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# EVALUATION OF MITOCHONDRIAL AND NEUROBEHAVIORAL DISORDERS IN BRAIN REGIONS OF OFFSPRING (F1, F2) AFTER GESTATING AND LACTATING FEMALE RATS EXPOSURE TO LOW-DOSE OF IMIDACLOPRID AND CYPERMETHRIN

*Souhila Haddad[1], Zeyneb Chouit[2], Dounia Djellal[2], Salim Gasmi[3], Messaoud Hachemi[4], Mourad Hanfer[5], Djamila Zama[6], Mohamed Kebieche[2], Rachid Soulimani[7]*

***Address(es):*** Salim Gasmi & Mohamed Kebieche,
[1]Laboratoire de Biomathématique, Biophysique, Biochimie et Scientométrie, University of Bejaia, Algeria.
[2]Faculty of natural and life sciences, LMAGECA and BMBP research laboratories, University of Batna2, 05078, Fesdis, Batna, Algeria.
[3]University of Larbi Tebessi, 12002, Tebessa, Algeria.
[4]University of Mohamed Seddik Ben Yahia, Jijel, Algeria.
[5]Veterinary department, University of Batna1, Algeria.
[6]University of Constantine, Constantine Algeria.
[7]Neurotoxicology, developpement & Bioactivity, LCOMS, Campus Bridoux, Université of Lorraine, France.

*Corresponding author: salim.gasmi@univ-tebessa.dz & m.kebieche@univ-batna2.dz

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**ABSTRACT**

The imidacloprid (IMID) and cypermethrin (CYP) pesticides are known to have neurotoxic effects and negative brain developmental consequences when used separately, but little is known about the consequences of using them as a mixture. That could be passed down from one generation to the next. In this context, we were interested in studying the effect of oral exposure of female rats during gestation and throughout the lactational period to real doses of IMID (1.2 mg/kg) and CYP (6.7 mg/kg) either alone or in a mixture on behavior and mitochondrial redox status. The first and second generation pups were followed from birth to juvenile age using a series of tests to assess reflex, coordination and general motor function, including surface righting, cliff aversion, negative geotaxis and the muscle strength test. At PND 21, cholinergic function, oxidative stress and mitochondrial integrity were assessed in the striatum and hippocampus. Our results showed that IMID and CYP, alone or in combination, induced decreased body weight gain, impaired neurobehavioral performance, and AchE inhibition. Oxidative stress markers including GSH level and SOD, CAT, GST and GPx activities showed a significant decrease. While, lipid peroxidation which was assessed by MDA assay, cytosolic calcium level, swelling and mitochondrial permeability recorded a significant increase. In conclusion, the disruption of mitochondrial redox homeostasis and the presence of neurobehavioral disorders even in the offspring of the F2 generation suggests that independent and combined exposure to IMID and CYP during a critical period of development has irreversible effects; long-lasting and persistent.

**Keywords:** Imidacloprid, Cypermethrin, Neurobehavior, Striatum, Hippocampus, Mitochondria, Oxidative stress, Rats

## INTRODUCTION

The fetal period and early life are important stages when optimal brain development is assured. This is when the basic structure of the brain is built. This construction involves a series of processes that are particularly vulnerable to chemical stressors including pesticides. Impairment of this process could disrupt homeostasis, thereby increasing the risk of neurodegenerative disorders **(Sunyer and Dadvand, 2019)**. Several studies have shown that exposure to pesticides is not only through inhalation, skin absorption or ingestion of contaminated food and water but also through the placenta and breast milk during critical periods of development **(Dewailly *et al*., 2014)**. The use of pyrethroids and neonicotinoids has registered a significant increase due to their efficiencies and wide range of application; they are also less toxic to non-target organisms and efficiently degradable. These two families are considered green pesticides compared to organophosphates. However, the effect of their low concentrations on wildlife, especially bees, as well as humans, remains of concern **(Zeljezic *et al*., 2017)**. The nervous system is the primary target of CYP, a pyrethroid that exerts a neurotoxic effect through voltage-gated sodium channels. Once this insecticide binds to the α subunit of the sodium channel, the latter remains in an open position, thus allowing a prolonged passage of sodium and a depolarization of the membrane and ends up disrupting neuronal transmission. Exposure to this pyrethroid also influences other channels such as voltage-gated calcium and potassium channels; it decreases dopamine and acetylcholine levels as it alters key enzymes involved in the synthesis and metabolism of neurotransmitters such as as adenosine triphosphatases and AChE **(Raszewski *et al*., 2016)**. In laboratory animals, intranasal exposure of mice to 5 and 20 mg/kg of CYP during gestation and lactation has been linked to mitochondrial dysfunction as a mechanism of developmental neurotoxicity **(Laugeray *et al*., 2017)**. Another study conducted on mice indicates that exposure to CYP results in the release of Ca2+ and free radicals leading to DNA damage and cell death **(Maurya, *et al*., 2014)**.

Neonicotinoids such as IMID act by binding to the α4 and β2 subunits of the nicotinic postsynaptic acetylcholine receptors (nAChRs) of mammals. The neurotoxicity of this class is manifested by the inhibition of acetylcholinesterase, thereby reducing the ability of this enzyme to break down acetylcholine in the synapses, which subsequently leads to continuous overstimulation of nerves and muscles. Prenatal exposure to its chemicals affects the formation of neural circuits in several regions of the brain that are involved in the regulation of depression, anxiety, memory and learning **(Sano *et al*., 2016)**.

**Osaka *et al*., 2016** reported the presence of metabolites of the neonicotinoid Thiamotoxam in the urine of young children. The increased resistance of pests to a single pesticide application has made it necessary to use a cocktail of these chemicals in order to increase its speed of action **(Shittu *et al*., 2021)**. Therefore, the combined effect of this mixture can lead to a joint interaction between the chemicals thus altering the absorption, biotransformation, distribution and elimination of one on the other and subsequently causing the appearance of new metabolites which could be more dangerous in comparison with the basic preparation, which makes the question of the risk for ecology and human health even more serious **(Aouey *et al*., 2017)**.

To our knowledge, the CYP and IMID cocktail has not been studied for its transgenerational neurotoxic effects in mammals. Therefore, the objective of this study was to evaluate the toxicity of these pesticides alone or in mixture by examining their effects on neuromotor development, mitochondrial integrity and oxidative damage in the striatum and hippocampus of pups of generations F1 and F2 born to mothers (F0) exposed during gestation and throughout the lactation period.

## MATERIALS AND METHODS

### *Chemical products*

Most of the chemicals used in this study were purchased from Sigma Aldrich, Germany. COMMANDO is the trade name of IMID, purchased from VAPCO, Jordan. CYRUX is the trade name of CYP, purchased from UPL Limited India. The doses were chosen by referring to the results of **(Jallow *et al*. 2017)** which found 1.2 mg/kg/d of IMID residues in cucumber and of **(Skretteber *et al*. 2015)** which found 6.7 mg/ kg of CYP residue in peppermint. To convert the concentration of pesticides in cucumber and peppermint to a daily dose in experimental animal studies, we used a conversion factor which is 0.05 in rats **(EFSA 2011)**.

### *Breeding and treatment of animals*

Wistar albino rats weighing approximately 200g were provided by the Pasteur Institute in Algiers. Upon receipt, the animals were placed in cages for a two-week acclimatization period. They were maintained under standard temperature and humidity conditions, with a temperature of 22± 2°C and a humidity of approximately 60%, with a periodic cycle (light/dark) of 12 hours. The rats had free access to food and water. All experimental testing was performed in accordance with international guidelines for the care and use of laboratory animals. After two weeks of habituation to the animal house, the females were bred with males (2:1). The next day, the rats were examined by demonstrating the presence of spermatozoa in the vagina; which announces the first gestational day G1. After fertilization, 4 females (F0) were placed individually in cages. They were then divided into 4 exposure groups:

- Control group-receiving corn oil
- IMID group receiving 1.2 mg/kg of IMID
- CYP group receiving 6.7 mg/kg of CYP
- Mixture group receiving a mixture (IMID at 1.2 mg/kg/day and CYP 6.7 mg/kg/day).

In this study, IMD and CYP were dissolved in corn oil and administered orally from day 1 of gestation to day 21 of lactation. Adult females of the F1 generation (offspring of F0 mothers) were mated with males (not exposed to pesticides) to generate the F2 generation.

*Sensory-motor development tests*

At birth, eye opening was monitored and body weight was measured from day 1 to day 21 of postnatal age.

*Evaluation of locomotor development*

*The turning reflex (surface righting)*

From day 3 to day 5 of postnatal age, the rollover reflex was performed as described by **Peiffer (2011)**. This test consists of placing the rat on its back on a horizontal board, the animal will then try to turn around by swinging to the right and to the left. In this test, the time it takes for the rat to get back on its four legs is timed.

*Cliff dislike*

From the 5th to the 7th day of postnatal age, Cliff aversion tests the young rat's reflex, strength and coordination. In this test, the rat's head and forelegs are positioned at the edge of a raised flat box. Two consecutive trials are carried out for each rat which will be scored by counting points and the time it takes to move away from the edge is timed **(Peiffer, 2011)**.

*Negative geotaxis*

On days 7 to 9 of postnatal age, negative geotaxis is performed to assess motor coordination, counting the time it takes for a rat placed on a 45o slope with its head pointing down to turn 180 o up **(Feather-Schussler and Ferguson, 2016)**.

*Suspension test (muscle strength)*

From the 10th to the 13th day of postnatal age, front-limb Suspension test reflects muscle strength and coordination. It consists of hanging the rat's front legs on a metal wire stretched over a stable object, recording the total time of the fall **(Albina *et al*., 2005)**.

### Biochemical analysis

At the end of the lactational phase (PND21), the first and second generation rats were sacrificed by decapitation after deep ether anesthesia; the brains were recovered and immediately washed with a cold phosphated saline solution (PBS), then dissected to separate the striatum and the hippocampus which are used to evaluate the effect of IMID and CYP alone or in mixture on the mitochondrial redox status and membrane integrity of this organ.

### *Separation of mitochondrial matrix from striatum and hippocampus.*

The extraction of the mitochondrial fractions was carried out according to the method described by **Sahu *et al*. (2014)**. Briefly, the hippocampus and the striatum are immersed and homogenized in a TSE buffer (10 mM tris, 250 mM sucrose, 0.1 mM EDTA, pH 7.2 at 4°C) to obtain a 10% homogeneous tissue. The homogenate recovered is centrifuged at 600 g/10 min in order to eliminate large cellular debris. The supernatant resulting from this centrifugation is recovered and then centrifuged at 10,000g/10min. The resulting supernatant is considered as the cytosolic fraction, and the pellet is resuspended in TS buffer (10mM tris, 250mM sucrose, PH 7.2) then centrifuged at 10,000g/10min. The pellet resulting from this last centrifugation is resuspended in the storage buffer (250 mM sucrose, 50 mM tris, PH 7.2) which will be used directly for the evaluation of the structural and functional integrity of the mitochondria; the rest is stored at -20°C for later analyses.
In order to burst the mitochondria and recover the mitochondrial matrix, we performed freeze-thaw combined with approximately 8 times homogenization; then, we performed a centrifugation at 10,000 g/10 min. The obtained supernatant was considered as the source of mitochondrial MDA, GSH, CAT, GST, GPx and SOD.

### *Determination of AChE activity and cytosolic calcium*

AchE activity was estimated according to the procedure of **Ellman *et al*. (1961)**, using acetylthiocholine as a substrate. 100 µl of cytosolic sample were added to 100 µl of DTNB (0.1 M, pH 8) + 1 ml of tris buffer (0.1 M, pH 7). Once the reaction was stable, 100 µl of acetylthiocholine substrate was added. Absorbance was monitored at 412 nm over 4 min for 20 min. AchE activity was expressed as IU/mg protein.
Calcium levels in the cytosol were measured following the recommendations of a commercial kit (SPINREACT, Spain).

### *Assessment of swelling and mitochondrial permeability*

Swelling of mitochondria was assessed in the striatum and hippocampus using the method of **Li *et al* (2014)** where the absorbance of mitochondria isolated from fresh tissue at 4°C is monitored at a wavelength of 540nm. The decrease in absorbance indicates swelling of the mitochondria, which is the result of loss of the mitochondrial permeability transition pore (MPTP). Mitochondrial permeability was evaluated according to the method designed by **Kristal *et al* (1996)**; this technique is based on the rate of passage of Ca++ followed by an increase in the size of the mitochondria detected every 30 s for 3 min at 540 nm.

### *Evaluation of markers of redox status in mitochondria of the striatum and hippocampus*

*Determination of glutathione and malondialdehyde acid levels*

The glutathione concentration was measured by the method of **Ellman (1959)**: 50 µl of trichloroacetic acid (TCA) (10%) + 50 µl of mitochondrial matrix are centrifuged at 1400 g/2 min. 50 µl of the supernatant are taken, to which 1 ml of phosphate buffer (PH=8) and 20 µl of DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) (0.01M) are added. After 15 minutes of incubation, the reading of the optical density was carried out at 412 nm with respect to the reagent blank prepared under the same conditions. The evaluation of the end products of lipid peroxidation was carried out by the analysis of side products such as malondialdehyde (MDA) which is commonly measured by its reaction with thiobarbituric acid (TBA). The intense absorption of this complex occurs at a wavelength of 532nm. MDA levels were assessed using the method of **Niehius and Samuelson (1968)**.

*Determination of CAT, SOD, GPx and GST activities*

The enzymatic activity of catalase was determined according to the method of **Clairbone (1985)**; this method is based on measuring the disappearance of hydrogen peroxide due to the activity of this enzyme. In a quartz cuvette, we put 1 mL of phosphate buffer (0.1 M, pH 7.4) + 0.950 mL of $H_2O_2$ (0.019 M) + 50 µl of the enzyme source (mitochondrial matrix). The reaction is monitored by recording the absorbance at 240 nm every 30 s for 3 minutes. The enzymatic activity is expressed in IU/mg of protein. The SOD activity was evaluated according to the method of **Beauchamp and Fridovich (1971)**. The SOD activity was evaluated according to the method of **Beauchamp and Fridovich (1971)**. Briefly, 50 µl of the enzyme source (mitochondrial matrix) were added to 2 ml of a reaction mixture which contains (sodium cyanide, NBT, EDTA, riboflavin and phosphate buffer at pH 7.8) in the presence of 100 µl of an electron donor such as methionine. The mixture is subjected to radiation from a 15 W lamp for 15 minutes. Absorbance

was measured at 560 nm. The GPx activity was carried out according to the procedure described by **Flohe and Günzler (1984)**. GPx is the main enzyme that removes hydrogen peroxide. 0.2ml of sample (mitochondrial matrix) was added to a tube containing 0.4ml of GSH (0.1mM) and 0.2ml of phosphate buffer (0.067M, pH 7.8). The mixture is incubated in a water bath at 25° C. for 05 min. $H_2O_2$ (1.3mM) was added to initiate the reaction. After 10 min, 1% TCA (tri-chloroacetic acid) was added to stop the reaction and the mixture is put on ice for 30 min and centrifuged for 10 min at 3000 rpm. A volume of supernatant is placed in a cuvette to which Na2HPO4 (0.32M) + DNTB (1mM) have been added. Absorbance was measured at 412nm every 30 seconds for 05min. The GST activity was measured according to the method of **Habig *et al*. (1974)**. The intensity of GST activity is directly proportional to the amount of 1-S-Glutathionyl 2-4dinitrobenzene formed from the conjugation between GST and CDNB (1-Chloro2,4-dinitrobenzene) which acts as a substrate. Absorbance was performed every 30s for 5min at 340 nm.

***Statistical analysis***

Data are presented as mean ± SD. In multiple comparisons, data were analyzed using a one-way ANOVA. Differences between mean values were considered significant at *p < 0.05; highly significant at **p < 0.01; and very highly significant at ***p < 0.001. The statistical study was carried out using Excel SPC software.

## RESULTS

***Sensory-motor development tests***

Pup maturation was assessed by body weight gain from day 1 (PND1) to day 21 (PND21). Independent and combined exposure of dams (F0) to IMID and CYP significantly reduced weight gain of first and second generation pups ($p \leq 0.05$ for IMID, $p \leq 0.01$ for CYP and $p \leq 0.001$ for MIX) (Fig 1).

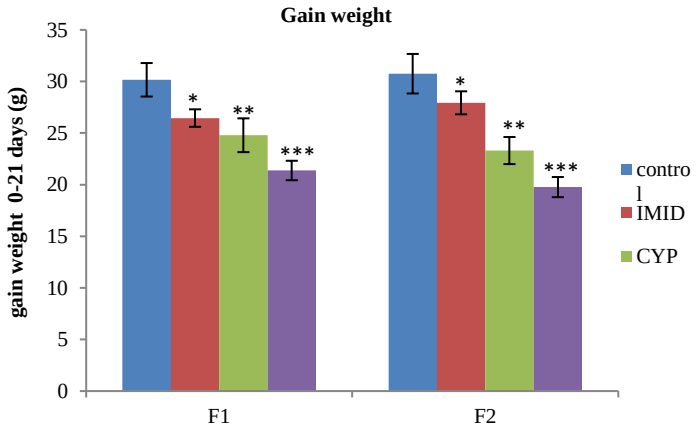



**Figure 1** Effects of gestational and lactational exposure to IMID, CYP and their mixture on weight gain in neonates of the first and second generation. *Values are means ± SD, (n= 10),* * Significant difference versus control ($p \leq 0.05$). ** Highly significant difference versus control ($p \leq 0.01$). *** Very highly significant difference versus control ($p \leq 0.001$).

Regarding eye opening, there were no significant changes in any of the treated groups compared to the control group (Fig 2).

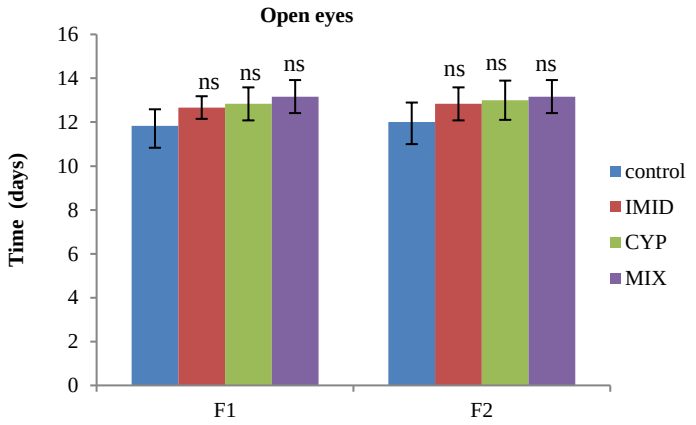



**Figure 2** Effects of gestational and lactational exposure to IMID, CYP and their mixture on eyes opening in neonates of the first and second generation. *Values are means ± SD, (n= 10), ns* no significant difference ($p > 0.05$).

*Evaluation of locomotor development*

Neuromotor development was also studied by several tests such as surface righting, Cliff aversion, negative geotaxis and front-limb suspension.

The development of the ability to turn over (surface righting) was significantly impaired by IMID and CYP either alone or in combination. The mean time needed to turn around was higher in F1 and F2 generation pups born to treated mothers compared to the control ($p \leq 0.01$ for IMID and CYP and $p \leq 0.001$ for MIX) (Fig 3).

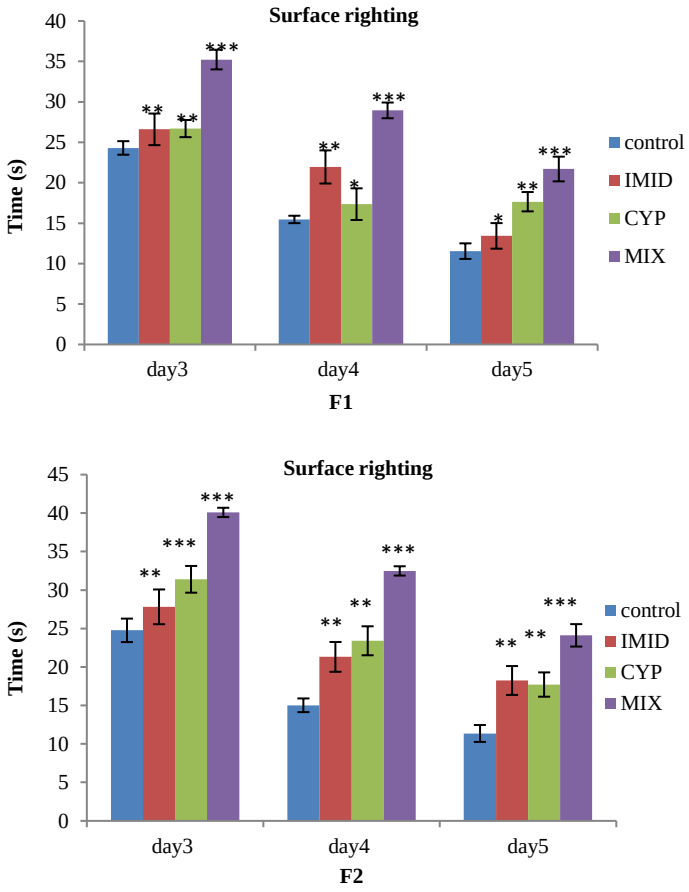
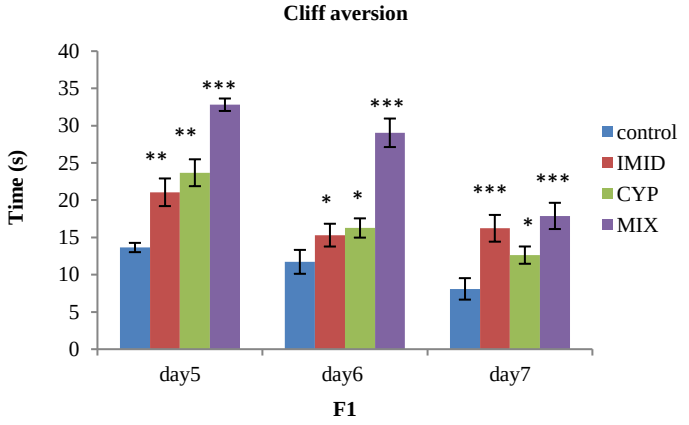



**Figure 3** Effects of gestational and lactational exposure to IMID, CYP and their mixture on surface righting reflex in neonates of the first and second generation. *Values are means ± SD, (n= 10),* * Significant difference versus control ($p \leq 0.05$). ** Highly significant difference versus control ($p \leq 0.01$). *** Very highly significant difference versus control ($p \leq 0.001$).

Cliff aversion in F1 and F2 offspring is shown in Figure 4. The pups born to the treated mothers took longer to move back on the platform during the three days of the test with a significant difference with the control, only in PND 5 the IMID did not exert any significant effect on the pups of the second generation.



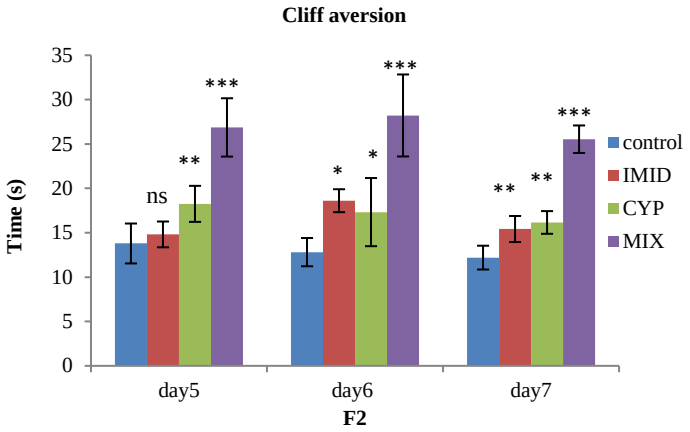



**Figure 4** Effects of gestational and lactational exposure to IMID, CYP and their mixture on cliff aversion in neonates of the first and second generation. *Values are means ± SD, (n= 10), ns* no significant difference ($p > 0.05$). * Significant difference versus control ($p \leq 0.05$). ** Highly significant difference versus control ($p \leq 0.01$). *** Very highly significant difference versus control ($p \leq 0.001$).

Pre- and postnatal exposure to IMID and CYP significantly impaired the ability of pups to exhibit reflex in the negative geotaxis test. However, in PND8 no significant difference was recorded in first and second-generation pups born to dams treated with CYP alone (Fig 5).

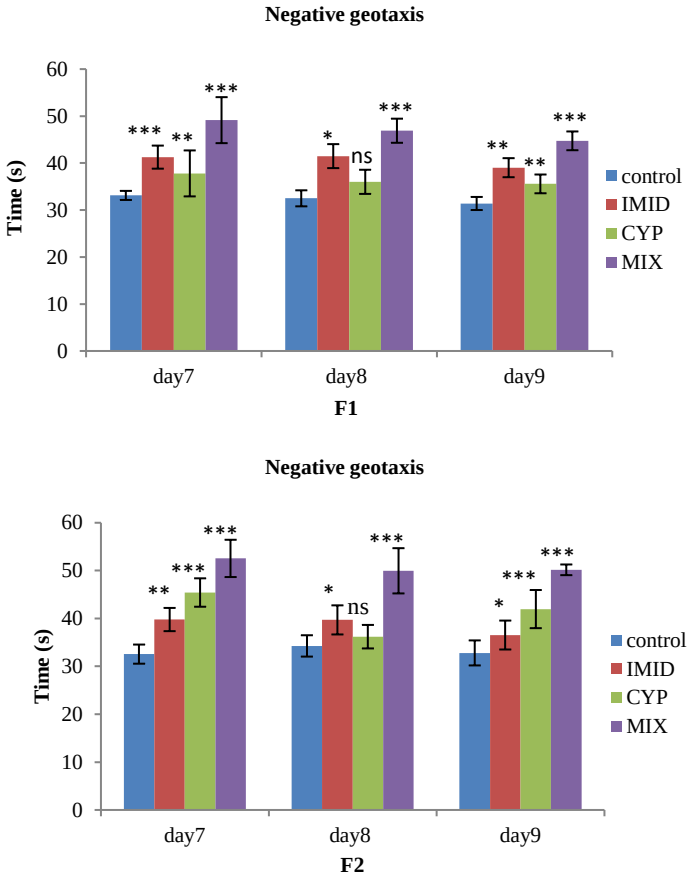



**Figure 5** Effects of gestational and lactational exposure to IMID, CYP and their mixture on development of negative geotaxis reflex in neonates of the first and second generation. *Values are means ± SD, (n= 10), ns* no significant difference ($p > 0.05$). * Significant difference versus control ($p \leq 0.05$). ** Highly significant difference versus control ($p \leq 0.01$). *** Very highly significant difference versus control ($p \leq 0.001$).

Coordination, development and motor strength were assessed by the front-limb suspension test. The results obtained showed that the fall latency was significantly affected, pups born to mothers treated with IMID and CYP either alone or in a mixture remained hanging from the wire longer than the controls (Fig 6).

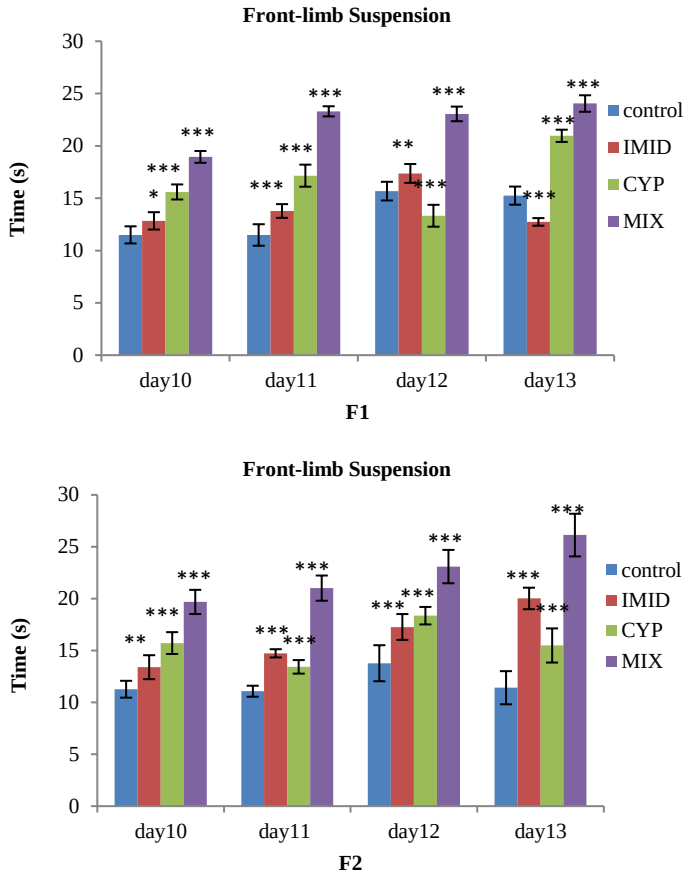
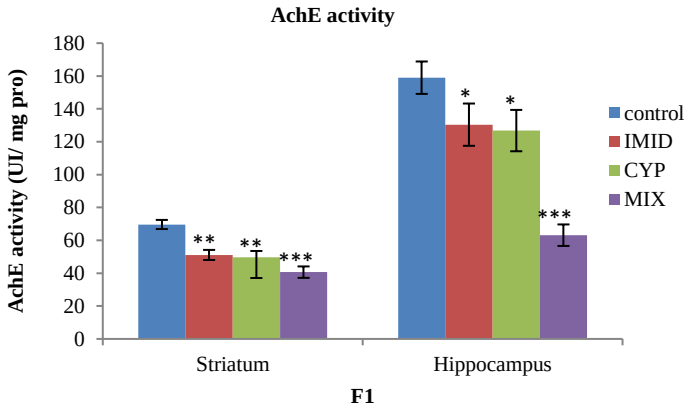



**Figure 6** Effects of gestational and lactational exposure to IMID, CYP and their mixture on coordination, development and motor strength in neonates of the first and second generation. *Values are means ± SD, (n= 10),* * Significant difference versus control ($p \leq 0.05$). ** Highly significant difference versus control ($p \leq 0.01$). *** Very highly significant difference versus control ($p \leq 0.001$).

***Evaluation of AchE activity in pups of the F1 and F2 generation***

Maternal exposure to IMID and CYP alone or in cocktail resulted in a significant decrease in AChE activity in the striatum and hippocampus of first and second generation juvenile pups (Fig 7).



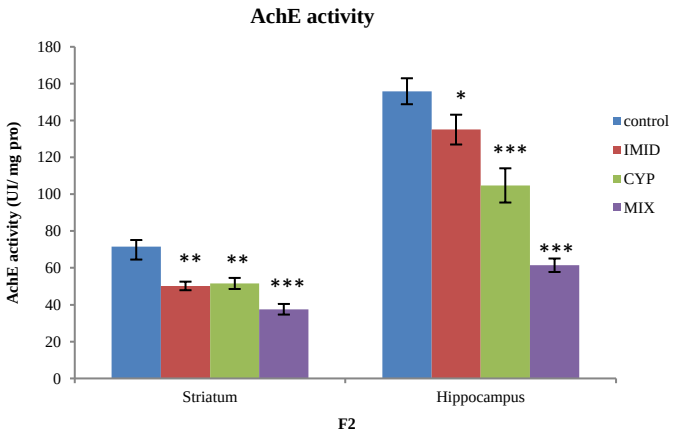



**Figure 7** Effects of gestational and lactational exposure to IMID, CYP and their mixture on acetylcholinesterase activity in striatum, and hippocampus of rats of the first and second generation observed at 21 days of age. *Values are means ± SD, (n= 10).* * Significant difference versus control ($p \leq 0.05$). ** Highly significant difference versus control ($p \leq 0.01$). *** Very highly significant difference versus control ($p \leq 0.001$).

***Evaluation of mitochondrial swelling and membrane permeability***

The results in Table 1, show a significant increase in mitochondrial swelling in the striatum and hippocampus of first and second generation pups born to dams treated during gestation and throughout lactation with IMID and of CYP, alone or in a mixture, compared to pups born to untreated mothers.

**Table 1** Effect of gestational and lactational exposure to IMID, CYP and their mixture on mitochondrial swelling in striatum, and hippocampus of rats of the first and second generation observed at 21 days of age. *Values are means ± SD, (n= 10).* ** Highly significant difference versus control ($p \leq 0.01$). *** Very highly significant difference versus control ($p \leq 0.001$).

| | **Mitochondrial swelling (optic density)** | | | |
|---|---|---|---|---|
| | **generations** | | | |
| | **F1** | | **F2** | |
| | **Brains regions** | | | |
| | *Striatum* | *Hippocampus* | *Striatum* | *Hippocampus* |
| *Control* | 0.297±0.020 | 0.262±0.017 | 0.293±0.014 | 0.270 ± 0.013 |
| *IMID* | 0.201±0.022** | 0.185±0.012*** | 0.213±0.008*** | 0.179±0.017*** |
| *CYP* | 0.214±0.022** | 0.170±0.015*** | 0.210±0.032*** | 0.192±0.021*** |
| *MIX* | 0.182±0.012*** | 0.118±0.014*** | 0.130±0.011*** | 0.098±0.006*** |

Maternal exposure to IMID and CYP, alone or in combination, significantly increased mitochondrial permeability in the striatum and hippocampus of first generation pups. This effect was transformed to the pups of the F2 generation born from the treated mothers even if there was no direct exposure of the F1 offspring (Tab 2).

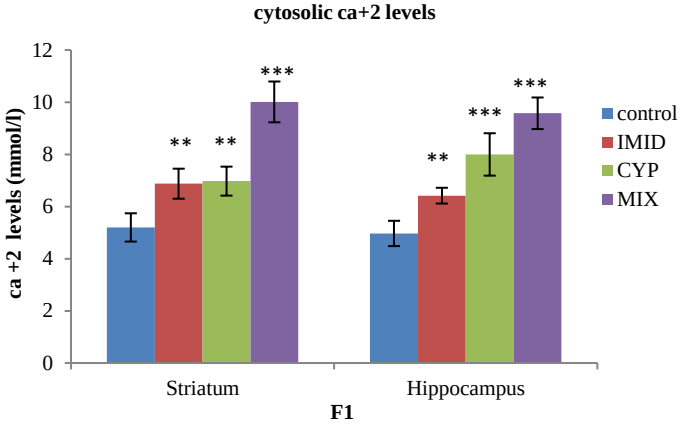
The results of changes in calcium concentrations outside the mitochondria are shown in Figure 8. After pre- and postnatal exposure to IMID and CYP either alone or in combination, cytosolic calcium levels increased significantly in pups born to treated dams relative to those born to control dams.

***Evaluation of mitochondrial redox status in the striatum and hippocampus of F1 and F2 generation pups***

With respect to antioxidant potency, CAT, SOD, GST, GPx activity and GSH level in the striatum and hippocampus of pups born to IMID and CYP-treated mothers, either alone or in mixture, recorded values significantly lower than the control. For lipid peroxidation, our results showed a significant increase in MDA in pups born to treated mothers compared to those of the control (Table 3).

**Table 2** Effect of gestational and lactational exposure to IMID, CYP and their mixture on mitochondrial permeability in striatum, and hippocampus of rats of the first and second generation observed at 21 days of age. *Values are means ± SD, (n= 10).* * Significant difference versus control ($p \leq 0.05$). ** Highly significant difference versus control ($p \leq 0.01$). *** Very highly significant difference versus control ($p \leq 0.001$).

| | **Mitochondrial permeability (ΔOD/Δt)** | | | |
|---|---|---|---|---|
| | **generations** | | | |
| | **F1** | | **F2** | |
| | **Brains regions** | | | |
| | *Striatum* | *Hippocampus* | *Striatum* | *Hippocampus* |
| *Control* | 0.017±0.006 | 0.019±0.003 | 0.022±0.001 | 0.020±0.002 |
| *IMID* | 0.027±0.007* | 0.031±0.003*** | 0.026±0.002** | 0.030±0.002*** |
| *CYP* | 0.031±0.008** | 0.037±0.005*** | 0.032±0.001*** | 0.037±0.001*** |
| *MIX* | 0.032±0.003** | 0.040±0.005*** | 0.031±0.001*** | 0.041±0.002*** |



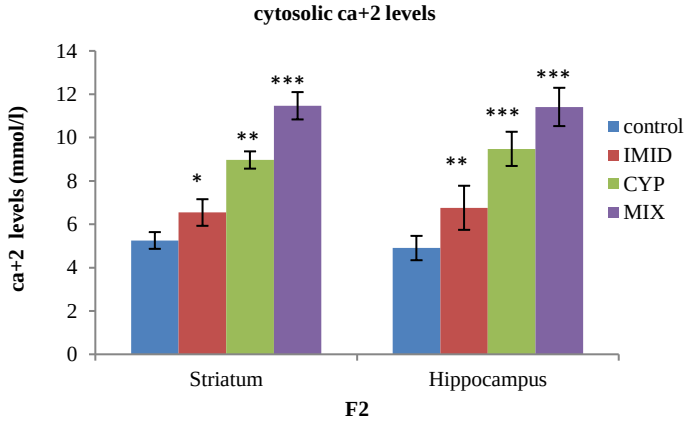




**Figure 8** Effects of gestational and lactational exposure to IMID, CYP and their mixture on cytosolic $ca^{+2}$ levels in striatum, and hippocampus of rats of the first and second generation observed at 21 days of age. *Values are means ± SD, (n= 10).* * Significant difference versus control ($p \leq 0.05$). ** Highly significant difference versus control ($p \leq 0.01$). *** Very highly significant difference versus control ($p \leq 0.001$).

The results in Table.4 show the persistence of disruption of mitochondrial redox homeostasis in the F2 generation pups even though there was no direct exposure of the F1 offspring. The pups in the treated groups recorded a significant decrease ($p \leq 0.001$) in the activity of CAT, SOD, GST, GPx, the level of GSH and significantly higher values of MDA ($p \leq 0.001$).

**Table 3** Effect of gestational and lactational exposure to IMID, CYP and their mixture on antioxidant enzymes and malondialdehyde levels in striatum, and hippocampus of rats of the first generation observed at 21 days of age. *Values are means ± SD, (n= 10).* * Significant difference versus control ($p \leq 0.05$). ** Highly significant difference versus control ($p \leq 0.01$). *** Very highly significant difference versus control ($p \leq 0.001$).

| | **Mitochondrial oxidative stress parameters** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **groups** | | | | | | | |
| | **Control** | | **IMID** | | **CYP** | | **MIX** | |
| | *Stria* | *Hippo* | *Stria* | *Hippo* | *Stria* | *Hippo* | *Stria* | *Hippo* |
| *MDA (nmol/mg Pro)* | 1.707±0.10 | 1.51±0.24 | 2.681±0.36*** | 2.025±0.27** | 2.13±0.09* | 2.48±0.21*** | 3.27±0.34*** | 3.43±0.22*** |
| *GSH (mmol/mgPro)* | 0.411±0.03 | 0.417± 0.03 | 0.356±0.03* | 0.351±0.03* | 0.33±0.02** | 0.31±0.05*** | 0.21±0.03*** | 0.33±0.02*** |
| *GST (UI/mg Pro)* | 50.52±4.58 | 49.68±1.43 | 42.797±2.82** | 38.498±1.41*** | 41.30±3.92** | 39.11±1.89*** | 30.19±4.94*** | 27.01±2.76*** |
| *GPx (UI/mg Pro)* | 0.274±0.02 | 0.28±0.01 | 0.237± 0.01** | 0.254± 0.01* | 0.25± 0.01* | 0.24± 0.02** | 0.20± 0.01*** | 0.24±0.02** |
| *CAT (UI/mg Pro)* | 0.090±0.00 | 0.08±0.01 | 0.069± 0.00** | 0.064± 0.013* | 0.07± 0.01* | 0.05± 0.00** | 0.03±0.01*** | 0.03±0.01*** |
| *SOD (UI/mg Pro)* | 10.45±1.10 | 10.02±0.24 | 8.80± 0.63** | 8.12± 0.84*** | 7.73± 0.76*** | 8.46± 0.74** | 5.16±0.72*** | 5.12±0.94*** |

*Stria : Striatum, Hippo : Hippocampus*

**Table 4** Effect of gestational and lactational exposure to IMID, CYP and their mixture on antioxidant enzymes and malondialdehyde levels in striatum, and hippocampus of rats of the second generation observed at 21 days of age. *Values are means ± SD, (n= 10).* * Significant difference versus control ($p \leq 0.05$). ** Highly significant difference versus control ($p \leq 0.01$). *** Very highly significant difference versus control ($p \leq 0.001$).

| | **Mitochondrial oxidative stress parameters** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **groups** | | | | | | | |
| | **Control** | | **IMID** | | **CYP** | | **MIX** | |
| | *Stria* | *Hippo* | *Stria* | *Hippo* | *Stria* | *Hippo* | *Stria* | *Hippo* |
| *MDA (nmol/mg Pro)* | 1.653±0.17 | 1.686±0.26 | 2.970±0.39*** | 2.704±0.29*** | 3.111±0.43*** | 2.729±0.31*** | 4.536±0.66*** | 3.950±0.17*** |
| *GSH (mmol/mgPro)* | 0.418±0.01 | 0.409±0.01 | 0.304±0.03*** | 0.342±0.03** | 0.292±0.04*** | 0.295±0.05*** | 0.197±0.02*** | 0.203±0.01*** |
| *GST (UI/mg Pro)* | 49.87±1.51 | 49.53±1.39 | 40.24±1.48*** | 36.65±1.52*** | 40.57±1.76*** | 35.20±3.37*** | 24.52±1.86*** | 20.99±4.98*** |
| *GPx (UI/mg Pro)* | 0.267±0.01 | 0.271±0.02 | 0.231±0.02** | 0.231±0.02** | 0.228±0.01*** | 0.232±0.01** | 0.197±0.02*** | 0.227±0.01** |
| *CAT (UI/mg Pro)* | 0.087±0.00 | 0.080±0.01 | 0.06±0.01 ** | 0.05±0.00 ** | 0.05±0.009*** | 0.05±0.01 *** | 0.029±0.01*** | 0.024±0.00*** |
| *SOD (UI/mg Pro)* | 10.98±0.89 | 11.08±0.88 | 7.22±0.84 *** | 6.01±0.96 *** | 6.04±0.99 *** | 5.51±0.72 *** | 3.91±0.70*** | 4.11±0.74 *** |

*Stria : Striatum, Hippo : Hippocampus*

## DISCUSSION

The ubiquity of pesticides in the domestic and agricultural environment increases the likelihood of exposure for pregnant women and nursing mothers, putting their children at risk **(Shittu *et al*., 2021)**. During fetal and childhood development, the environment can play an important role in the onset of many chronic diseases, including neurodevelopmental abnormalities. It has also been shown that these adverse effects can be transmitted to subsequent generations without the need for additional exposure **(Blanc *et al*., 2020)**. The results of the present study demonstrated that exposure to a mixture of pesticides during a critical developmental period even at low doses that have been adapted for human consumption affects neuronal development and alters the mitochondrial redox status of 1st generation pups, these negative effects were also transmitted to the 2nd generation pups. It is well known that IMID, which is a member of the neonicotinoid family, acts in a similar way to nicotine by binding to postsynaptic nicotinic acetylcholine receptors (nAChRs). Hyperactivity of these receptors influences food intake and decreases body weight, which explains the reduction in weight gain in F1 and F2 offspring born to dams treated with IMID and CYP either alone or in combination. This reduction indicates reduced growth, which testifies to the vulnerability of developing rats to pesticides, this could be explained by the placental transfer of the compound from the mother to the fetus, this parameter is an important factor in determining developmental toxicity **(Syed *et al*., 2016)**. Our results agree with those of **Burke *et al*. 2018** and **Elser *et al*. 2020** which showed weight reduction in the offspring of mice exposed during gestation and throughout lactation to 0.5 mg/kg IMID and 10 mg/kg CYP, respectively.

On the other hand, several studies have reported that exposure to pyrethroids and neonicotinoids affects neurobehavioral performance, hence the delay of the righting reflex (surface righting), disorders in the development of coordinated movement and the sense of movement. equilibrium that were recorded after pre and postnatal exposure to IMID and CYP either alone or in cocktails. This confirms the negative impact of these two pesticides on neuromotor development, motor functions and the vulnerability of the developing brain to xenobiotic insults **(Syed *et al*., 2016)**. These neurobehavioral deficits could be the result of dysfunction in multiple areas of the central and peripheral nervous system; a recent study showed that fetal and lactational exposure to a neonicotinoid, clothianidin, inhibits the maturation of immature neurons in juvenile progenitor cells and the viability of adult progenitor cells **(Maeda *et al*., 2021)**. Our results are in agreement with the work of **Laugeray *et al*., 2017** who conducted a study in mice where females received 5 and 20 mg/kg/day of CYP throughout gestation and up to the 15th day. of lactation and those of **Elser *et al*. 2020** who showed that maternal exposure to CYP influences the development of GABAergic neurons. The delay in the formation of these neurons can have a significant impact on their functions in the mature brain, which will subsequently cause neurodevelopmental problems in children. Moreover, a significant correlation between impaired memory performance, developmental/coordination problems and the application of ACh receptor antagonists has been demonstrated **(Haense *et al*., 2012)**. It is now known that neonicotinoids and pyrethroids have the ability to inhibit AChE which leads to the accumulation of acetylcholine in synapses, subsequently causing persistent stimulation of cholinergic neurons **(Arora *et al*., 2017)**. Overall, our results agree with those of **Sinha *et al*. 2006** who tried to understand the mechanism of toxicity of a pyrethroid-based mosquito repellent at different stages of development of the central nervous system of rats and those of **Liu *et al*., 2018** who confirm that thiamotoxam, another pesticide of the neonicotinoid family, has the ability to block the transfer of information between neurons and ultimately paralyze target organs. These results indicate that there is a link between cognitive delay in children and prenatal exposure to pesticides **(Shelton., 2014; Gunier *et al*., 2017)**.

As the brain consumes a large amount of oxygen, contains high amounts of polyunsaturated fatty acids (PUFAs) and has low levels of antioxidant enzymes; which makes it very vulnerable to oxidative stress **(Sharma *et al*., 2014)**, which is defined by an imbalance between the amounts of oxidant and antioxidant compounds, thus promoting the excessive generation of free radicals or slowing down their elimination **(Bragante *et al*., 2022)**. Exposure of F0 generation rats to IMID and CYP, alone and in mixture, during gestation and throughout the lactation period resulted in increased cytosolic calcium levels in first generation rat pups. and the second generation. This dysregulation is due to the overload of the mitochondria with calcium, which is known as an activating factor of several enzymes such as nucleases, proteases and phospholipases which will cause the oxidation of lipids and proteins of the mitochondrial membrane, thereby promoting the opening of the mitochondrial permeability transition pore (mPTP) (**Panel *et al*., 2018)**. This mitochondrial depolarization should produce ATP depletion, followed by swelling and finally the release of cytochrome c which then promotes cell death signaling **(Hosseini *et al*., 2013)**. Our results are in agreement with those of **Muhammed *et al*. 2020** which prove that CYP has the ability to cause neurodegenerative disorders through mitochondrial dysfunction, caspase activation, genomic DNA damage, inhibition of AchE activity. Since neonicotinoids have the same structure as nicotine and also share agonist activity at nicotinic acetylcholine receptors (nAChRs), our results are consistent with those of **Önal *et al*., 2004** which indicate that maternal exposure to nicotine alters the normal development of the different regions of the brain, in particular the CA1 of the hippocampus, by modifying its ultrastructure by the condensation of nuclear chromatin, the dilation of the rough endoplasmic reticulum and mitochondrial swelling.

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According to our results, it seems that CYP is more neurotoxic than IMID. But in general, even as an insect-specific insecticide, IMID could alter the behavior and mitochondrial integrity of non-target species. Our results are in agreement with the work of **Liu *et al*. (2018)**; which prove that pyrethroids like deltamethrin have a higher level of neurotoxicity compared to organophosphates and neonicotinoids due to their lipophilicity which allows them to accumulate in the cell membrane by disrupting its structure **(Aouey *et al*. 2017)**. It is important to note that our results showed the persistence of neurobehavioral disorders and mitochondrial dysfunction even after cessation of exposure in F2 generation pups. A study that was conducted in Brazil, Colombia and Spain reported the presence of CYP residues in human breast milk indicating that IMID and CYP can be transferred from mother to offspring possibly through the placenta and through breast milk **(Elser *et al*., 2020; Corcellas *et al*., 2012 ; Djellal *et al*., 2022 ; Gasmi *et al*., 2022)**. This proves that neonicotinoids and pyrethroids have transgenerational consequences that manifest later in life. This transmission could be explained by epigenetic mechanisms **(Yuan *et al*., 2016)**. These results indicate that organisms may be subject to molecular changes accumulated over generations and/or that adaptive mechanisms may be able to reset their physiological state after a number of generations **(Beck *et al*., 2017)**.

## CONCLUSION

It can be concluded that maternal exposure to IMID and CYP, alone and in cocktails, during gestation and throughout the lactational period, even at low doses, affects not only the behavior and brain function of the generation F1, but these effects are also transformed to the next generation (F2) even though there was no direct exposure of the F1 offspring. This proves that independent and combined exposure to IMID and CYP during a critical period of development has long-lasting, even persistent, irreversible effects. From here, we can say that exposure to Imidacloprid and Cypermethrin pesticides causes several pathological effects at the level of the brain, which leads to its dysfunction, which was confirmed by previous experiences, especially with regard to behavior, memory and intelligence. This study also confirmed the hypothesis of transmission of these toxics through breastfeeding.

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