosomal dominant frequency of 18–50% (Piaceri et al. 2011). In fact, identification of these mutations has articulated the hypothesis of amyloid cascade in AD pathogenesis. Interestingly, these mutations were not implicated in the sporadic form of AD. In fact in sporadic AD, aging remains the main risk factor (Ikeda et al. 2010). Furthermore, free radical and mitochondrial theory of aging is consistent with the hypothesis of early involvement of mitochondrial dysfunction and oxidative stress in pathogenesis of AD (Harman. 1995; Swerdlow et al 2011). Besides aging, carriage of the APOE ε4 allele of alipoliprotein gene (APOE) has been identified as a major risk factor in both, sporadic and familial AD (Ikeda et al. 2010; Piaceri et al. 2011). The fact that (APOE) is coding for the most important cholesterol carrier has reinforced the hypothesis of considering AD as a metabolic disorder (Leduc et al. 2010). This hypothesis was further supported by results from

epidemiological studies establishing diabetes, insulin resistance, hypercholesterolemia and obesity as risk factors for sporadic AD (Li et al. 2015; Sridhar et al. 2015).

Epidemiological studies revealed also a strong link between exposure to environmental pollutants and AD prevalence. In several epidemiological studies, blood levels of organochlorine pesticides have been found to be directly linked to an increased risk of AD (Singh et al, 2013; Richardson et al, 2014) while exposure to other pollutants like lead (Pb) and PCB has been related to behavioural deficits and disturbance of cognitive function (Martin et al. 2012). Induction of epigenetic effects, neuroinflamation, oxidative stress and mitochondrial dysfunction in the brain are the most proposed mechanisms of environmental neurotoxicity proposed in literature (Kanthasamy et al. 2012). Recently, it has been proposed that xenobiotics could induce neurotoxic effects, indirectly through their interactions with the function of peripheral organs. Mumaw and collaborators (2016), reported that exposure to air pollution could induce neuroinflamation through the axis of lung-brain. According to this study on rats, exposure to O_3 (ozone) which is not able to reach the brain, has modulated lung to secrete circulating cytokine independent signals able to activate microglia in the brain, thus, inducing proinflamatory and neurotoxic effects. In the same context, chronic alcohol exposure has been reported to induce insulin resistance and disturb PI3/AKT pathway in both liver and brain. It induces also steatohepatitis leading to production of toxic lipids such as ceramides, and through the axis of liver, ceramides activate pro-inflammatory cytokines and increase lipid adducts and insulin resistance in the brain leading to impairment in cognition and motor function in rats as reported by De la monte et al. (2009; 2012).

PFOS is a persistent organic pollutant (POP) known to be hepatotoxic, particularly through alteration of lipid metabolism in liver, and neurotoxic, particularly during development. In this chapter we will try to discuss mechanisms of PFOS neurotoxicity and spot the light particularly on the possible toxic effects that could be induced through the axis of brain-liver and its implications in the pathogenesis of AD.

2. Perfluorooctane sulfonic acid (PFOS)

Polyfluoroalkyl chemicals (PFASs) have been manufactured since the 1950 and used extensively for their inertness and heat stability in many industrial and commerce applications, such as surfactants, lubricants, paper and textile coatings, polishes, food packaging and fire-retarding foams (Lau et al. 2007).

Because of their high stability, most of PFASs persist in environment and organisms, and can be transported to remote locations. Perfluorooctane sulfonic acid (PFOS) particularly, meets all POPs criteria and was included in Stockholm convention in 2009. Since then, its use has been restricted in many parts of the world, yet due to its high persistence and bioaccumulation general population is still exposed to this chemical with an estimated blood concentration around 130ng/ml in 1970. Recently, it is estimated that these concentrations have been dropped to the range of 5-40 ng/ml whereas levels of bioaccumulation in populations of occupational exposure are reported to be 3 to 5 times higher (Mariussen et al. 2012).

Even though levels of PFOS exposure have been decreased importantly during last decades, its toxicity is attracting a lot of attention, particularly developmental neurotoxicity because of the ability of PFOS to cross brain blood barrier, placenta and breast milk. In fact, breast milk is considered one of the main depuration routes of PFOS, and blood concentrations of PFOS could drop to over than 95% in a year in breast feeding mothers. Fromme and collaborators (2010) indicated that even that the breast milk PFOS concentrations are very low (0,047 ng/ml), this kind of intake could lead to a body burden in nurslings at the age of six months similar to or higher than that found in adults. The developing brain is more sensitive to environmental toxicants, since interaction with critical phases of neurodevelopment could lead to serious alterations that may persist on adulthood and contribute to the establishment of neurodisorders later in life.

3. Epidemiological studies

Epidemiological studies concerning PFOS neurotoxicity are limited; in 2008, Fei and collaborators reported that children from mothers with high serum PFOS concentrations are slightly delayed in time of sitting without support, PFOS concentrations in umbilical cord serum were also negatively associated with birth weight and head circumference. A positive correlation between the serum levels of perfluoroalkyl compounds (PFCs) in children and teenagers and the prevalence of ADHD was also reported by previous studies (Stein and Savitz. 2011; Hoffman et al. 2010).

4. Experimental studies and proposed mechanism of PFOS neurotoxicity

Toxicity of PFOS has been studied in different species but mostly in rodents with different exposure paradigms and doses.

Rodents' exposure to PFOS on adulthood according to several studies has induced no behavioural effect. Two studies have reported slight effects on motor activity after exposure to 1mg/kg PFOS for 4 weeks or 6 mg/kg for 28 days (Fuentes et al. 2007; Butenhoff et al. 2012). On the other another hand, studies on gestational and lactational exposure indicate an alteration in neuromotor development which is in accordance with reports from epidemiological studies.

In the study of Butenhoff and collaborators (2009), gestational and lactational exposure to 1mg/kg of PFOS increased motor activity and reduced habituation of pups in PD17, however, these effects disappeared later on adulthood. Johansson and collaborators (2008) have also reported a lack in habituation and hyperactivity after a one dose exposure at PD10. However, these alterations persisted on adulthood; furthermore, the cholinergic system was affected; manifested as a hypoactive response to nicotine exposure compared to a hyperactive response in controls. Later, it has been reported that an increase in expression of proteins involved in neural growth and synaptogenesis; Tau, CaMKII, GAP-43, and synaptophysin in the hippocampus of the neonate mouse one day after exposure to PFOS (11.3 mg/kg) (Johansson et al. 2009; Lee et al. 2013). Interestingly, gestational and lactational exposure to PFOS altered synaptogenesis via a decrease in levels of synaptophysin and synapsin in hippocampus and an increase in the levels of synaptophysin and a decrease in the levels of synapsin in cortex (Zeng 2011a). In these studies, it is appearing that PFOS exposure altered synaptogenesis but in different manners indicating that mechanisms of neurotoxicity are dependent on the type and level of exposure. Alteration of neurotransmission was reported also in *in vitro* studies, exposure to PFOS (50-250 μM) was reported to promote differentiation of the PC12 cell into the cholinergic phenotype at the expense of the dopaminergic phenotype (Slotkin et al. 2008). In their study on neural stem cells, Ibrahim and collaborators (2013) showed that PFOS at only 100nM was able to alter cell viability whereas the 12.5nM concentration increased neural differentiation; the effect was attributed to the peroxisome proliferator activated factor (PPARγ) activation. Also in the study of Liao and collaborators (2009), low dose exposure to PFOS at 1 μM increased the inward glutamate currents whereas higher concentrations (10 and 100 μM) dose-dependently reduced the inward glutamate currents. The effect was attributed to disturbance of Ca^{+} homeostasis. PFOS at 30 μM was able to induce elevated intracellular concentrations of $Ca2^{+}$. The increase intracellular $Ca2^{+}$ appeared to be of both extracellular origin involving voltage gated Ca2+ channels, and intracellular origin such as activation of ryanodine receptor and inositol

phosphate-3 (IP3)-receptors (Liu et al. 2011). According to the same study, the disturbance of the Ca2+-homeostasis was followed by an increase in oxidative stress and an increased expression of calcineurin (a Ca2+ activated protein phosphatase). Cytotoxicity and oxidative stress may also be induced as a consequence of inflammatory responses; PFOS is known to induce such immune responses. In prenatally PFOS-exposed rats it was observed an increase in inflammatory response in the juvenile rat brains as shown by an increase in the mRNA levels of proinflammatory cytokines, such as interleukin 1β, tumour necrosis factor α, AP-1, NF-kappa-B and CREB (Zeng et al. 2011b). On the other scenario, PFOS could induce apoptosis via activation of protein kinase C (PKC) and extracellular signal-regulated kinases (ERK) pathways which are linked to caspase3 activation and DNA fragmentation (Lee et al. 2013).

It is important to mention that studies above used doses that are at list 10 times higher than doses of exposure in general population. In fact, a typical blood concentration of 5ng/ml corresponds only to 10nM. In other hand, possible effects of mixtures should not be ignored. Several organohalogen compounds, pesticides and heavy metals in environment are established as neurotoxicants and it is plausible that their combination may reach concentrations that can increase harmful effects (Mariussen et al 2012).

5. Possible links between PFOS hepatotoxicity and neurotoxicity effects: implications on the aetiology of Alzheimer's disease

Liver has the highest level uptake and storage for PFOS (Maestri et al. 2006). It is also considered as the main target for PFOS toxicity. Its toxicity is mainly linked to its ability to activate peroxisome proliferator activated receptor (PPAR) leading to disturbance of lipid metabolism, steatosis and hepatomegaly (Shipley et al. 2004). Recent studies indicate that toxicity of PFOS could be independent of PPAR activation; PPARa null mice exposed to PFOS has shown the same profile of toxicity as the wild type (Rosen et al. 2010). Other than disturbance of lipid metabolism, PFOS is known to disturb thyroid hormone homeostasis (Yu al. 2009), induces in liver inflammatory responses, oxidative stress and apoptosis. Furthermore, several epidemiological studies have linked diabetic prevalence to environmental factors and pollutants exposure including PFOS (Lin et al. 2009). Experimental studies have shown that PFOS is able to disturb glucose homeostasis. Exposure of adult mice to 100ug/kg PFOS for 5 weeks elicited insulin-resistance phenotype. In cultured mouse hepatocytes under low glucose conditions, PFOS also stimulated hepatocyte glucose production and also enhanced glucagon-induced glucose production. Interestingly, Ziquan

and collaborators (2011) found that gestational and lactational exposure to PFOS at a dose of 0,5 and 1,5mg/kg exhibited on adulthood a reduced glucose tolerance, and higher levels of serum insulin, implying insulin resistance, and impaired glucose homeostasis.

In the brain, for a long time, it has been thought that insulin does not play any role and that glucose uptake is independent on insulin signalling; however, the abundant expression of insulin receptors on different brain regions remained unexplained. Until the past few years, when it has been confirmed that insulin plays a pivotal role in regulation of long term potentiation (LTP); the physiologic process responsible on learning and memory formation (Blázquez et al. 2014; Lee et al. 2016).

Disturbance of insulin and glucose homeostasis experimentally altered learning and memory in mice and could play a role in AD pathophysiology. In fact, several epidemiological and experimental studies have established a link between diabetes and AD. Furthermore, insulin resistance is reported to be a hallmark of AD that appears independently on diabetes (Li et al. 2015).

One of the most plausible mechanisms is the interference of insulin with extracellular proteolytic Aβ degradation that occurs via the insulin-degrading enzyme (IDE); under insulin resistance conditions, insulin may competitively inhibit the insulin-degrading enzyme, thus impairing degradation of Aβ, increasing its neurotoxicity and promoting AD (Li et al. 2015) (Fig.3). Besides Aβ, insulin resistance or deficiency also increases tau protein phosphorylation through activation of glycogen synthase kinase3 (GSK3β) (Llorens-Marítin et al. 2014) (Fig.4).

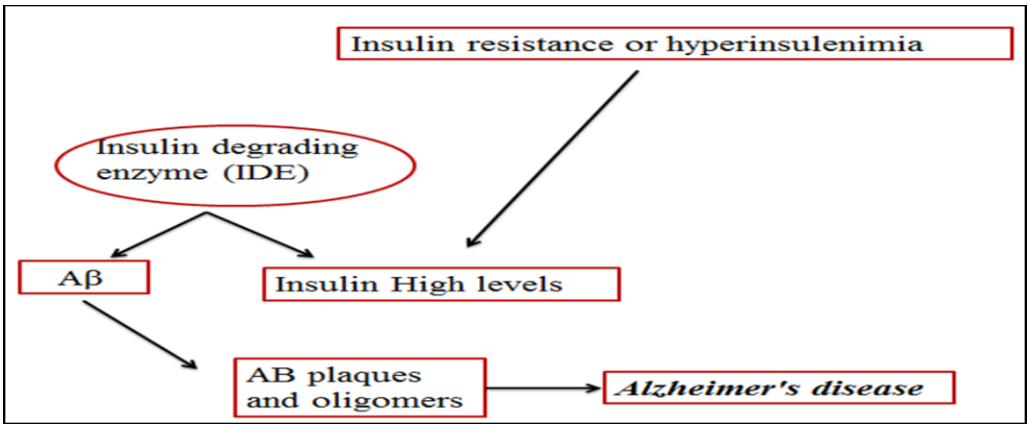

Fig. 3 Effect of insulin resistance on Aβ pathway. *Insulin resistance lowers the expression of Aβ-degrading IDE. Reduced IDE then leads to an increased Aβ levels and accumulation of Aβ oligomers. Hyperinsulinemia exacerbates also IDE deficiencies because excess insulin occupies IDE binding sites rendering them unavailable for Aβ. The increased amyloidogenic processing that occurs in insulin resistance combined with decreased Aβ clearance by IDE results in a deleterious positive-feedback cycle as Aβ oligomers contribute to insulin resistance in the brain. As Aβ levels continue to rise, insulin resistance worsens leading to further production of the toxic peptide.*

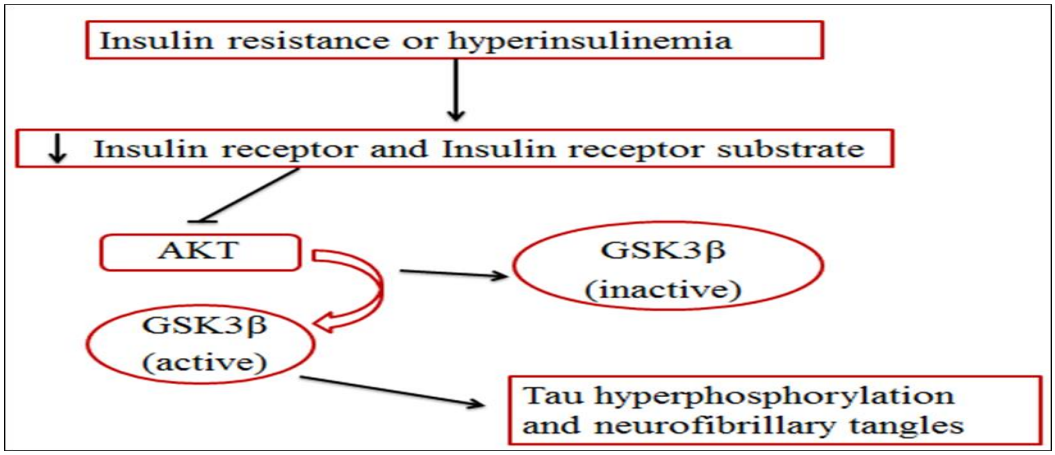

Fig.4 Effect of insulin resistance of Tau/Ptau pathway. *Under normal conditions, insulin signalling via insulin receptor and AKT leads to the inactivation of GSK3β, whereas insulin resistance leads to GSK3β dephosphorylation and activation. As GSK3β is also the main kinase of tau, its hyperactivation will lead to tau hyperphosphorylation, and production of neurofibrillary tangles.*

Beside insulin resistance, AD is characterised by disturbance of lipid metabolism, particularly cholesterol. It has been proposed that disturbance of cholesterol homeostasis interferes with the process of APP proteolysis and Aβ transport (Burns et al. 2011), and modulates as well inflammatory pathways (Gee et al. 2005).

As it was already mentioned above, PFOS alters glucose and lipid metabolism in the liver and induces insulin resistance. Starting from these concepts, it would be quite interesting to investigate whether PFOS has the same profile of toxicity in brain as in liver. It is already reported that exposure to PFOS increases PPARγ expression in the brain (Wang et al. 2010; et Ibrahim et al. 2013) and promote inflammation, however its effect on insulin signalling and lipid and glucose metabolism are not yet investigated.

PFOS is a persistent organic pollutant able to cross blood brain barrier placenta and breast milk, leading particularly to neurodevelopmental toxicity. Several mechanisms are established like alteration of synaptogenesis and neurotransmission, calcium homeostasis, and induction of apoptosis pathways, oxidative stress and inflammation. In liver, PFOS is known mainly by disturbance of lipid metabolism through PPAR activation. Induction of Insulin resistance and disturbance of glucose homeostasis are also reported. PFOS PPARy-activation is already reported in the brain, indicating that brain and liver could share some mechanisms of toxicity toward PFOS. Alterations of insulin and glucose homeostasis in the brain whether directly or through the axis of liver are plausible mechanisms by which PFOS could alter brain function and probably trigger or promote propagation of neurodegenerative diseases like AD. An investigation in this contest could contribute to a better understanding of PFOS neurotoxicity and its implications in the aetiology of Alzheimer's disease.

Chapter III

Neurobehavioral deficits and brain oxidative stress induced by chronic exposure to a low dose mixture of persistent organic pollutants (POPs) in adult female rats.

1. Aim of the study

Taking in consideration that oxidative stress and mitochondrial dysfunction are early hallmarks of neurodegenerative diseases (Manczak et al. 2006; Swerdlow 2009) and that POPs are well-known by their prooxydant effects and mitochondrial alteration, the present work aims to evaluate if neurobehavioral and oxidative stress effects might be induced after a chronic exposure in adult age to a low dose of POPs mixture consisting of two PAH; benzopyrene, a highly prooxydant and carcinogenic compound (Saunders et al. 2006) and naphthalene, a relatively less toxic PAH, and two pesticides, Endosulfan , an organochlorine pesticide , which is banned or use restricted from almost all the parts of the world but still found in nature due to its high persistence and non-authorized use (ATSDR. 2013), and chlorpyrifos, an organophosphorous pesticide, still in debate to be classified or not as a POP (Giesy et al. 2014a) 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 no-observed-effect level (NOEL) without taking in consideration possible interactions with other chemicals present in environment (Giesy et al. 2014b).

2. Materials and methods

2.1. Chemicals

The mixture used in this study consisted of two pesticides: Endosulfan (35%) and Chlorpyrifos (48%) as commercial forms , Thiodan 35 EC® and Dursban® (India) respectively, and two PAHs: α -Benzopyrene (95%) and Naphthalene (99.5%).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 real exploration study on pesticides in vegetables in Algeria (data not published), while residue levels of PAHs were

taken from the study of Martorell et al. (2010). The daily food consumption was determined according to the study of (Serra-Majem et al. 2003).

The dose of each pollutant was calculated according to the equation: $\mathbf{D = \Sigma\ C \times QC}$

Where C: concentration of pollutant µg / g of a given food.

QC: amount of food consumed / days by an adult woman.

D: dose of pollutant consumed per day µg / kg.

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 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 α –Benzopyrene (0,002µg/kg).

2. 2. Animals and exposure protocol

Twenty eight female *Wistar albinos* rats, weighing 200–250g, were obtained from Pasteur Institute (Algeria). Upon arrival, the rats were housed, 5 per cage. Animals were maintained under a daily light/dark cycle with 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 90 days between 9:00 and 10:00 am.

2.3. 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 six consecutive days and the probe trial conducted in the 6th day.

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 4 groups, to avoid circadian variation. Tests were recorded and all the variables were analysed by the same experimenter, using the video tracking program Etho-Vision® from Nolduls Information Technologies, USA.

Morris Water Maze: Spatial learning and retention were tested in a water maze according to a test modified from the procedure of (Morris 1981; Brits-Bromley 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 60s, being each trial separated by a 60s intertrial 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 analysed as the measures of water maze performance.

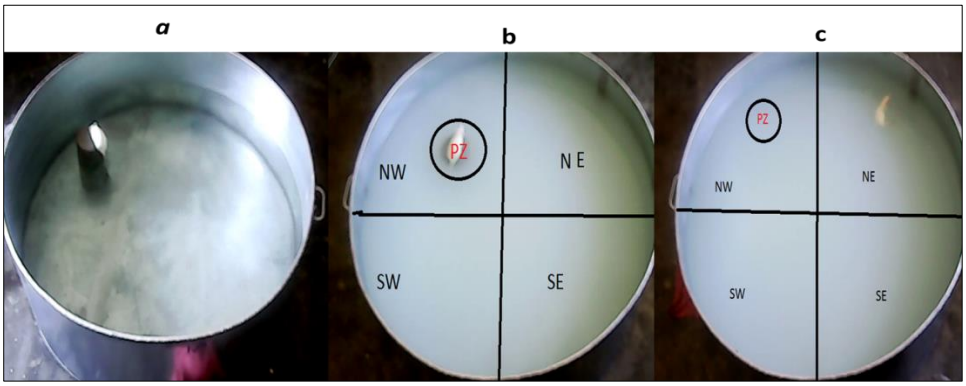

Fig.1 Test of Morris water maze. (a) *Day 1: habituation session where the platform is clearly obvious to the animal.* ***(b)*** *From day 2 until day 6: training sessions where the animal learns to find the position of the hidden platform. The swim-path length and the latency to find the escape platform reflect the capacity of the animal for the acquisition of the information.* ***(c)*** *Day 7: probe trial where the platform is removed and the animal will undergo a free swimming session of 60s. Cumulative time in the quadrant where was the platform and the frequency to pass by the platform zone reflect the memory retention of the animal.*

Open-field: This test was performed to assess the general locomotor activity (Walsh and Cummins 1976; Riebe et al. 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 70% ethanol.

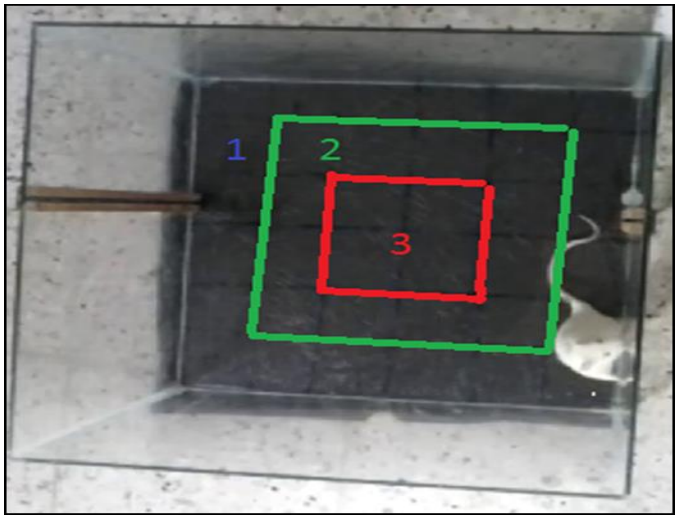

Fig.2 Test of open field. *(1) Peripheral area. (2) Intermediate area. (3) Centre area.*

Light dark Box test: This test was performed to assess anxiety behaviour (Riebe et al. 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 ×50cm); 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 70% ethanol solution between trials.

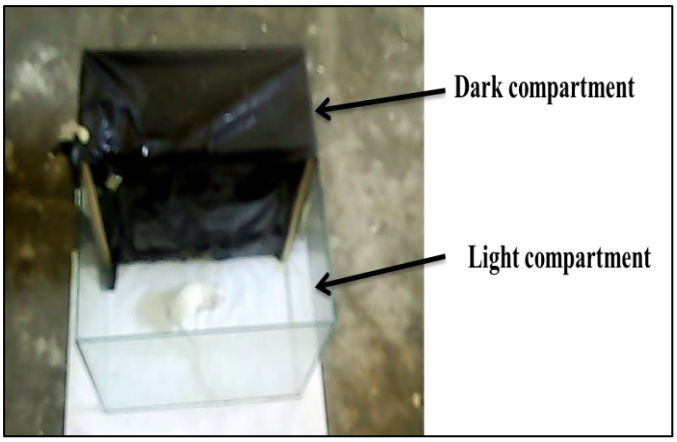

Fig.3 *The light dark box test.*

2.4. Tissue samples

On the 90th 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 (Clayton and Doda. 2001) with slights modifications. Briefly, the hemisphere was washed in cold buffer, PH 7.4 (20 mM tris-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 at 4 C°. After that, the pellet was recentrefuged in the same conditions. Supernatants from the two centrifugations were mixed and centrifuged at 15000 g for 20 min.

The supernatants was considered as a cytosolic fraction and conserved at -20C° until ulterior determination of CAT, SOD, GST activities, while the resultant pellet was washed twice with Tris buffer (20 mMTtis-HCl, 250 mM sucrose) pH,7.4 at the same conditions, resultants mitochondrial pellets were suspended in 300 µl of phosphate buffer and frozen at -20 C° until their 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.

On the other hand, left hemispheres were dissected immediately after sacrifice to 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

2.5. Biochemical analyses

MDA levels: Lipid peroxidation was evaluated by the malondialdehyde (MDA) assay according to the method of Ohkawa and collaborators (1979). MDA is one of the terminal products of polyunsaturated fatty acid (PUFA) decomposition under the effect of free radicals released during stress. In an acidic and hot medium a molecule of MDA is condensed with two molecules of thiobarbituric acid (TBA) to form a coloured pink complex (reading at 530 nm).

Briefly, 200 µl of the cytosolic or mitochondrial fraction were added to 0,5 ml of 20% trichloroacetic acid and 0,7 ml of thiobarbituric acid (TBA) 0.67%. The mixture was heated to 100 ° C for 45 minutes, cooled on ice then centrifuged at 3000g for 15 minutes, then was taken and used to determine the absorbance. The concentration of MDA was deduced from a standard curve established under the same conditions using the 1,1,3,3 tetratoxypropaneas a standard molecule.

*Glutathione levels:*The GSH quantification is based on the Ellman's colorimetric method (1959). Oxidation of GSH with 5, 5'-Dithiobis 2-nitrobenzoic acid (DTNB) releases the thionitrobenzoïc acid (TNB) that absorbs at 412 nm. 50 µl of cytosolic or mitochondrial fraction were added to 1 ml of phosphate buffer (0,1 M, pH=8) and 20µl of DTNB (0,01M), followed by addition of 2ml of ethanol. The mixture was incubated for 15 min, than the absorbance was determined at (412 nm). The concentration of GSH was deduced from a standard curve established under the same conditions using molecular GSH as a standard molecule.

Catalase activity: CAT activity was determined according to the method of Clairborne (1985).

Catalase breaks hydrogen peroxide into water and molecular oxygen according to the following equation:

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_2O + O_2$$

In this analysis, CAT activity is measured as the rate of H_2O_2 disappearing from the medium in time unit at 25°C. For that 50 µl of cytosolic or mitochondrial fraction were added to 1ml of phosphate buffer (KH_2PO_4, 0.1 M, pH 7.2) and 950µl of 30% H_2O_2 freshly prepared. The absorbance was determined at 560 nm in the interval of two minutes. The enzyme activity is calculated in international unit (IU) according to the following equation:

UI/mg= (2.3033/T) × (logA1/A2) /mg protein.

A1: Absorbance in the first minute.

A2: Absorbance in the second minute.

T: Time interval (two minutes).

SOD activity: The SOD activity was evaluated by the method of Beauchamp and Fridovich (1971).

In the cell, SOD catalyses the dismutation of superoxide into molecular oxygen or hydrogen peroxide according to the following equation:

$$M^{(n+1)+}\text{-SOD} + O_2^- \rightarrow M^{n+}\text{-SOD} + O_2$$
$$M^{n+}\text{-SOD} + O_2^- + 2H^+ \rightarrow M^{(n+1)+}\text{-SOD} + H_2O_2.$$

Where: M = Cu (n=1) ; Mn (n=2) ; Fe (n=2) ; Ni (n=2).

The Principale of the method is based on the ability of the enzyme to inhibit the reaction between nitrobluetrazolium (NBT) and the superoxide anion produced by photo reaction of oxygen and riboflavin in the presence of a donor of electrons as methionine. The reduction of NBT by the superoxide anion to Formazan can be monitored by spectrophotometer at 560 nm. One unit of SOD activity is defined as the amount of enzyme capable of inhibiting 50% of the NBT reduction. For that, 50 µl of cytosolic or mitochondrial fraction were added to a mixture composed of 2 ml of the reactive medium (Sodium cyanide 10^{-2} M, solution of NBT at 1.76 × 10^{-4} M, EDTA 66 mmol, Methionine 10^{-2} M, Riboflavin 2 µmol, pH 7.8) . This mixture was exposed to light of a 15 Watt lamp for 30 min to induce the photoreaction of riboflavin. Reduction of NBT into Formozan gave a blue colour. The colour was measured by

spectrophotometer at 560nm. The enzymatic activity is calculated in terms of IU / mg of proteins according the following equation:

$$\% \text{ inhibition} = (DO_{control} - DO_{sample} / DO_{control}) \times 100.$$

GST activity: The activity of GST was evaluated according to the method of Habig et al. (1974).

It consists in providing the enzyme with a substrate, generally chlorodinitrobenzene (CDNB), which reacts readily with many forms of GST and GSH.

Briefly, a mixture of 1700 µl of phosphate buffer (0.1M, pH 6.5) and 100 µl of 20 mM CDNB was prepared, and was incubated at 37 ° C for 10 min. The reaction was triggered by the addition of 100 µl of a solution of 20 mM GSH and 100 µl of cytosolic fraction to this mixture. A control was prepared under the same conditions with phosphate buffer. The enzyme was followed as the increase in the absorbance measured by spectrophotometry at 340 nm every minute for 5 minand calculated according to the following equation:

$$K = (DO_{control} - DO_{sample}) \times 100 / (9.6 \times 0.05).$$

The mitochondrial and cytosolic proteins concentration was measured by the method of Bradford, (1976) using e bovine serum albumin (BSA) as a standard.

Aiming to understand the contribution of pesticides versus HAP in the neurotoxic effect of the studied mixture as well as the effect of chronic mode of exposure versus the acute mode we conducted a complementary study where the exposure was acute and the mixture was consisted of the same compounds but with different proportions. Two proportions were used, the doses of pesticides of the first mixture (Mixture 1) were those of the mixture D folded by 1000, while the doses of HAP were the same as the mixture D. In the second mixture (Mixture 2), doses of pesticides were the same as the mixture D and doses of HAP were those of the mixture D folded by 1000. After 16 h, rats were sacrificed. Tests were run following the same protocols as the first study. We evaluated specifically MDA and GSH levels in whole brain mitochondria and in the cytosol of cortex, hippocampus, striatum and cerebellum.

2. 6. Statistical analysis

Data from behavioural and biochemical tests were analysed using a one-way analysis of variance (one-way 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. 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.

3. Results

3.1Behavioural tests

Morris Water maze test: In MWM, all groups tended to decrease 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 however, the group treated with D has shown instead a significant decrease in travelled distance and a non-significant decrease in latency time (Tab.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. 4), and a significant decrease in the cumulative time in the probe zone was observed in the group treated with D×100 (Fig. 5).

Table1 *Effects of the POPs mixture on memory and learning in the 5 th acquisition session in MWM*

Variable	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 t-test was used for multiple comparisons.***P<0.001**P<0.01*P<0.05 as compared to control.*

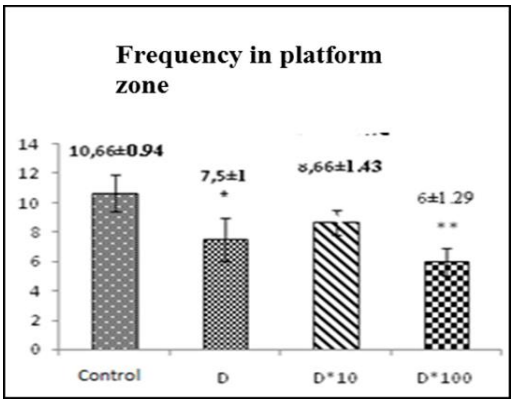

Fig.4 *Effect of the POPs mixture on frequency of crossing the platform zone in the probe test of MWM.*

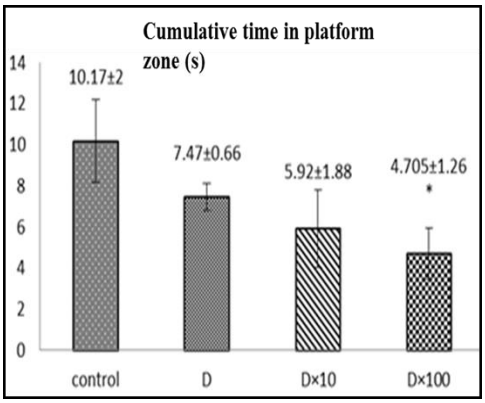

Fig.5 *Effect of the POPs mixture on cumulative time passed in the platform zone in the probe test of MWM.*

*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*

Open field test: In the OF test (Tab. 2), a significant effect of the treatment on the number of rearing was observed by one way ANOVA. Post hoc Bonferroni's t-test revealed a highly significant decrease in rearing 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 tended to increase 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 aria 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 as revealed by one way ANOVA.

Table2 *Effects of POPs mixture on locomotor activity and anxiety assessed in the open-field.*

Variable	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.*

Light dark box test: In LDB, one way ANOVA revealed a significant effect of the treatment on the latency time and the time spent in the light compartment. Post hoc Bonferroni's t-test showed that only exposure to the dose D×100 and D has induced a significant increase in these two parameters (Tab.3). On the other hand, one way ANOVA has revealed no significant effect of the treatment at any dose on the number of transitions and the number of entries to each compartment.

Table3 *Effects of POPs mixture on anxiety assessed in the Light Dark Box*

variable	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.*

3.2. 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. 6). In Cerebellum, MDA also showed a highly dose dependent increase in all treated groups, however, in hippocampus Bonferroni's 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, we did not notice an obvious effect of studied mixture on lipid peroxidation since MDA levels were normal in all treated groups compared to control as revealed by one way ANOVA (Fig. 7).

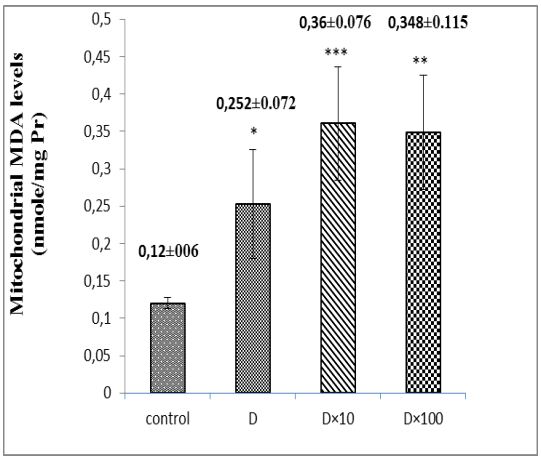

Fig.6. *Effect of the POPs mixture on whole brain mitochondrial MDA levels.*

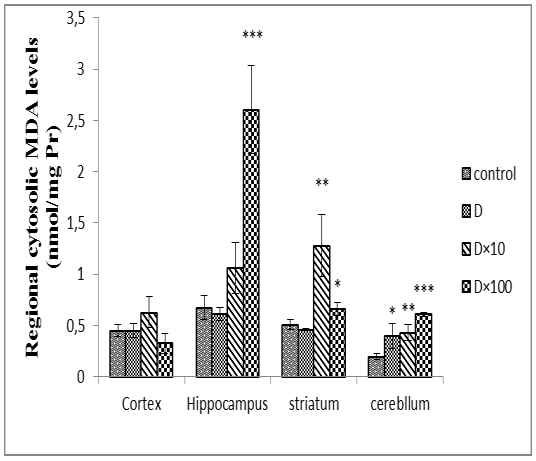

Fig.7 *Effect of the POPs mixture on regional cytosolic MDA levels.*

*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.*

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.8) 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 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.9).

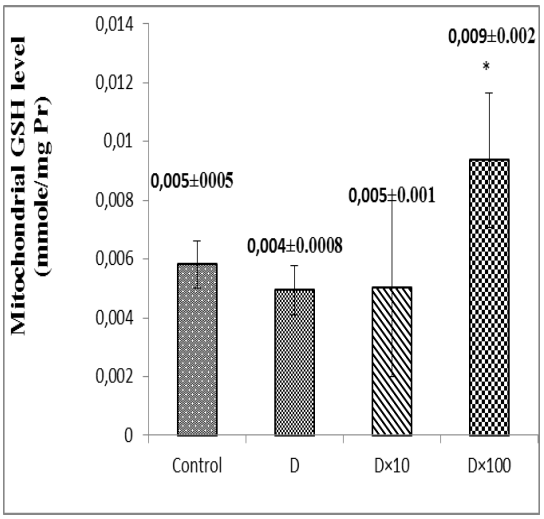

Fig.8 *Effect of the POPs mixture on whole brain mitochondrial GSH levels.*

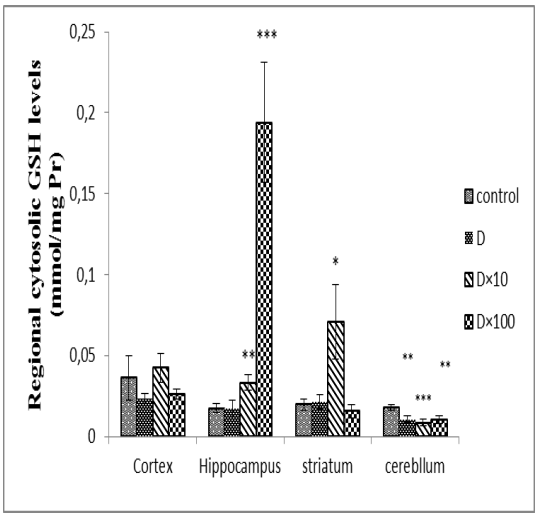

Fig.9 *Effect of the POPs mixture on cortex, hippocampus and striatum cytosolic GSH levels.*

*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.*

On the other hand, GSH and MDA levels are correlated in hippocampus and striatum, where Pearson test revealed a strong positive correlation ($r=0.88$, $P \approx 0$). 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, ($r=0.53$, $P=0.020$).

In cortex and similarly to MDA, GSH levels were not affected in all treated groups compared to control as revealed by one way ANOVA (Fig.6).

Antioxidant enzymes activity: Levels of antioxidant enzymes activity are presented in (Tab. 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 Bonferroni's 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's 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.

In contrary to the study of chronic exposure, acute exposure to the mixture with high doses of pesticides (D×1000) has caused death of 2/5 animals whereas the mixture with the high dose of PAH has induced no mortality.

Results of whole brain mitochondrial and regional cytosolic MDA and GSH levels are presented in (Tab. 5) where an increase in MDA levels was observed in mitochondria but only in the group treated with mixture 1 with the high proportion of pesticides; however, this increase was not significant as revealed by Bonferroni's t-test.

One way ANOVA revealed a non-significant effect of both mixtures on MDA levels in cortex. However, in striatum, both mixtures 1 and 2 have induced a statistically significant increase while in hippocampus only mixture 2 with the high proportion of HAP induced a significant increase in MDA as revealed by Bonferroni's t-test.

Regarding GSH, a non-significant increase was noticed in mitochondria of groups treated with both Mixtures 1 and 2. No significant effect was observed also in striatum and hippocampus. However and contrary to the first study where a decrease in GSH was observed in cerebellum, acute exposure to both mixtures 1 and 2 has induced a significant increase in GSH in this region as revealed by Bonferroni's t-test. Furthermore, in this study the mixture 1 was able to alter cortex by inducing a highly significant decrease in GSH levels.

Table 4 Effect of POPs mixture on antioxidant enzymes activity.

variable	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/mgPr)	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.*

Table 5 Effect of acute exposure to POPs mixtures 1 and 2 on whole brain mitochondrial and regional cytosolic levels of MDA and GSH levels

variable	control	Mixture 1	Mixture 2
MDA levels (nmol/mg)			
Whole mitochondria	0.0257±0.013	0.0541±0.002	0.0158±0.0046
Cortex	0.162±0.028	0.155±0.034	0.130±0.027
Hippocampus	0.0189±0.0036	**0.0145±0.0052****	0.039±0.005
Striatum	0.0199±0.0071	0.103±0.021	**0.0779±0.015*****
Cerebellum	0.0091±0.004	**0.128±0.042***	**0.0191±0.0028***
GSH levels (mmol/mg)			
Whole mitochondria	0.0038±0.0005	0.0063±0.0009	0.0050±0.01
Cortex	0.0079±0.0007	**0.0038±0.0006****	0.0063±0.01
Hippocampus	0.0037±0.0002	0.0043±0.0018	0.0037±0.0002
Striatum	0.0190±0.0071	0.0062±0.0004	0.0168±0.002
Cerebellum	0.0014 ± 8.10^{-6}	**0.0026 ± 0.0002*****	**$0.0016\pm6\times10^{-6}$***

*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.*

4. 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, neighbouring 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 organ-ism. In contrast to naphthalene, which is less toxic but wide-spread 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 frequent-ly 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 decrease locomotor activity (Saunders et al. 2006). The same result was observed in juvenile and adult rats after post-natal 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 (Terry 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 as 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 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 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 besides 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, it 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 mech-anisms between POPs (Lukaszewicz-Hussain 2008). 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 expo-sure 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 environ-mental dose, indicating that mitochondria is a privileged tar-gets to the mixture toxicity.

In fact, it is proven 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 (Hatcher et al. 2007; Hargreaves 2012) 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 environ-mental mixture D folded by 1000 increased instead GSH levels.

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).

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 benzopyrene 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 O2° in mitochondria (Massicotte et al. 2005) probably by respiratory chain enzymes which are known to be altered by OP and OC (Hatcher et al. 2007; 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 Ojha 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 CAT and SOD activities have increased when animals were exposed to chlorfenvinphos, even in a dose two times lower than LOEl (little observable effect level). On the other hand, it is well established that high levels of H2O2 inhibit CAT activity, which may explain the significant de-crease 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 in-crease of oxidative damage in the brain and disturbance of neurobehavioral abilities, such mild cognitive impairment (Praticò et al. 2002; Grova et al. 2007; 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 further targets, particularly those related to neurotransmission will provide a better image about the mechanisms of the toxic effect of complex environmental mixture of POPs.

5. Conclusion

Chronic exposure to the POPs mixture, used in this study, was able to induce neurobehavioral abnormalities and disturbed oxidative stress parameters in different brain regions of adult female rats. The results of the present study indicate that adult 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 oxidative stress induction and neurobehavioral alterations. Actually, the role of alteration of GSH homeostasis in oxidative stress induction remains unclear and requires more investigations. In this context, further research is required to well understand patterns of the brain response to dietary exposure to POPs mixture and their implications in aetiology of neurodegenerative diseases.

Chapter IV

Neurobehavioral deficits and brain oxidative stress induced by developmental exposure to a low dose mixture of persistent organic pollutants (POPs).

1. Aim of the study

The present study focuses on determination of the pro-oxidant and neurobehavioral effects of developmental exposure to an environmental mixture of POPs consisted of two pesticides (chlorpyrifos and endosulfan) and two PAHs (naphthalene and α-benzopyrene). The experiment design was carried out to evaluate the maturation of the early brain functions of the second adult generation, which had accumulated both the effects of prenatal exposure and effects of postnatal exposure via lactation. Two experimental aspects have been targeted in this work, namely neurobehavioral and neuronal redox status.

2. Materials and methods

2.1 Study design and animal exposure

We used sexually mature *Wistar albino* female and male rats provided from Pasteur institute (Algiers). (200 - 290 g). Rats were acclimatised for two weeks in standard cages at 5 rats /sex/cage, under normal light/dark cycle (light on from 8:00 p.m. to 8:00 a.m.), with free access to food pellets and tap water. After two weeks of habituation, two female rats were mated with one male overnight and were examined the following morning for copulatory plugs. We singly housed female rat on the day on which a vaginal plug was present. One week before the delivery, we supplied a cotton nest square as source of nesting material. The day of parturition was considered to be the postnatal day 0 (PD 0). At PD 4 we recorded sex and individual pup weight. We reduced litters to eight pups, equally composed of both sexes. We treated dams daily with the POP mixture from the previous study with same doses D, D×10, D×100, where the environmental dose D consisted of chlorpyrifos (5,2µg/Kg/day), endosulfan (2,6µg/Kg/day), Naphtalene (0,023µg/Kg/day) and α-benzopyrene (0,002µg/Kg/day). The control group treated only with corn oil. The treatment started from the day 0 of gestation until day 24 of lactation using oral gavage to the dams.

2.2 Developmental and behavioural tests

We used a battery of tests that provides an assessment of neurodevelopmental milestones throughout the neonatal period of the 2end postnatal week. Then, we evaluated cognition, anxiolytic behaviour as well as muscular strength in the period of juvenile life (PD32-PD35).

Test of *Surface righting reflex :* It is an early developed reflex that allows the neonate animal, in a situation of imbalance or after a fall, to recover its normal quadruped position.

Procedure: Each animal was observed from the 7th day of postnatal age (PD7) to PD14 according to the method described by Heyser (2004) with modifications. The pup was initially deposited on the back on a horizontal board. No impulse should be given to it, which may facilitate its returning. The duration of the test is limited to 60 seconds. Initially placed on the back, the pup will swing to the right and to the left, trying to turn around and recover on its four legs. The time necessary for the animal to recover on its four legs is recorded. The time limit for the test is 60 seconds. If the pup does not respond in 60s, the test is stopped.

Negative geotaxis Test: The purpose of this test is to evaluate the vestibular function (balance of the axis of the body) and the motor coordination according to the method described by Heyser (2004).

Device: We used for this test a board (45 × 40 cm) inclined 45 ° to the horizon and covered with glass paper to prevent the animal from slipping (Fig1).

Procedure: Animals were subjected to this test from PD7 to PD14. Initially, each animal received a trial of (60s), in which we measured the time taken by the pup to complete a 180 degree turn when placed in a head-down position on the plywood surface of 45 degrees. The animal passes this test when it makes a rotation of 180 ° with respect to its initial position. The maximal duration of the test has been set to 60.

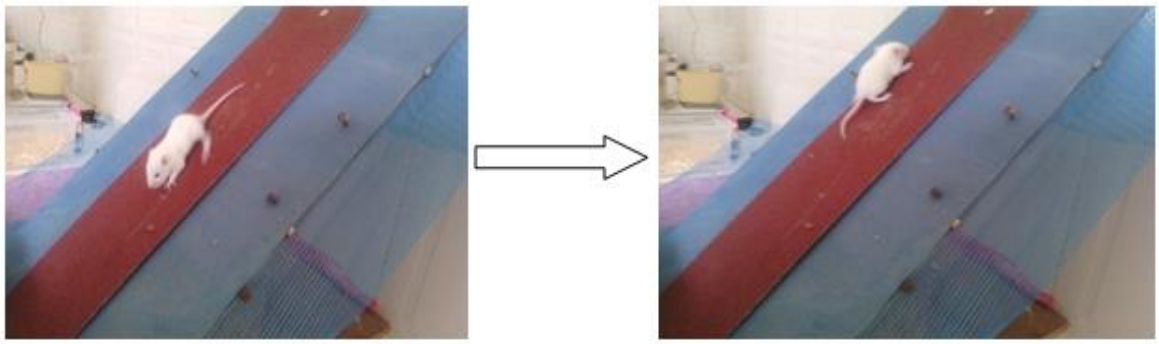

Fig.1 Negative geotaxis test

Test of Cliff Avoidance reflex: the purpose of the test is to evaluate the reflex of avoidance reaction of the animal with respect to a vacuum space. This reflex involves also the muscular strength and coordination of the anterior and posterior limbs of the pup according to the method described by Heyser (2004) with modifications.

Device: The material used to perform this test consists of a wooden block (L: 20 cm, 1: 10 cm, h: 10 cm) and a pile of wood shaving to receive the animal in case of a fall (Fig2).

Procedure: This test was performed starting from PN7 to PN14. The animal was initially placed at the edge of the platform, the head and forelegs in the void. The apprehension of the fall pushes animal to try to move away from the edge, either by retreating, or by rotating to the right or to the left in an attempt to restore himself entirely on the block of wood. An animal that fails to fully recover on the surface of the platform is considered unsuccessful. The time taken by the animal to find itself completely on the block (the head and the four legs) was registered. If the pup loses the pose and slips off the box or does not respond within 60 second, the test is stopped.

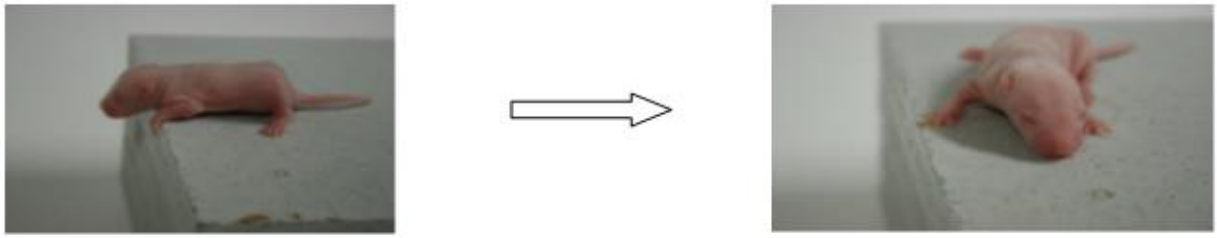

Fig.2 Cliff aversion test.

The Forelimb Grip-strength test: the purpose of this test is to evaluate the development of the physical resistance and muscular strength. This test was performed from PD7 to PD14, while the pups are still blind, where only the muscular force influences the performance of the animal. Because when they have their eyes open, they become able to perceive their environment and thus judge the danger that represented the fall, because pups may choose to drop if they consider that the fall is not dangerous or attempt to remain suspended as long as possible to avoid it, according to (Nevins et al. 1993; Heyser et al. 2004).

Device: A metal rod (0.5 mm in diameter) is stretched between the two poles of a grid about 20 cm above the table (Photo3).

Procedure: This test was performed from PD7 to PD14. The pup was suspended by its frontal legs holding the metal rod in a container with cotton to prevent it from falling out of the stem. The maximum duration or the animal remained hooked to the stem on two consecutive tests was recorded without any time limit.

Fig. 3 Forelimb Grip-strength test.

The inverted screen test: is a test aims to evaluate the muscle strength of all four limbs. This is a pure test of strength, although as for any test motivational factors could potentially play a role (Deacon 2013a).

Device: The inverted screen is a 43 cm square of wire mesh consisting of 12 mm squares of 1 mm diameter wire (Figure 4). It is surrounded by a 4 cm deep wooden beading (which prevents the occasional rat which attempts to climb on to the other side).

Procedure: this test has been carried out at PD32. Before the start of the experiment animals have been brought to the experimental room 5-20 min before. The rat was placed in the center of the wire mesh screen. The screen then, has been rotated the screen to an inverted position over 2 sec, with the rat's head declining first and steadily 40-50 cm above a padded surface. The time of the rat stayed attached to the screen before it fallen was noted. If the rat did not fall after 60 min the experiment is ended.

Test of Successive Alleys: the purpose of this test is to evaluate the anxiolytic and depressive behaviors of the animal. (Deacon 2013b).

Device: The successive alleys apparatus consists of four successive linearly connected alleys of putatively increasing anxiogenic character and is made of painted wood. (Fig5). The rationale for the different wall heights was: those of alley 1 need to be high to maximise its non-anxiogenic properties. Also high walls create a darker environment. The walls of alley 2 had to appear less safe than alley 1 to mice, but to still provide some degree of protection. Alleys 3 and 4 had just enough height to maximize anxiety but still provide a grip if the rat is in danger of falling off.

Procedure: the test was performed on PD33. Before the start of the experiment animals have been brought to the experimental room 5-20 min before. The rat is placed at the closed end of alley 1 facing the wall, and the total time spent in each alley is recorded for five minutes. When the mouse places all four feet on to the next alley it is considered to have entered it. Between trials the apparatus has been cleaned with 70% ethanol.

Alley	Length	Width	Wall height	Color
1	25.0	8.5	25.0	Black
2	25.0	8.5	5.0	Grey
3	25.0	3.5	0.8	White
4	25.0	1.2	0.2	White

Fig. 4 Successive Alleys test and dimensions of the alleys of the device (cm).

The Novel object recognition test (NOR): the purpose of the test is to evaluate the cognition and working memory of the rat. It is based on a spontaneous behaviour: the main assumption at the base of this test is that access to novelty (e.g. an object or an environment) can elicit approach behaviours in animals. NOR is consisting of two trials. In the first trial the animal is exposed to one or two identical objects (sample object). Following the sample object exposure, the animal is returned to his home cage for a retention period. In the second trial, which follows the retention time, the animal is returned to the environment (arena) and presented with a familiar (sample) and a novel object. When the subject 'remembers' the previous exposure to the familiar object, it will explore the novel object to a greater degree than that of the familiar one (Moscardo et al. 2012).

Device: the test was performed in a transparent, square, open glass arena (50 cm × 50 cm × 40 cm). Two identical white plastic cubes and a third one in pink were used as the sample and novel object respectively. A video camera attached on the wall above the room has been used to record the test session for ulterior data analysis. (Fig5).

Procedure: the test was consisted of two days. In the first day, animal went through a simple habituation session in the empty arena for ten minutes. The next day (PD35) animals went through to sessions, in the first one the animals were exposed to the sample objects (white cubes) for ten minutes than retrieved to their home cage. After an interval time of 45 minutes, animals went through another session, but one of the white cubes has been changed with the novel object (pink cube) the session time was also ten minutes. The two sessions were videotaped and cumulative time spent by animals exploring with the novel object was calculated.

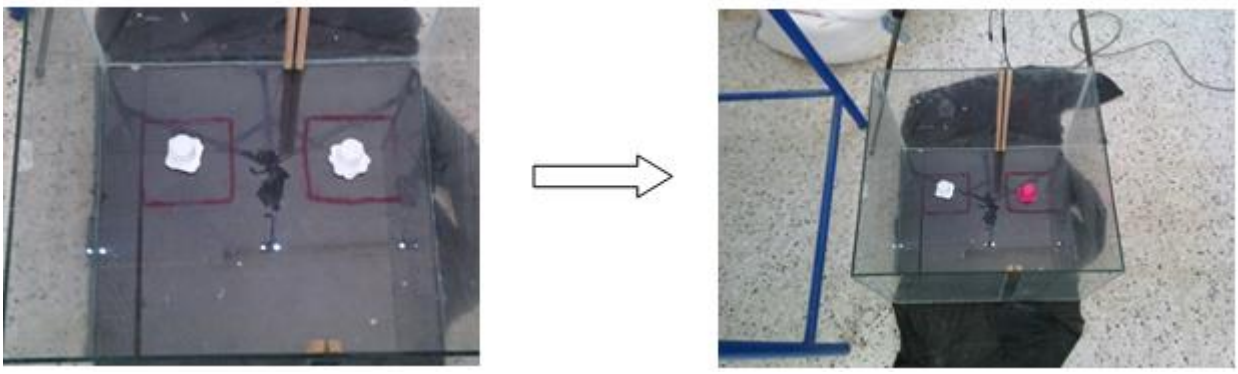

Fig.5 Novel object recognition test.

2.3 Biochemical analysis

At the end of the study (PD40), the animals were sacrificed by decapitation, we used the whole brain for extraction of the cytosolic and mitochondrial fraction and the quantification of the oxidative stress parameters according same protocols mentioned in the previous chapter.

2.4 Statistical analysis

Developmental motor and behavioural data were analysed by two-way repeated measures ANOVA with dose and time as main factors and is expressed as group means ± standard deviation of the mean. When ANOVA was significant, controls versus treatment group comparisons were made by the Bonferroni multiple range tests. Antioxidant parameters data were analysed by one way ANOVA. Group comparisons were made using the Bonferroni post hoc test when ANOVA was significant. The criterion for statistical significance was $P < 0.05$ for all parameters

3. Results

3.1 Developmental and behavioural tests

Body weight: we measured the gain weight in pups every day during the second postnatal week, than, each four days until PD44 (Fig 6). ANOVA and Post hoc analysis revealed that only groups treated with the high and low doses expressed a significant decrease in body weight during the first days of second postnatal week. In the same groups, we noticed also a significant decrease between PD 18 and PD24. At PD24 we noticed also a significant decrease in both males and females exposed to D×10. Between PD28 and PD32 we noticed a significant decrease in males of the three exposed groups, than the weights levels returned to normal for the rest of the study. Interestingly in female rats between PD 32 and PD 40 we noticed an increase in body weight compered to control. This increase was significant at PD32 in females treated with D×10 and at PD 36 in females treated with D. by the end of analysis at PD44, weight of females treated with D returned to normal compered to control, and we noticed a decrease but insignificant in weight of females exposed to D×10 and D×100.

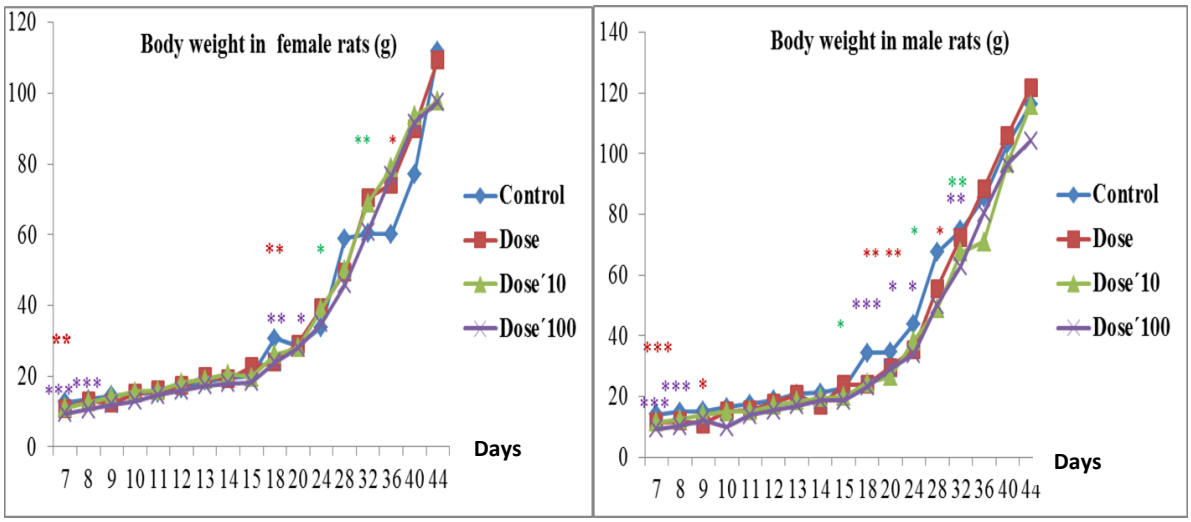

Fig.6 Effect of developmental exposure to the studied POPs mixture on the wain eight of exposed animals from PD7 to PD44.

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=8). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect (***) denotes very highly significant effect (p <0.001)

Righting surface reflex: according to ANOVA analysis we noticed a significant decrease in the development righting reflex as reflected by an increase in the time needed for pups to place the four lambs under the body (Table 1 and 2). In the group treated with D×100, Post hoc analysis revealed a significant decrease in performance in male pups starting from PD9 and persisted until PD13, while in female pups from the same group the effect was significant only on PD10, 12 and 13. In the group treated with D×10 the decrease in performance was significant only on PD 10 (P<0,05) and PD 13(P<0,01) in female pups and only on PD13(P<0,05) in male pups. In the group treated with the environmental dose D the decrease in performance was significant in male pups on PD8 and 9 (P<0,05) and at PD10 (P<0,05) in female pups as compared to the control group.

Table 01: Effect of developmental exposure to the POPs mixture on maturation of the surface righting reflex in female pups during second postnatal week.

	Latency of the surface righting reflex in female pups (seconds)			
PD	**control**	**Dose**	**Dose´10**	**Dose´100**
7	1.618±1.31	1.891±0.41	1.713±0.16	4.688±1.003
8	0.746±0.18	2.015±0.98	1.166±0.32	1.717±0.88
9	1.026±0.29	1.2±0.22	1.121±0.17	1.762±0.655
10	0.38±0.06	**1.043±0.50***	**0.854±0.22***	**1.43±0.28*****
11	0.787±0.16	0.597±0.16	0.939±0.3	1.372±0.69
12	0.485±0.09	0.613±0.25	0.56±0.25	**1.533±0.54***
13	0.247±0.06	0.508±0.19	**0.536±0.08****	**0.866±0.20*****
14	0.375±0.142	0.506±0.18	0.452±0.27	0.513±0.10

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=8). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect. (***) denotes very highly significant effect (p <0.001)

Table 02: Effect of developmental exposure to the POPs mixture on maturation of the surface righting reflex in male pups during second postnatal week.

	Latency of the surface righting reflex in male pups (seconds)			
PD	**control**	**Dose**	**Dose´10**	**Dose´100**
7	0.83±0.22	2.52±0.9	1.695±0.24	2.99±0.56
8	0.786±0.24	**2.5±0.81***	1.123±0.31	1.87±1.04
9	0.85±0.21	**2.17±0.9***	1.92±0.31	**1.82±0.60***
10	1.021±0.63	1.125±0.47	0.9±0.3	**1.326±0.46****
11	0.615±0.37	0.674±0.34	0.947±0.3	**1.14±0.28****
12	0.481±0.26	0.581±0.115	0.705±0.1	**1.271±0.34***
13	0.328±0.11	0.572±0.171	**0.527±0.21***	**0.91±0.115***
14	0.253±0.05	0,47± 0.12	0.36±0.05	0.54±0.171

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=8). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect (***) denotes very highly significant effect (p <0.001)

Negative geotaxis test: ANOVA analysis revealed that exposure to the POP mixture during development altered the development of the reflex of negative geotaxis in both female and male pups over the second postnatal week (table 3 and 4). Post hoc test revealed that only the highest dose D×100 altered significantly the development of the reflex from PD7 and until PD14, pups spent a longer time to accomplish the test, the effect was significant on PD 7, 9 and 11 in female pups (P<0, 05). In male pups, the effect was more severe where we noticed a significant effect on PD 7 (P<0,05) and (P<0,01) on PD 8, 9, 13 and 14. The environmental dose D induced a significant decrease in performance only on PD9 (P<0,05) in both male and female pups with the intermediate dose D×10 did not affect the reflex during all tested time points in both sexes as compared to control.

Table 03: Effect of developmental exposure to the POPs mixture on maturation of the negative geotaxis reflex in female pups during second postnatal week.

	Latency of negative geotaxis reflex in female pups (seconds)			
PD	**control**	**Dose**	**Dose´10**	**Dose´100**
7	24.793±23.47	53.938±10.10	43.43±16.56	**60±0***
8	18.811±13.72	32.33±11	27.26±13.58	46.66±13.77
9	21.03±5.48	**49.5±14***	18.48±4.17	**47.05±13.78***
10	28.87±15.36	25.73±11.42	14.43±5.07	38.30±14.69
11	10.271±4.50	7.33±1.80	8.8±1.26	**37.56±14.62***
12	9.976±2.2	6.1±1.58	10.11±4.62	9.91±2.95
13	5.283±0.97	4.173±1.10	4.56±2.81	8.52±2.98
14	3.22±0.75	3.406±1.44	3.86±0.58	6.17±3.27

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=8). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect (***) denotes very highly significant effect (p <0.001)

Table 04: Effect of developmental exposure to the POPs mixture on maturation of the negative geotaxis reflex in male pups during second postnatal week.

	Latency of negative geotaxis reflex in male pups (seconds)			
PD	**control**	**Dose**	**Dose´10**	**Dose´100**
7	28.91±20.72	46.91±17.45	53.24±11.25	**60±0***
8	17.19±8.84	43.2±21.2	42.25±18.3	**51.76±8.85****
9	7.35±2.05	**44.48±20.69***	26.81±13.58	**46.59±17.87****
10	26.34±13.17	11.50±2.92	20.64±9.12	41.53±18.46
11	6.91±2.02	6.10±1.083	20.56±15.05	32.08±18.60
12	9.87±4.34	5.49±1.39	15.41±6.95	18.59±13.80
13	4.5183±1.17	3.47 ±1.02	6.61±1.75	**8.63±1.54****
14	3.91±0.64	3.52±0.705	3.03±1.077	**8.368±2.37***

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t (n=8). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect (***) denotes very highly significant effect (p <0.001)

Test of Cliff avoidance reflex: ANOVA analysis revealed a significant decrease in performance of the test, translated by an increase in the time taken by pups to retreat from the edge of the cliff (Table 5 and 6). Post hoc analysis revealed that D×100 induced a high significant effect on female pups starting from PD 9 (P<0,001) until PD13 (P<0, 05). However, in male pups of D×100 the decrease in performance was significant only on PD11 and 13 (P<0,05) . D×10 induced also a significant decrease in performance (p<0,05) in female pups on PD10 and 11. In male pups of D×10 the effect was significant (p<0,05) on PD 11 and

13 same as in male pups of D×100. Interestingly, the dose D induced a high significant effect (P<0,001) but only on PD9 and only in female pups. The decrease in performance on the rest of time points of the study was insignificant (P>0,05) in both male and female pups.

Table 05: Effect of developmental exposure to the POPs mixture on maturation of the cliff aversion reflex in female pups during second postnatal week.

	Latency of cliff Aversion reflex by female pups (seconds)			
PD	**control**	**Dose**	**D´10**	**D´100**
7	36.84±23.16	31.873±14.53	42.22±19.49	60±0
8	38.17±21.83	40.498±7.031	33.96±17.36	53.66±10.55
9	14.563±4.28	**60±0*** **	32.3±18.46	**60±0*** **
10	10.328±3.045	28.4±11.06	**32.01±12.92***	**33.311±11.35***
11	10.325±1.965	28.04±21.3	**17.93±4.46***	**38.11±16.38****
12	10.888±3.635	21.77±5.61	10.416±3.51	**16.6±5.54***
13	4.336±1.243	6.728±1.09	8.45±2.48	**17.51±6.99***
14	5.181±2.98	10.45±38	3.033±0.68	6.50±0.60

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=8). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001).

Table 06: Effect of developmental exposure to the POPs mixture on maturation of the cliff aversion reflex in male pups during second postnatal week.

	Latency of cliff aversion reflex by male pups (seconds)			
PD	**control**	**Dose**	**D´10**	**D´100**
7	30.44±21.54	51.63±8.36	47.4±12.6	60±0
8	32.006±21,51	33.35±8.88	34.23±17.17	42.11±14.94
9	39.69±20,65	52.39±9.43	38.15±15.38	60±0
10	13.22±4,48	28.14±9.51	36.11±16.95	31.693±14.77
11	9.08±1,87	16.85±14.38	**37.66±16.4***	**25.021±11.29***
12	6.85±3,15	31.87±18.75	15.36±4.03	9.98±5.94
13	3.23±1003	5.703±1.13	**9.73±2.2***	**10.93±8.38***
14	3.85±1,47	16.44±10.52	3.26±1.09	4.99±1.71

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=8). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001).

Forelimb Grasp test: ANOVA analysis revealed a significant decrease in the muscular strength of treated pups compared to control (Table 7 and 8). Post hoc analysis revealed a significant decrease in female pups treated with D×100 at PD12 (P<0,05) and PD13(P<0,01). While in male pups the effect was significant only at PD9 (P<0,05). The intermediate dose D×10 induced a significant effect only in male pups at PD 8 (P< 0,05). And in the group treated with the environmental dose D, the decrease in performance was only significant in female pups at PD12 (P<0,05).

Table 7: Effect of developmental exposure to the POPs mixture on muscular strength in female pups during second postnatal week.

	Muscular strength of female pups (second)			
	control	**Dose**	**D´10**	**D´100**
7	7.53±3.3	8.2±3.86	3.56±1.23	2.066±0.95
8	5.28±2.81	9.233±4.56	1.51±0.58	1.55±0.31
9	3.63±1.97	4.35±1.21	1.91±0.65	1.816±0.62
10	7.48±4.85	7.11±2.61	2.91±0.55	2.566±1.13
11	6.66±1.57	7.83±2.26	11.28±4.52	4.316±0.97
12	12.29±2.04	**6.60±2.13***	11.75±5.5	**6.295±2.04***
13	10.45±2.53	9.716±3.3	9,6±1,7	**2.6±0.86****
14	10.08±2.65	9.463±3.06	10.55±2.88	5.366±2.6

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=8). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001)

Table 8: Effect of developmental exposure to the POPs mixture on muscular strength in male pups during second postnatal week.

	Muscle strength of male pups (second)			
PD	**control**	**Dose**	**D´10**	**D´100**
7	9.45±5.06	5.7±1.63	3.016±0.88	2.65±0.55
8	2.566±0.63	9.53±4.43	**0.75±0.3****	3.85±3.95
9	9.018±4.08	2.966±1.11	2.65±0.85	**0.83±0.21***
10	8.633±4.3	10.333±6.55	7.13±3.27	2.73±1.82
11	8.305±2.86	11.66±5.62	6.16±3.45	2.65±1.28
12	10.241±3.77	8.65±4.8	11.83±5.13	4.76±1.82
13	9.85±3.38	9.490±1.66	8.13±1.27	6.76±3.5
14	17.8±7.8	14.55±3.11	14.38±3.28	8.216±3.52

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=8). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001)

Muscular strength test: at PD 32, in the test of inverted screen ANOVA analysis revealed a significant effect of the treatment on the muscular strength of treated rats compared to control (Figure 7). Post hoc analysis revealed that D×100 and D induced a significant decrease in the time spent by rats hugged on the inverted screen (P<0,01) and (P<0,05) respectively, while the treatment with the intermediate D×10 did not induce any significant effect.

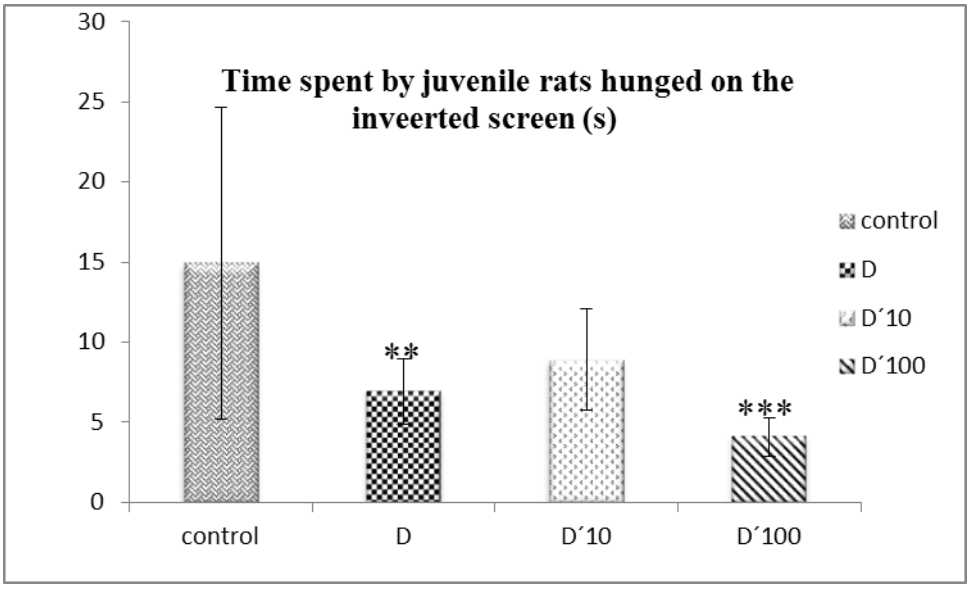

Fig.7 Effect of developmental exposure to the studied POPs mixture on the muscular strength of juvenile rats at PD32 in the inverted screen test.

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=6). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001

Successive alleys test: at PD 33, ANOVA analysis revealed a difference in the anxiolytic behaviour of juvenile rats treated with the POPs mixture compared to control group (Figure 8). Exposure to the highest dose D×100 induced an increase in the cumulative time spent by male pups in the closed section of the device (alley 1), although, the effect was insignificant (P>0, 05) as revealed by Post hoc analysis. The intermediate and the low dose affected only female juveniles and induced an increase in the time spent in the closed ally (alley 1) reflecting thus, an anxiolytic behaviour. However, the effect was significant only in the group treated with the intermediate dose D×10 (P<0, 01). In contrary, in the group treated with the high dose D×100, we noticed a significant decrease in time spent by female juveniles in the closed alley (1) and an increase in the time spent in the open alley (2) as compared to control which reflects an anti-anxiolytic behaviour.

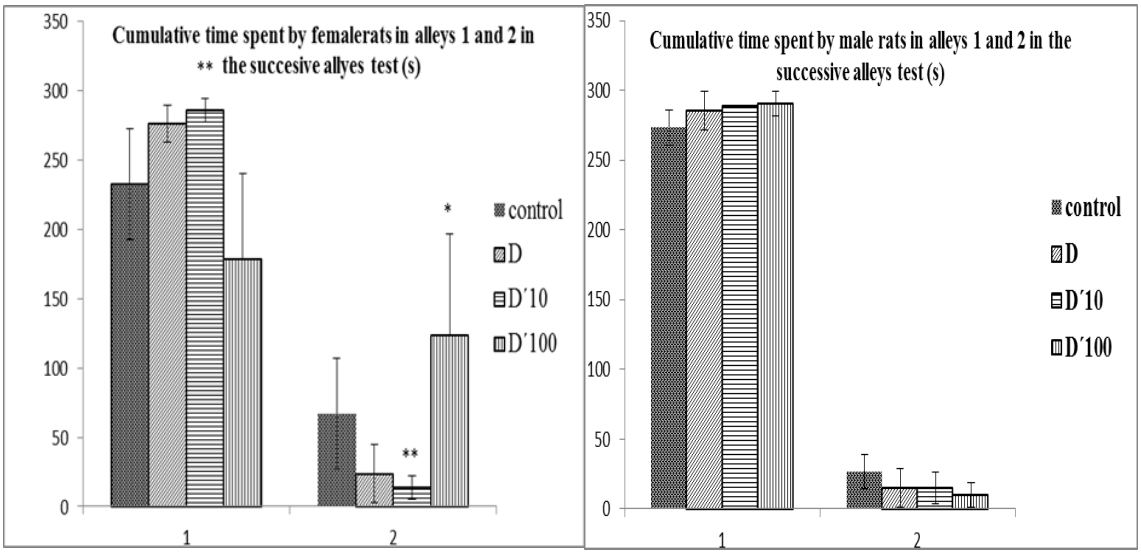

Fig.8 Effect of developmental exposure to the studied POPs mixture on anxiolyitic behavior in the succesive allyes test at PD33.

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=6). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001).

Novel object recognition test: at PD 35, we noticed that mice treated with the intermediate and the high dose abnormally spent a shorter time in exploring the novel object than the time thy spent in exploring the old objet (Fig.9), in contrary to the rats in the control group and the group treated with the low dose, thy spent a longer time exploring the new object.

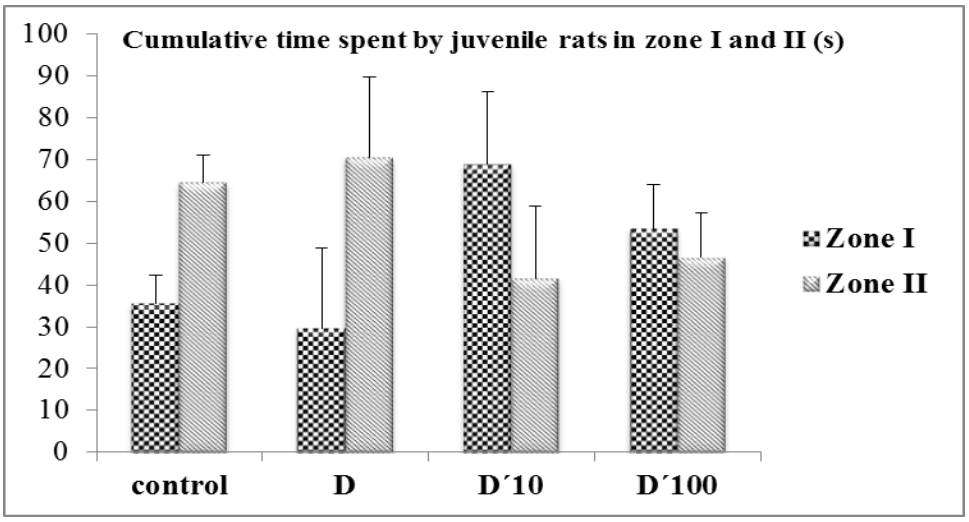

Fig.9 Effect of developmental exposure to the studied POPs mixture on working memory of juvenile rats in NOR test at PD35.

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n= 3females+3males). (P <0.05), (*) denotes significant effect (p<0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001).

3.2 Oxidative stress parameters

MDA levels: ANOVA analysis revealed a significant change in mitochondrial and cytosolic MDA levels (Figure 10 and 11). Post hoc analysis revealed that in mitochondria, only exposure to D×100 induced a significant increase, but selectively to female rats (P<0,05). In cytosol, we noticed a significant increase in MDA levels of males treated with D×100 and D×10 (P<0,01) while in females the effect was less significant (P<0,05). The environmental dose D induced instead an increase in MDA levels selectively in female rats (p<0,05).

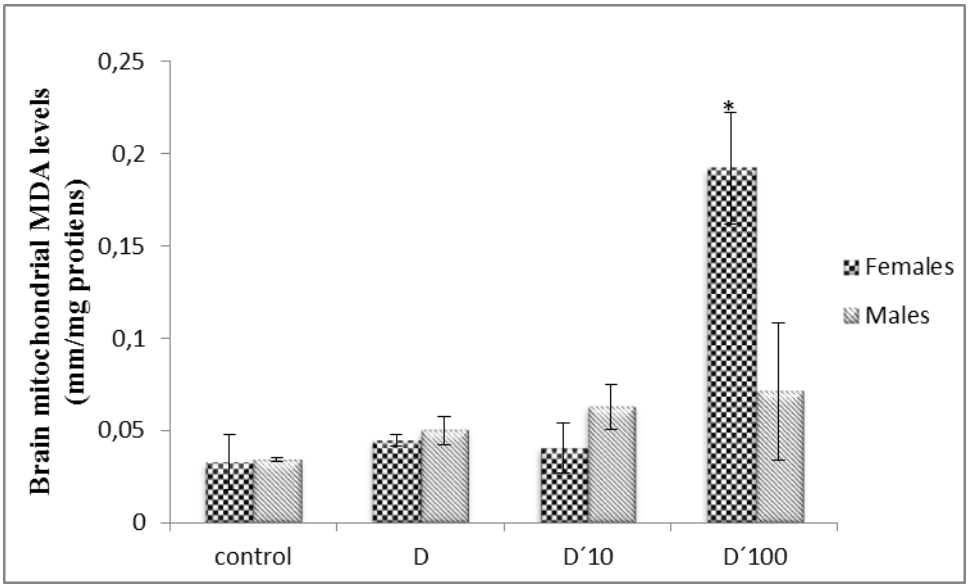

Fig.10 Effect of developmental exposure to the POPs mixture on mitochondrial MDA levels of female and male rats at PD 45.

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=4 females, 4 males). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001).

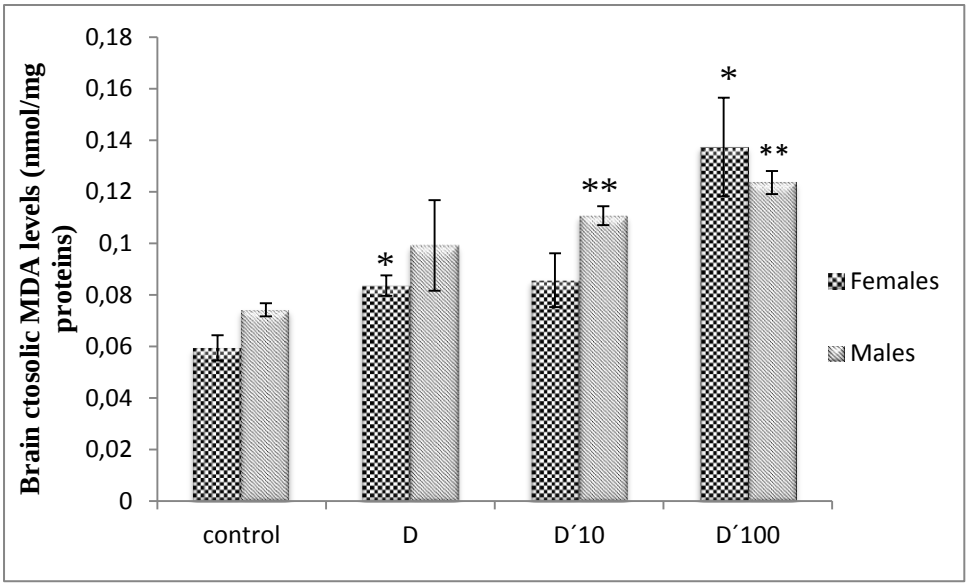

Fig.11 Effect of developmental exposure to the POPs mixture on cytosolic MDA levels of female and male rats at PD 45.

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=4 females, 4 males). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001).

GSH levels: ANOVA analysis revealed also a significant effect on GSH levels in both cytosol and mitochondria (Figure 12 and 13). According to Post hoc analysis, exposure to the high dose D×100 induced a sever decrease in mitochondrial GSH levels (P<0,01) but only in male juveniles. Exposure to D×10 induced instead an increase in mitochondrial GSH levels also selectively to male juveniles (p<0,01), while the environmental dose D did not induce any change in mitochondrial GSH. Same in cytosol, D did not induce any significant change in GSH levels. In the group treated with D×10 we registered an increase in GSH of males and females but it was statistically insignificant (p>0,05), while D×100 induced a very significant increase and in both male and female rats (P<0,01).

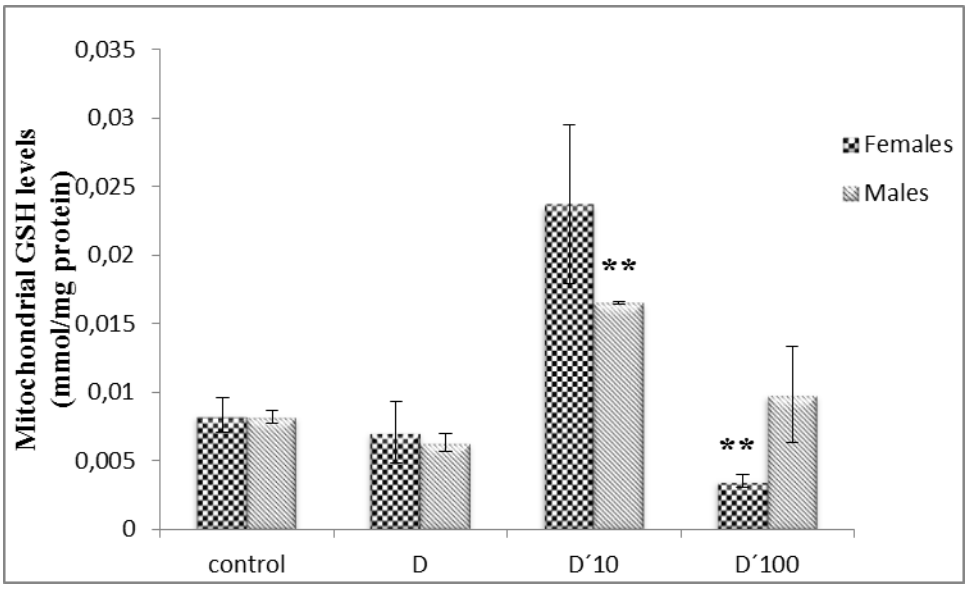

Fig.12 Effect of developmental exposure to the POPs mixture on mitochondrial GSH levels of female and male rats at PD 45.

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=4 females, 4 males). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001)

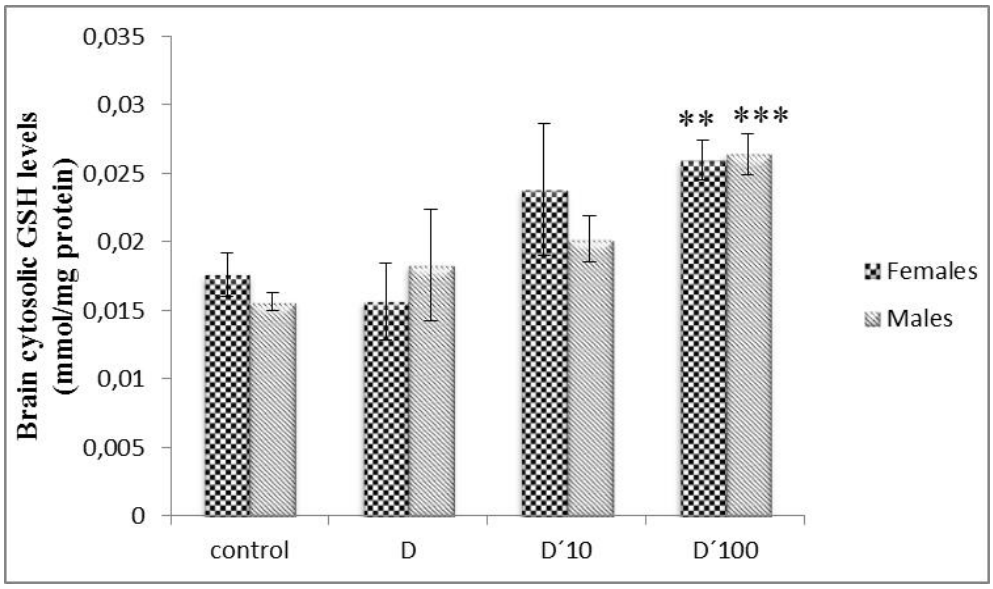

Fig.13 Effect of developmental exposure to the POPs mixture on cytosolic GSH levels of female and male rats at PD 45.

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=4 females, 4 males). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001)

SOD activity: ANOVA analysis revealed a significant change in SOD activity (Figure 14). Posthoc analysis revealed that exposure to the low dose D did not affect SOD activity and increased significantly only in male rats of the group exposed to the highest dose D×100

(P<0,05). We noticed also an increase in SOD activity in female rats of D×100 and both male and female rats of D×10, however the effect was statistically insignificant (P>0,05).

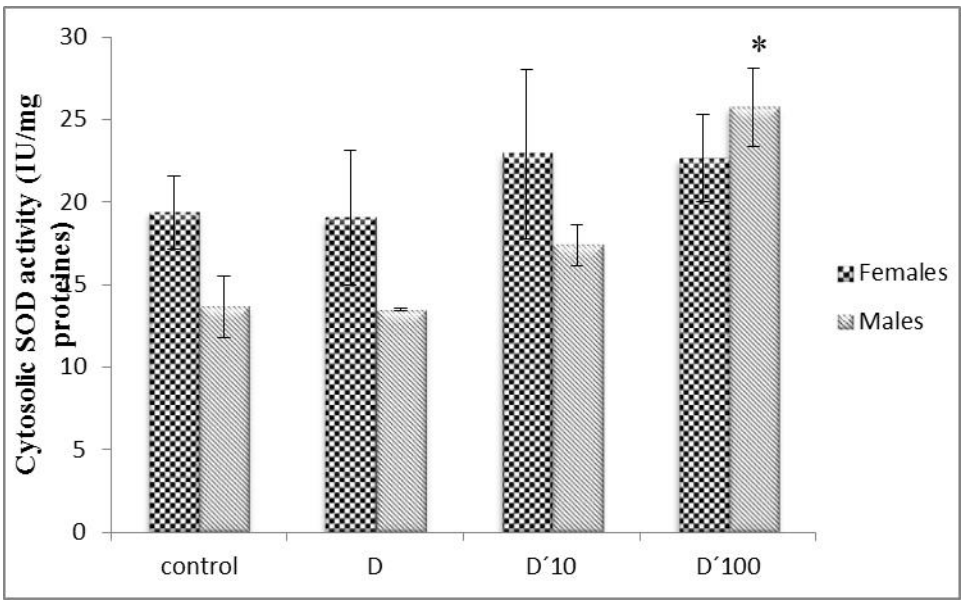

Fig.14 Effect of developmental exposure to the POPs mixture on cytosolic SOD activity of female and male rats at PD 45.

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=4 females, 4 males). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001)

CAT activity: ANOVA analysis revealed that exposure to the POPs mixture altered CAT activity (Figure 15). Post hoc analysis revealed that D, D×10 and D×100 reduced significantly CAT activity in male rats (D , D×100 p<0,05), (D×10 P<0,01). CAT activity in female rats has not been changed in both groups D and D×10 (P>0,05), in contrast to female rats of the group D×100, where we noticed a significant increase in CAT activity (p<0,05).

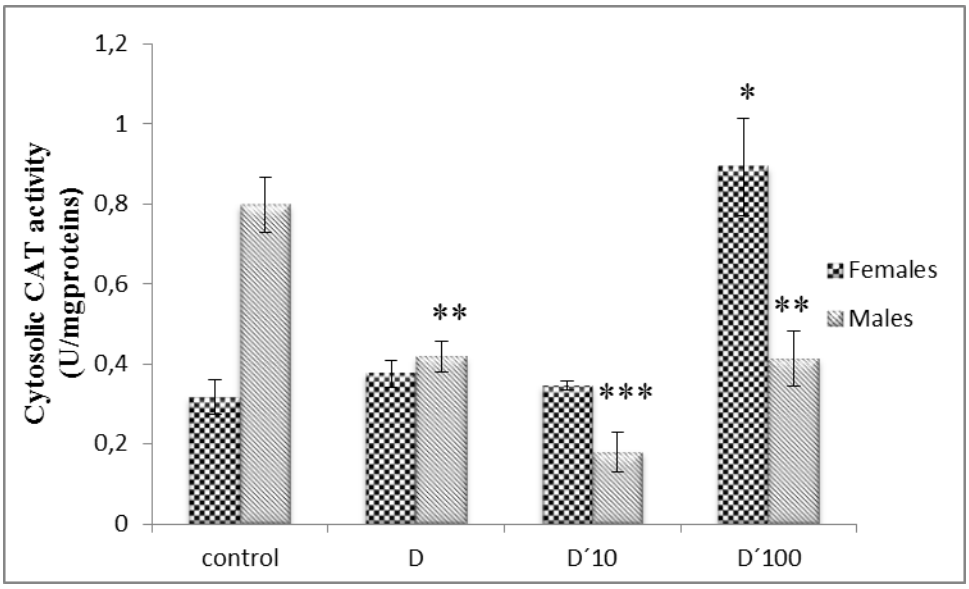

Fig.15 Effect of developmental exposure to the POPs mixture on cytosolic CAT activity of female and male rats at PD 45.

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=4 females, 4 males). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001).

GST activity: ANOVA analysis revealed a significant effect in GST activity. Post hoc analysis revealed that only female rats exposed to D×100 expressed a significant increase in GST activity (P<0,05), we noticed an increase as well in male rats from the same group however the effect was insignificant (p>0,05). D×10 and D did not induce any significant effect on GST activity neither in female or male rat.

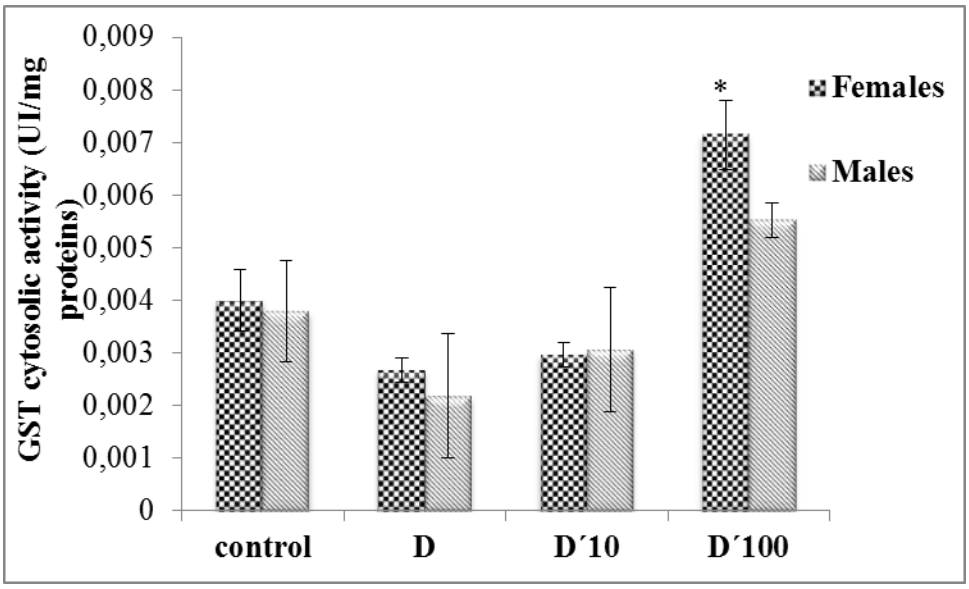

Fig.16 Effect of developmental exposure to the POPs mixture on cytosolic GST activity of female and male rats at PD 45.

The results are expressed as average ± standard deviation comparison between the treated and control made by Bonferoni t test (n=4 females, 4 males). (P <0.05), (*) denotes a significant effect (p <0.01), (**) denotes a highly significant effect, (***) denotes a very highly significant effect (p <0.001).

4. Discussion

In 1990, Barker and his colleagues introduced for the first time the concept of developmental origins of diseases based on conclusions from epidemiological studies indicating that low birth weight is correlated with the development of heart diseases later in life (Osmond & Barker 2000). This concept has been expended to include exposure to pollutants and its possible implications in the developmental basis of neurodegenerative diseases. In fact, several epidemiological studies have reported that developmental exposure to pollutants and particularly POPs alters cognition and memory in children. Experimental studies revealed several mechanisms of toxicity that could alter brain development and pave the way to neurodegeneration later in life. Induction of oxidative stress is one of the most proposed mechanisms (Gupta et al. 2004); however, most of these studies used high doses of exposure which does not reflect in general human developmental exposure to POPs. In the present study we used a mixture of organochlorine and oraganophosphorous pesticides as well as PAH with environmental doses (D). To evaluate the effect of the dose, we used also an intermediate and a relatively high dose of the same mixture (D×10 and D×100 respectively).

In general, developmental exposure to the POPs mixture used in this study induced some developmental deficits and altered brain redox homeostasis later in life. Some of the effects were irreversible, sex dependent and do not follow a monotonic pattern for the dose.

Exposure to the POPs mixture in its three doses reduced pups gain of weight, which is an overall sign of developmental toxicity that could be an origin to the development of diseases later in life. weight loss and structural malformations are strongly associated with neurological disorders, and may indicate a prenatal developmental defect that cannot be reversed nutritionally. Indeed, developmental exposure to certain PAHs such as benzopyrene as well as OCs and OP has been reported to reduce birth weight and induce neurobehavioral disorders and alter intellectual development (Gilmore et al. 2006; Perera et al. 2009; Peiffer et al. 2011).

Measure of primitive reflexes in neonates is a good indicator on the effects of xenobiotics on the development of nervous system. Surface righting reflex and cliff Avoidance reflex are primitive and reflect well the development of sensorimotor system. We noticed that exposure to the POPs mixture in the three doses altered both reflexes but in varying degrees. The highest dose (D×100) induced the most sever effect over almost all the 2nd postnatal week.

In the same context, the negative geotaxis test showed that the pups took a longer time to perform the 180° turn in all groups of the study but particularly in the rats treated with the highest dose (D×100) in both sexes and less in the group treated with D, while in the group treated with D×10 we did not notice any effect as compared to the control. The aim of this test is to evaluate the vestibular function and the motor coordination of the pups. During test performance, we noticed that all the pups developed the antigravity reflex (negative geotaxis), however the treated pups failed down before the continuation of the 180 ° turn indicating that the deficit is specific to the development of motor coordination.

As for the Forelimb Grip-strength test, it aimed to evaluate muscular strength of posterior paths of pups. Effect of the pops mixture on muscular strength during the 2nd post natal week seemed to be slight compared to the effect on other reflexes and its presented significance only in the group treated by D×100.

The peak in brain growth in the human occurs near term (38–40 week gestation). In contrast to humans, rodents experience their peak brain growth postnatally particularly from PD7 to PD14 (Neal et al. 2004). In terms of brain growth velocity, germinal matrix composition,

neurochemical expression, electroencephalographic patterns, and synapse formation, the PD 9, rat brain is estimated to be roughly equivalent in neurodevelopment to that of a full-term human infant. Thus in this study, the disturbances noticed in terms of primitive reflexes reflect in fact alterations in the processes of brain development. Interestingly, we noticed that the environmental dose D altered the primitive reflexes, specifically at PD9, which is a key time point in brain development (Dobbing et al. 1982; Neal et al. 2004). In agreement with the actual results, it has been reported that lactatioanal exposure to benzopyrene at 2 and 20 mg/kg affects the neuromaturation of pups by significantly decreasing their surface righting reflex and negative geotaxis reflex (Bouayed et al. 2009). In human, Engel et al. 2007 reported that maternal organophosphate metabolites and organochlorine levels were associated with anomalies in primitive reflexes in a multi-ethnic cohort of neonates (n=311).

It is well known that alterations in brain development could induce delayed effects later in life. In the present study, although that the effect of the POPs mixture on the muscular strength during the second postnatal week was marginal, we noticed that the effect persisted in juvenile rats treated with the high and the low dose as reflected by the test of the inverted screen. At the same time, the POPs mixture in its three doses did not alter learning and memory of the juvenile rats as revealed by NOR test. Using the test of successive alleys, we noticed anxiolytic behaviour selectively in females of both groups treated with the intermediate and the high dose. Interestingly, in the group treated with D×10, female rats spent a long time in the closed alley and less time in the open alley reflecting thus, an anxiolytic like behaviour. In contrast, female rats treated with the D×100 expressed an anti-anxiolytic effect as they spent less time in the closed alley and a longer time in the open alley which is more likely a depressive behaviour. These results indicate again that for the same mixture, the dose is a key factor in determining mechanisms of neurotoxicity.

During development, oxidative stress alters signalling pathways leading to the disturbance of processes of brain maturation (Kaindl et al. 2006). In the adult brain, oxidative stress is implicated in triggering and promoting neurodegeneration. In the present study we assessed brain redox status in adult rats that were exposed to POPs during their foetal and neonatal life. We noticed that developmental exposure to the POPs mixture induced a state of oxidative stress in brain cytosol and mitochondria of adult rats. This state might partially explain the neurodevelopmental and behavioural abnormalities.

In mitochondria, only the high dose D×100 selectively in female rats increased lipid peroxidation and reduced GSH levels, which is a prove on potential mitochondrial dysfunction that will eventually lead to neurodegeneration. In cytosol, we noticed an increase in lipid peroxidation in all treated groups but in varying levels. We noticed also an increase in GSH levels. However the increase was significant only in the group treated with the highest dose D×100. The increase in GSH level in this group might be a response of adaptation, as it was accompanied by an increase in GST activity. In fact, GST plays a key role with GSH in the short term and long term adaptive responses to oxidative stress (Sthijns et al. 2016). SOD and CAT are the main antioxidant enzymes. It has been reported that developmental aspects as well as sex differences play a major role in determining the activity of these enzymes (Giergiel et al. 2013). Interestingly in the present study, we noticed that CAT activity in male controls was higher than CAT activity in female controls, in contrast to SOD activity that was higher in females compared to males. In the treated groups, we noticed an increase in SOD activity that reaches significance in males treated with the high dose. On the other side, exposure to the POPs mixture in its three doses reduced CAT activity in male rats and increased it in females treated with D×100. It has been reported that activity of the enzymes of the antioxidant system in the brain of female rats is depending on the influence of steroid hormones (Pajovic et al. 2008) what could explain the sex difference in response noticed in this study. POPs are also known to be endocrine disruptors. Endosulfan owns an estrogen like structure and it has been reported to modulate expression of estrogen dependent genes (Varayoud et al. 2008) leading to the alteration of processes like synaptogenesis and neurotransmission (Briz et al. 2011). On the other side, chlorpyrifos exposure has been correlated with decreased levels of testosterone. Developmental exposure to this pesticide has been reported to induce anti-androgenic effect and hypothyroidism in female rats (Jeong et al. 2006). Indeed most of neurodevelopmental effects of chlorpyrifos have been reported to be gender selective and related to endocrine disruption (Saunders et al. 2012). Ricceri et al. 2006 reported that developmental exposure to chlorpyrifos induced an anti-anxiolytic behaviour in female rats which is in accordance with the same effect noticed in female rats exposed to the high dose D×100. We noticed also only in this group mitochondrial oxidative stress that was manifested by an increase in lipid peroxidation and a decrease in GSH levels. From this level of investigation, it is not clear whether the anti-anxiolytic behaviour and the mitochondrial oxidative stress are related or independent effects. Mitochondrial oxidative stress could be a strong basis of initiation and progression of neurodegenerative diseases such as AD later in

life. Interestingly, female selectivity noticed in this study correlates with the reports from epidemiological studies indicating that female gender is a risk factor of AD (Tejada-Vera. 2013). Whether the early mitochondrial oxidative stress noticed in this study selectively in female rats would be developed to an AD like pathology in the aging brain, it remains a very tempting question to be investigated in the future.

5. Conclusion

Developmental exposure to the studied POPs mixture at low doses altered neurodevelopment induced behavioural deficits in juvenile life and oxidative stress in early adulthood. Further studies are required to define whether oxidative stress is a delayed effect of developmental exposure, or it was induced during development and contributed to the neurological disturbances induced during development and juvenile life. It would be quite important to continue the evaluation of effects of exposure on the aging brain since it will permit to understand further how developmental exposure to POPs is implicated in the aetiology of neurodegenerative diseases.

Chapter V

Neurotoxic effects induced by perfluorooctane sulfonate (PFOS) exposure in adult age; implications for Alzheimer's disease pathogenesis.

1. Aim of the study

For a long time, it has been thought that blood brain barrier (BBB) protects brain from exogenous and endogenous fluctuations. Today it is proved that brain is not that mush protected, it could be easily affected by peripheral changes like fluctuations in glucose metabolism and disturbances in insulin signalling. Moreover, many xenobiotics could cross the BBB and alter brain integrity. PFOS is one of the xenobiotics known to reach the brain and induce toxic effects especially during development; able to reach the brain and alter its integrity. Recently, it has been reported that developmental exposure to PFOS induced in the adult age an increase in βamyloid and Ptau (Zhang et al. 2016), two key factors in AD pathogenesis. PFOS is mainly known to alter lipid and glucose metabolism as well as insulin signalling. Since AD is strongly related to metabolic disorder, we attempted in the present study to evaluate the relationship between peripheral and central effects of PFOS and its implications on AD main pathways, APP/βamyloid pathway and tau/Ptau pathway. The study has been conducted in the University of Rhode Island (USA), department biochemical and pharmaceutical sciences, where bimolecular and biochemical tests have been conducted in the lab of neurodegeneration (dr. Zawia lab), and animal samples have been provides from the lab of dr. Slitt.

2. Materials and Methods

2.1. Animal exposure

Female mice of C57BL/6 strain were imported from Jaxon labs and housed at the animal facility of University of Rhode Island (URI, USA). 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. All experimental assays were carried out in conformity with international guidelines for the care and use of laboratory animals. Mice were fed a standard chow low-fat diet (lfd) or 60% Kcal high-fat diet (hfd). After four weeks, PFOS was introduced to the diet for another 10 weeks. Mice were divided into two main groups – diet alone or diet containing 0.003% PFOS (~360 µg/kg/day). This resulted in a total of four

treatment groups as follows: i) **lfd**, ii) lfd+PFOS **(lfdp)** , iii) **hfd** , iv) HFD+PFOS **(hfdp)**. By the end of exposure the mice were sacrificed and their brains dissected and cortices were stored at −80°C until its ulterior use.

2.2 Sample preparation and Western blotting

Brain cortical tissues were lysed homogenized in RIPA lysis buffer (lysis buffer: 150 mM NaCl, 25 mM Tris-HCl at pH 8.0, 1% NP-40, 10 mM NaF, 1mM Na3VO4), containing 1% protease inhibitors cocktail (Sigma-Aldrich, MO). The samples were vortexed for 5min prior to centrifugation at 10,000 x g for 20 min at 4°C to pellet the cell debris, the supernatant obtained was used for Western blot analysis. Protein concentration was determined by using a BCA kit (Pierce Biotechnology Inc. Rockford, IL). Immunoblotting was determined following overnight exposure at 4°C to the primary antibodies diluted at 1:1000: mouse anti-Tau 46 (Sigma Aldrich, MO), rabbit anti p-Thr-181tau, (Cell Signaling technology, MA), rabbit anti-CDK5, (Cell Signaling technology, MA), rabbit anti-glycogen synthase kynase3 (Cell Signaling technology, MA), rabbit anti- insulin degrading enzyme (IDE) (Sigma-Aldrich, MO), rabbit anti-amyloid precursor protein (Cell Signaling technology, MA). On the following day, membranes were washed and exposed for 1 h to goat anti mouse/goat anti rabbit IRDye® 680LT infrared dye (LI-COR Biotechnology, NE), diluted at 1:10,000. The images were developed using Odyssey infrared imaging system (Model- 9120, LI-COR Biotechnology, NE). As a control for equal protein loading, membranes were stripped and reprobed for 2 h with mouse glyceraldehydes 3 phosphate dehydrogenase (GAPDH) antibody or B actin antibody diluted at 1:5000 (Sigma-Aldrich, MO) at room temperature followed by washing and re-exposure to goat anti mouse IRDye® 680LT infrared dye. After transferring to a polyvinylidene fluoride (PVDF) membrane, the gel was stained with Bio-safe Coomassie blue stain (Bio-Rad, Hercules, CA) to assess the loading of the samples. The protein expressions of the various protein analysed in the present study where normalized against βactin or GAPDH, which is normally used as reference protein product.

2.3 Total RNA isolation, synthesis of complementary DNA, and real-time polymerase chain reaction

Total RNA was extracted from cortex tissue using Trizol reactive according to the manufacturer instructions. Briefly, 25 mg of the tissue were harvested and homogenised in 1 ml of Trizol, then centrifuged for 5 min at 12000g, 4 C°. 0.2 ml of chloroform were added to the recuperated supernatant and incubated for 3 min. the sample was then centrifuged for 15

min at 12,000g. The aqueous phase was recuperated in new tubes and homogenised with 600µl of isopropanol and precipitated at -20 C° for 2 hours. Isopropanol was removed after a centrifugation at 12000 g, 4 C° for 10 min, and the RNA precipitate was recuperated and washed three times in cold ethanol 75%, than air dried and recuperated in 30µl of RNAase free water. RNA purity and concentration were checked using Nano drop, than the RNA was stored at -80 C° until its ulterior use.

Complementary DNA (cDNA) was synthesized from 1.5 µg of total RNA using the iScript cDNA kit (Bio-Rad, CA) according to the manufacturer instructions. Briefly, 4µl of iScript reaction mix were added to 1µl reverse transcriptase and 1,5 µg of total RNA, the volume was completed to 20µl with nuclease free water. The reaction mixture was then incubated in thermal cycler (5 min at 25°C, 20 min at 46°C and 1 min at 95°C). cDNA was then amplified using real-time PCR. The SYBR Green qRT-PCR assays were performed in 12 µl reactions in replicates using 1.5 µl of cDNA template, 1×SYBR Green master mix (Thermo Fisher scientific, USA), 0.4 µM forward and reverse primers, and deionized water. The following primer pairs were used: APP forward primer 5′-GTG GCC AGG TGG AAG TAA AA-3', APP reverse primer 5′-TGG AAG ACA CAT TGC TGA GG-3′; Bactin forward primer: 5′-TGG TGA AGC AGG CAT CTG AG-3′, Bactin reverser primer 5′-CCA TTG CAG CTG TCG AAA TA-3′; Amplification was undertaken on an ABI PRISM 7500 machine (Applied Biosystems, Foster City, CA, USA) with sequence detection software version 1.3, and expression was reported relatively to β actin mRNA with the $^{2-\Delta}$ ΔCt method.

2.3 Sandwich ELISA measurement of Aβ40 and Aβ42

Aβ40 and Aβ42 were detected in cortex by ELISA using human Aβ40/Aβ42 assay kit JP27713 (IBL, Gunma, Japan). All the buffers used in this experiment belong to the kit. Briefly, 100 µg of total protein samples were placed in each well of a 96-well plate coated with monoclonal antibody specific for the Aβ35–40 and Aβ35–42 (1A10) and was incubated overnight at 4°C. On the following day, the 96-well plate was given extensive washings with EIA buffer for 10 times followed by addition of 100 µl of labelled antibody to each well containing sample or standard and incubated at 4°C for 1 h. The wells were again washed 10 times with EIA buffer followed by the addition of 100 µl TMB solution and were incubated in the dark for 30 min at room temperature. The reaction was terminated by adding 100 µl of $1NH_2SO_4$ and the colorimetric absorption was registered at 450 nm. The levels of Aβ40 and

Aβ42 in the test samples were calculated relatively to the standard curve generated for each plate.

2.4 Statistical analysis

Western blot bands were quantified by using the LI-COR Odyssey infrared image system. All measurements were made in triplicate and all values are presented as mean standard error of the mean. The significance of the difference among means of the experimental groups was obtained with one-way analysis of variance, the Tukey-Kramer multiple-comparison post-hoc test, and the student Newman-Keuls comparison post-hoc test, using Graph pad Prism 3.0 computer software (La Jolla, CA, USA). The level of significance was set at $P<0.05$.

3. Results

3.1 APP/ β amyloid pathway

To evaluate APP expression we quantified levels of APP protein by western blot and APP mRNA by rtPCR (Fig 1). ANOVA test revealed a significant difference between the groups of the study in both APP protein levels and APP mRNA. By the post hoc test we noticed that PFOS exposure induced a significant increase ($P<0,001$) in APP mRNA in both groups receiving normal diet and high fat diet. However on proteomic level, PFOS induced a significant increase, only in the group receiving the high fat diet, while, the group receiving the high fat diet without PFOS exposure didn't show any increase in APP proteins neither mRNA. These results indicate that PFOS is able to raise APP mRNA levels independently on the effect of high fat diet, however to increase APP proteins; PFOS exposure is dependent on the simultaneous exposure to high fat diet.

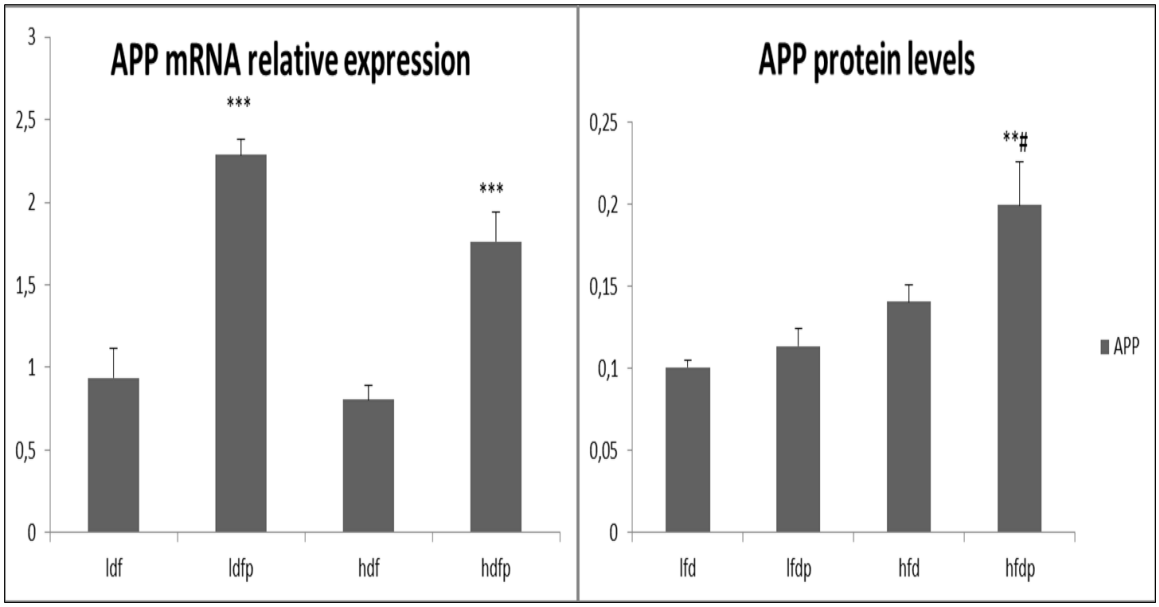

Fig.1. Effect of PFOS and high fat diet exposure on levels of cortex of relative APP mRNA and protein levels.

*Results are presented as mean ± SD. n=5.Newman-Keuls was used for multiple comparison*p<0.05, **p< 0.01, ***p<0.001 compared to the control group.#p<0.05 compared to LFDP group.*

β Amyloid 40 and 42 are the harmful end product of APP processing. The sedimentation of these peptides leads to the formation of neurotoxic β amyloid plaques, which is the main hallmark of Alzheimer's disease.

We quantified Aβ40 and Aβ 42 levels in the brain cortex by the Elisa test (Fig.2). Statistical analysis revealed that PFOS didn't induce any significant change in AB levels compared to control (p>0, 05). However, we noticed a slight but significant decrease in the group receiving only the high fat diet compared to control and groups treated with PFOS. Thus, high fat diet alone had a negative effect on AB formation, and the simultaneous exposure to PFOS has reversed the AB levels back to normal compared to control.

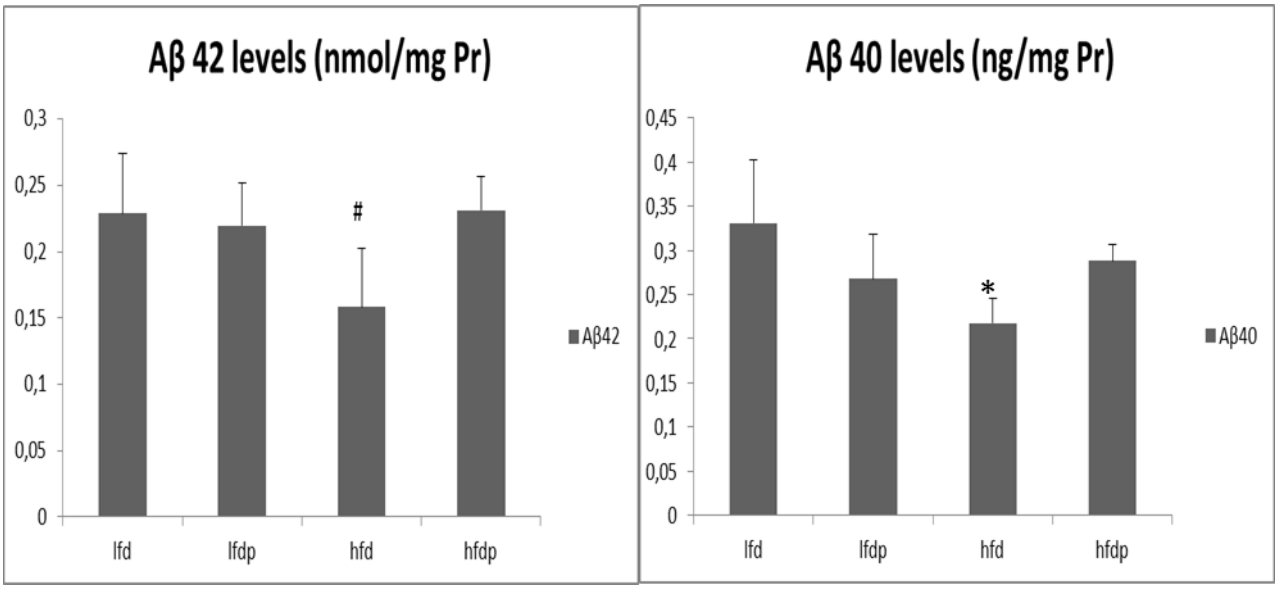

Fig.2 Effect of PFOS and high fat diet exposure on levels of Aβ 40 and Aβ42 in brain cortex

*Results are presented as mean ± SD. n=5. Newman-Keuls was used for multiple comparison *p<0.05 compared to the control group.#p<0.05compared to LFDP group.*

Insulin degrading enzyme (IDE) is a key enzyme in the APP/ β amyloid pathway because Aβ is one of its main substrates beside insulin. Insulin is an antagonist of (IDE), thus any perturbation in insulin will affect both Aβ levels and IDE activity and/or levels.

In this study we evaluated IDE levels by western blot (Fig.3). We noticed a significant increase in its levels by ANOVA test. Post hoc test revealed that this increase is highly significant in the group receiving PFOS and normal diet and in the group receiving only the high fat diet. Indicating that both factors; PFOS and the high fat diet are able to increase IDE levels independently from each other. However, PFOS and the high fat diet together exercised an additive effect on the increase of IDE level compared to the group receiving PFOS or high fat diet only.

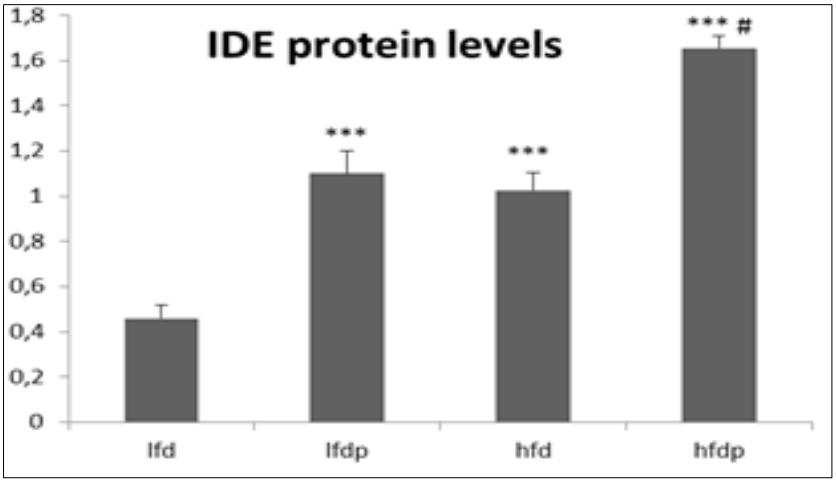

Fig3. Effect of PFOS and high fat diet exposure on cortex relativelevels ofIDE protein.

*Results are presented as mean ± SD. n=5. Newman-Keuls was used for multiple comparison*p<0.05, **p< 0.01, ***p<0.001 compared to the control group.#p<0.05 compared to LFDP group.*

3.2 Tau/Ptau Pathway

Tau is microtubule stabiliser protein assigned to back up the neuron structure. In the case of Alzheimer's disease, hyperphosphorylation of tau leads to the destabilisation of the microtubule and the collapse of the neuron structure. In this study, we quantified by western blot levels of tau, P tau and the main two tau kinases; CDK5 and GSK3β.

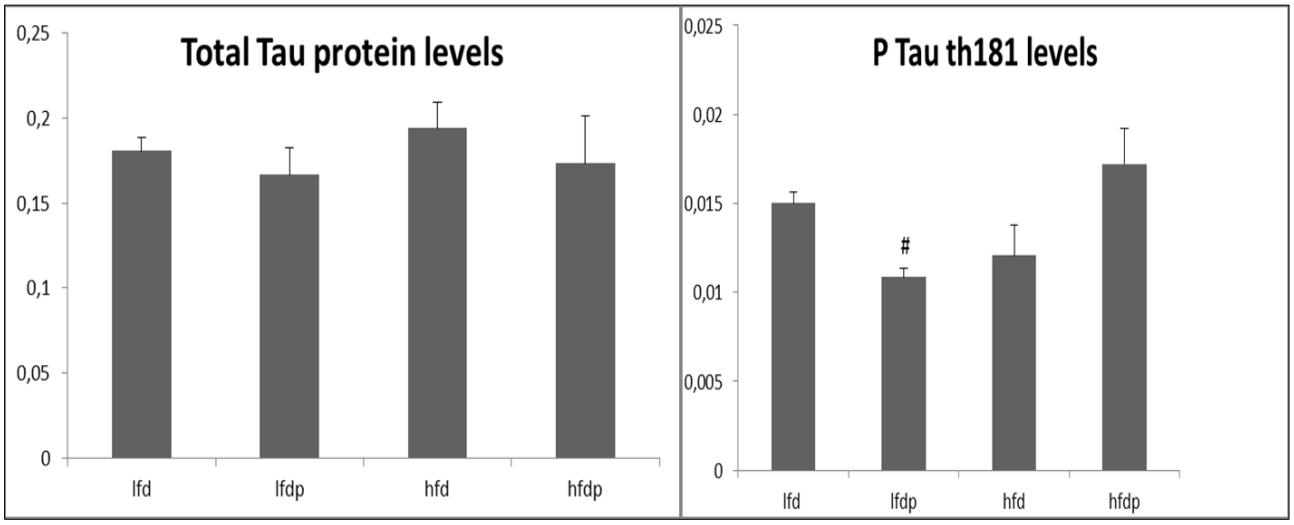

Fig4. Effect of PFOS and high fat diet exposure on cortex relative levels of total Tau and Thr181Ptau.

*Results are presented as mean ± SD. n=5. Newman-Keuls was used for multiple comparisons.*p<0.05 compared to the control group.#p<0.05compared to HFD group.*

ANOVA test did not reveal any difference in tau levels between the control and treated groups (P>0, 05). On the other side, we noticed a significant decrease in Thr181Ptau levels but only in the group treated with PFOS alone (LFDP) when compared to the group receiving simultaneously the high fat diet and PFOS (HFDP) as revealed by the post hoc test. In the groups receiving both PFOS and high fat diet levels of P tau were similar to+ those of control indicating that the high fat diet reversed the effect of PFOS on tau phosphorylation, at least on the site Thr181.

CDK5 levels did not change in the group treated with only PFOS compared to control. However we noticed a significant increase in both groups receiving high fat diet and high fat diet with PFOS (Fig. 5).

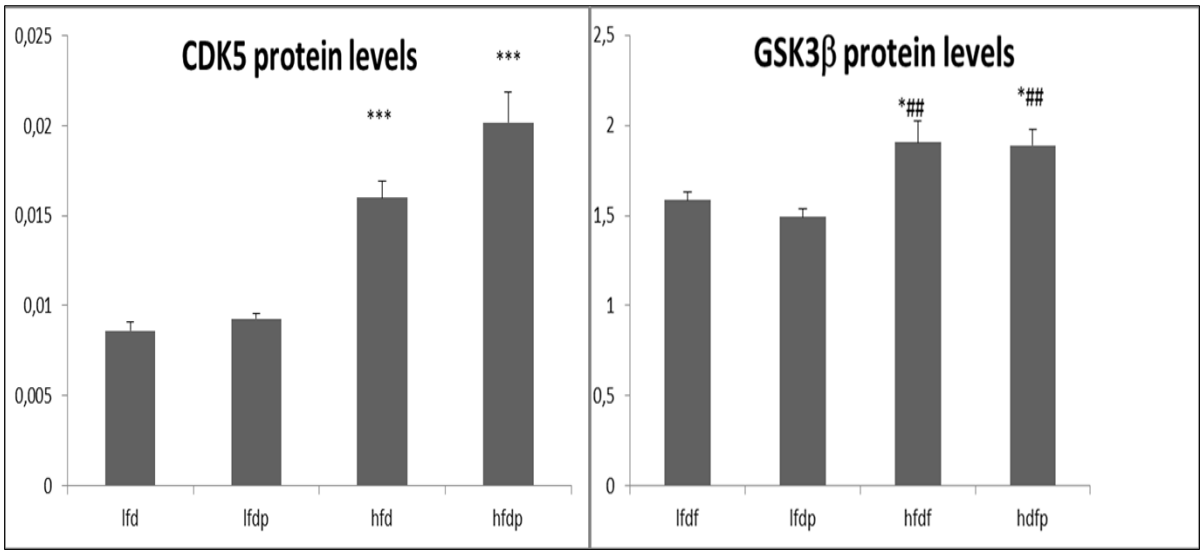

Fig.5 Effect of PFOS and high fat diet exposure on cortex relative levels of kinasesCDK5 and GSK3β.

*Results are presented as mean ± SD. (n=5) *p<0.05, **p< 0.01, ***p<0.001 compared to the control group.##* p <0.01 compared to the LFDP group.

Furthermore, post hoc test has revealed no significant change in CDK5 levels between these two groups indicating that the effect on CDK5 is more likely dependent on the high fat diet and independent on PFOS.

ANOVA test revealed also a significant change in GSK3β levels (Fig. 5). The post hoc test revealed a significant increase in the groups receiving high fat diet and high fat diet with PFOS. The increase seems to be dependent only on the high fat diet. Howe ever in the group treated only with PFOS we noticed a slight decrease in GSK3β levels, this decrease looks to be in correlation with the decrease in Thr181Ptau levels noticed in the same group, indicating that the two effects could be related to each other.

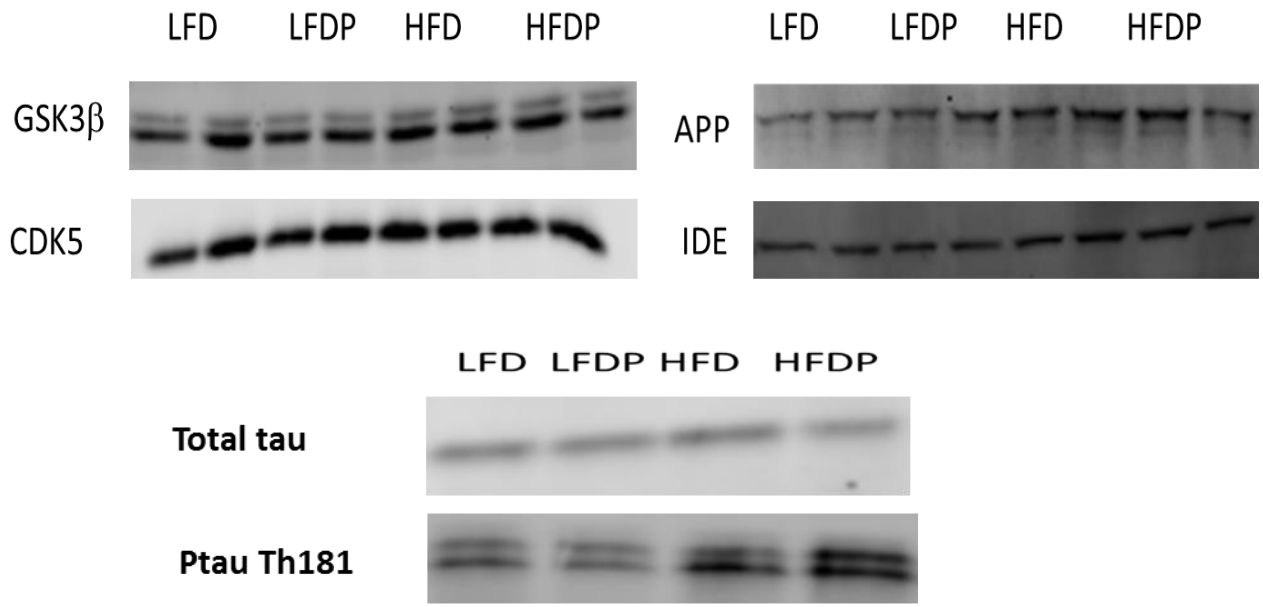

Fig.6 Presentation of Western blot analysis of proteins GSK3β, CDK5, APP and IDE ,taotal Tau and Ptau Th181

4. Discussion

PFOS is a POP able to cross the blood brain barrier (BBB) and alters brain integrity particularly during development by several mechanisms (Lee et al. 2013). Some of these mechanisms are directly related to AD (Johansson et al. 2008; Zhang et al. 2016). PFOS also is able to alter glucose metabolism and insulin signalling in peripheral tissues (Lin et al. 2009; Lv et al. 2013). It happens that alteration of glucose metabolism and insulin signalling are now considered as AD biomarkers (Li et al. 2015).

In the present study we found that adult exposure to PFOS is able to alter some AD biomarkers, furthermore some of these alterations are related to metabolic deregulations

induced by PFOS and the high fat diet. In a parallel study (data not published) carried out by the team of Dr. Slitt (department of biochemical and pharmaceutical sciences) on blood of the same animal cohort used in the present study, PFOS induced a hypocholestremia in the group receiving normal diet; however, exposure to the high fat diet alone or with PFOS induced a hyperinsulenemia in treated mice. In the same context, fasting blood glucose, glucose intolerance and gain of weight have been noticed in both groups receiving high fat diet (HFD) and high fat diet with PFOS (HFDP). However, PFOS alone induced instead a decrease in fasting blood glucose but only during the first two weeks of exposure.

APP expression, its processing and degradation play a key role in AD pathogenesis (Thinakaran et al. 2008). In the present study, exposure to PFOS induced an increase in levels of APP mRNA in both groups treated with the normal and the high fat diet. However, the increase in the APP protein appeared only in the HFDP group. Aβ40 and Aβ42 as pathologic end products of APP degradation are reported to be increased in high fat diet conditions, but only in transgenic mice with human APP gene, and not in the wild type mice (Maesako et al. 2015). The results of this study did not reveal also, a significant increase in Aβ levels, however, in the group receiving only the HFD we noticed a slight decrease in Aβ levels accompanied by a slight increase in levels of APP protein but not its mRNA. Probably the increase in cholesterol levels due to the high fat diet has led to an alteration in APP processing. Howland and collaborators (1998), indicated that cholesterol altered APP trafficking and decreased levels of Aβ in a mouse model of AD. Bodovitz and collaborators (1996), reported that cholesterol alters *in vitro* APP trafficking by increasing membrane rigidity. We noticed also an increase in blood cholesterol in both HFD and HFDP groups, while the LFDP has shown instead a decrease in blood cholesterol levels as we mentioned above; this could explain also why we noticed an increase in APP protein level in the group HFDP but not in the LFDP even though both groups have shown an increase APP-mRNA.

Levels of Aβ in high fat diet and its modulation by cholesterol may be dependent on the model of the study; Lin and collaborators (2016), reported instead an increase in Aβ levels but only in transgenic mice and not in the wild type mice. Rabbits are a common model of the increase of Aβ due to high fat diet and hypercholesterolemia (Sparks et al.1994). One of the proposed mechanisms is the decrease in IDE levels or activity, the enzyme responsible on Aβ degradation (Prasanthi et al. 2009). Insulin modulates IDE levels. Hyperinsulinemia could increase IDE levels, but also decrease it Aβ degrading activity due to competition with insulin

(Li et al. 2015). We noticed an increase in IDE levels in all treated groups compared to control. Although, we did not measure insulin in brain or blood, other insulin resistance signs in blood have been noticed in a previous study, like the increase in fasting blood glucose, glucose intolerance and gain of weight in HFD and HFDP groups. Contrarily, in the LFDP, the increase in brain IDE level was accompanied by a decrease in fasting blood glucose in the first two weeks of the study, then the effect diminished by the end of the study. PFOS was reported to increase insulin sensitivity in the liver; this could explain the decrease in fasting blood glucose. Then, the increase in IDE in the brain could be a response to PFOS actions in peripheral tissues, and a response to the direct effect of PFOS on the brain, for example, PFOS is an activator of peroxisome proliferator activating receptor (PPARγ) in the brain , and IDE expression was reported to be regulated by PPARγ (Du et al. 2009) .

Tau hyperphosphorylation is the other key player in AD pathophysiology. Although, we did not notice any change in tau levels in all groups of the study, we noticed an increase in P tau levels especially in the HFDP group. There are many kinases for tau, which among them GSK3β and CDK5 are the most important (Engmann et al. 2009). GSK3β activity is directly inhibited by AKT, a key enzyme in the insulin signalling pathway (Hur et al. 2010). In high fat diet and insulin resistance conditions, GSK3β activity has been found to be up regulated due to a deficiency in AKT activity, an increase in total GSK3β levels was also reported (Niu et al. 2016). In the present study, high fat diet induced also an increase in total GSK3β levels, PFOS did not brought any additional effects. CDK5 levels also did not change in PFOS exposure with normal diet and increased in the high fat diet and high fat diet with PFOS. There are growing evidences that GSK3βand CDK5 activities are linked to each other (Plattner et al. 2006; Engmann et al. 2009) which could explain the correlation between their levels in this study.

5. Conclusion

As it was already reported, high fat diet was able to increase levels of GSK3β and IDE, an evidence of a state of insulin resistance in the brain. Low dose exposure to PFOS on adulthood altered both Amyloid pathway and Tau pathway through mechanisms that could be related to disturbance of metabolism and insulin signalling. Some of PFOS effects were directly modulated by the exposure to the high fat diet. Since PFOS acts directly on lipid metabolism in the liver, these results suggest strongly the implication of liver-brain axis in the neurotoxicity of PFOS. To investigate further this hypothesis, it would be quite interesting to

evaluate some of the PFOS direct effects in the brain like PPARγ and its regulated gene products.

Conclusion

Exposure on adulthood to the environmental POPs mixture used in this study altered cognition and induced oxidative stress in different brain regions. Mitochondria, particularly, was a privileged subcellular organelle target. Developmental exposure to the same mixture altered neurodevelopment and induced also oxidative stress later in juvenile life. In both models, some but not all of the behavioural alterations were correlated with the state of oxidative stress noticed in the brain, thus, we suggest the involvement of additional mechanisms of toxicity other than oxidative stress. On the other side, exposure to a low dose of PFOS (a POP characterised by disturbance of metabolism and liver function), altered brain integrity through modulation of the expression of neurotoxic proteins related particularly to Alzheimer's disease. Moreover, these effects were strongly correlated with alterations of glucose and lipids levels in the blood, what suggest that PFOS exerts at least a part of its neurotoxicity through the axis of liver-brain.

In the present study, exposure to environmental levels of POPs either during development or on adulthood was able to alter relatively, brain integrity. Neurotoxicity was manifested as an early and late disturbance of behaviour, induction of oxidative stress and alteration of metabolism. It happened that all these effects are reported to be risk factors for neurodegeneration particularly related to Alzheimer's disease and dementia.

Results of the present study are in accordance with epidemiological reports linking POPs exposure to the aetiology of neurodegenerative diseases. In fact, today in the world there is a great concern regarding the silent epidemia of POPs exposure and its potential consequences on human health. The unauthorised use of some pesticides like endosulfan and the uncontrolled uses of others like chlorpyrifos put Algerian population specifically in a great danger. According to the WHO, 35.6 million people are currently living with dementia worldwide and the number will nearly double every 20 years, reaching 115.4 million in 2050, with the majority living in developing countries including Algeria (WHO 2012). It is estimated that by 2050 four fifths of the people older than 60 years will be living in developing countries (Mavrodaris et al. 2013). In Algeria, it is estimated that the aging rate will reach 7.3% of total population by 2030 (CRIS 2012).While the Algerian population is on its way to aging, a rise in chronic diseases is inevitable. The exposure of today to POPs could magnify tremendously the prevalence of Alzheimer disease and dementia tomorrow. By that time, the risk will exceed the mental health of individuals to threaten the mental health of the

whole society. It is important to take these facts in consideration and work farther to understand better effects of POPs on the brain and its implications in the aetiology of neurodegenerative diseases. At the same time, it became a necessity to develop methods and strategies to decrease or even, eliminate the exposure to POPs in order to protect our brains, in order to protect our future.

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