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The effect of chronic vitamin deficiency and long term very low dose exposure to 6 pesticides mixture on neurological outcomes – a Real-Life Risk Simulation approach

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Highlights:

- Chronic exposure to vitamin deficiency and a very low dose of pesticides mixture determine stimulation of SNC
- Chronic vitamin deficiency decreased locomotor and spatial orientation activity in rats
- Exposure to very low doses of a mixture of 6 pesticides caused an inhibition of the central nervous system
- Chronic vitamin deficiency and exposure to a very low dose of pesticides mixture affect long-term memory

Abstract

We investigate the long-term effect of very-low dose exposure to a mixture of six pesticides associated with hydrophilic vitamin deficiency on the neurobehavioral outcomes of rats. Two hundred Wistar rats were divided into four groups, two control groups, a vitamin
sufficient control group and a vitamin deficiency control group and 2 test groups, a vitamin
sufficient test group, and a vitamin deficiency group. The test groups were exposed for 9 months
to a mixture of diquat, imazamox, imazethapyr, tepraloxydin, bentazone and acifluorfen in doses
of 0.01xNOAEL (mg/kg bw/day). After 9 months of exposure, the behavior changes were
evaluated by open field test and elevated plus maze test and the memory was assessed by passive
avoidance test. Chronic vitamin deficiency decreased locomotor and special orientation activity
and increased anxiety-like behavior in rats. Exposure to very low doses of a mixture of 6
pesticides caused central nervous effects, manifested as decreased locomotor activity, and
increased anxiety levels. Vitamin deficiency and low dose chronic pesticides mixture exposure
thus affected the central nervous system, especially long-term memory.

Abbreviations:

AD – Alzheimer’s disease
ADI – acceptable daily intake
ALS - amyotrophic lateral sclerosis
B1 – thiamine
B12 – cyancobalamin
B6 – pyridoxine
DALYs – disability-adjusted life-years
NOAEL – no-observed adverse effects levels
PD – Parkinson’s disease

Keywords: mixture, vitamin deficiency, pesticides, real-life risk simulation, hormesis, open field
test, elevated plus maze test; passive avoidance test
Introduction

In 2015, the neurological disorders were the leading cause of disability-adjusted life-years (DALYs) and the second leading cause of death in the world: An increase of 36.7% in the number of deaths from 1990 to 2015 and an increase of 7.4% in the number of DALYs (Feigin et al., 2017) was observed. Neurodegenerative diseases such as Alzheimer's disease (AD) and other dementias, Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) represent an important part of neurological disorders that affect especially the aging population. In recent years, a lot of studies investigated the potential contributing factors for the developing these diseases (Androutsopoulos et al., 2011; Dardiotis et al., 2013, 2018a,b; Kostoff et al., 2018a; Hakansson et al., 2003; Lin et al., 2016; Zaganas et al., 2013) in order to find preventive or therapeutic solutions for their symptoms and if possible reverse the disease (Costa et al., 2017; Kostoff et al., 2018a; Misik et al., 2016). Neurodegenerative diseases’ etiology and progression is a multifactorial process that encompasses interaction between genetic factors and environmental factors that lead to mitochondrial dysfunctions, impair protein dynamics, oxidative stress that leads at the fine to neuronal death (Sheikh et al., 2013). Environmental factors can influence the development and progression of the neurological diseases according to the particular time of exposure and the level of exposure (Olsson et al., 2016; Hernandez et al., 2016; Huang et al., 2014). Epidemiological and animal studies showed the association of exposure to different xenobiotics such as pesticides, heavy metals, endocrine disruptors chemicals, chemicals or mixtures of chemicals generated during food processing with the development and progression of several neurological disorders (Andrade et al., 2017; Baltazar et al., 2014; Chen et al., 2019; Dardiotis et al., 2013; Erkekoglu and Baydar, 2014; Ijomone et al., 2018; Masuo and Ishido, 2011; Petrakis et al., 2017).

Deficiencies in vitamin intake especially from the B series such as B12 (cyanocobalamin), B6 (pyridoxine), B1 (thiamine) and folate are associated with neurological dysfunctions and congenital defects (Selhub et al., 2010). The exact mechanism is not
completely elucidated but one explanation is related to the association between increased production of homocysteine and cognitive impairments and memory decline in the elderly (Nurk et al., 2005). In homocysteine metabolism, vitamin B has an important role as cofactors in the chemicals reactions of methionine metabolism. A deficiency in these cofactors determines an excessive production of homocysteine (Kamat et al., 2016). Hyperhomocysteinemia and deficiency in B vitamins are associated with neurodegenerative disorders including vascular dementia, Parkinson’s disease, multiple sclerosis, and Alzheimer’s disease (Bowyer et al., 2018; Duan et al., 2002; Hamel and Logigian, 2018). It has also been shown that low consumption of vitamin K diet determines mild cognitive impairment in aged rats (Carrie et al., 2011).

Nutritional status and vitamin deficiency can also influence the metabolism of several xenobiotics by acting on the cytochrome P-450 enzymes. These interactions can lead to increased neurotoxicity for several classes of chemicals known for their neurotoxic potential (Guengerich et al., 1995).

Pesticides are compounds from several chemical classes designed to control insects and pests, especially to protect plants or plants products during production, storage, and transport. The human exposure to pesticides is not singular, each individual is exposed daily in real life to a mixture of pesticides from several sources with food products, water consumption, and environmental exposure. Despite the set of safety levels by regulatory agencies for each pesticide, the epidemiological and biomonitoring studies continue to associate exposure to these products with several diseases including cancer (Parrón et al., 2014), neurodegenerative diseases being one group of them (Mostafalou and Abdollahi, 2018; Baltazar et al., 2014; Taghizadeh et al., 2019; Zaganas et al., 2013). One of the explanations of these findings are associated with the fact that the safety levels are based on animal studies that analyze one chemical and follow in general one critical effect, while in the real-life the humans are exposed to mixtures of chemicals that in some situations can lead to “cocktail” effects determined by additive or synergic interactions that can potentiate the effect (Hernandez et al., 2013; Minigalieva et al., 2017; Sakita
et al., 2017; Shukla et al., 2017). Several studies proposed new methodologies for real-life risk simulation in order to better understand the real risk for the general population that is exposed to several chemicals from several sources in a long-term regimen (Docea et al., 2016; Tsatsakis et al., 2016, 2017, 2018, 2019a; Tsatsakis and Lash, 2017; Kostoff et al., 2018b; Colosio et al., 2012). Several animal studies already showed non-monotonic dose-response effects and side effects on different targets after long term exposure to mixtures of chemicals in doses below the NOAEL (no-observed adverse effects levels) (Docea et al., 2018, 2019; Tsatsakis et al., 2019b).

Diquat is a contact herbicide also used as seed desiccant and aquatic weed control agent. Its primary toxicity in humans is associated with the formation of reactive oxygen species during his metabolism that is associated with diquat hepato-, nephro- and neurotoxicity (Curcic and Djukic et al., 2006; Dinis-Oliveira et al., 2006; Djukic et al., 2012; EFSA, 2015). Imazamox is an herbicide that belongs to imidazolinone class acting by inhibition of acetohydroxyacid synthase, an important enzyme in the biosynthesis of branched-chain amino acids in plants (EFSA, 2016a). This biosynthesis pathway is not present in mammals, so it is considered to be safe for non-target species, including humans. Animal studies revealed low toxicity, the critical effect used for the set of acceptable daily intake (ADI) doses are decreased bodyweight, decrease body weight gains and decreased food consumption (EFSA, 2016a). Imazethapyr is a herbicide with imidazolinone chemical structure extensively used in agriculture, especially in the United States of America (National Agricultural Statistics Service, 2005). It is known for its low acute toxicity, but the information regarding long-term chronic effects are limited even if the general population can be exposed to imazethapyr by food or drinking water from groundwater contamination (USEPA, 2002). Tepraloxydin is an herbicide from cyclohexanedione class that acts by inhibiting acetyl CoA carboxylase in plants. Its toxicity to non-target organism is manifested in mammals, especially as hepatotoxicity (EC, 2004). Bentazone is an herbicide that inhibits plant photosynthesis and is classified as moderately hazardous for humans by WHO (EFSA, 2015b). Long-term exposure can determine biochemistry and hematological abnormalities, especially at
liver and kidney level (EFSA, 2015b). Diphenolic ether herbicide acifluorfen acts on target organism by inhibiting the protoporphyrinogen oxidase (PPO) enzyme that catalyzes the chlorophyll and heme biosynthesis. In non-target organisms such as mammals, long-term exposure could result in liver and kidney disturbances, teratogenic effects, and possible carcinogenic effects (USEPA, 1987).

This study presented one part of a big experiment and aimed to investigate the long-term effect (9 months) of a mixture of six pesticides in doses 100 times below NOAEL on neurobehavioral outcomes of rats and to check the hypothesis that long-term deficiency in soluble vitamins can potentiate the mixture neurotoxicity. The mixture contains diquat, imazamox, imazethapyr, tepraloxydin, bentazone, and acifluorfen, herbicides usually found as contaminants in food or drinking water (USEPA, 2002).

**Material and Methods**

**Animal study**

Specific-pathogen-free male Wistar rats (n=200) included in the study were obtained from Affiliated Unit "Stolbovaya" of Scientific Center for Biomedical Technology of the Federal Medical and Biological Agency. The rats were 30 days old, with an initial body weight of 90.9±0.9 g at the beginning of the study. The animals were acclimatized for one week to the new housing conditions before study begins. During the experiment, the animals were housed in plastic cages (2 animals per cage) with wood shavings in a steady heated (21 ºC - 23 ºC) air-conditioned room with natural light settings.

The animals were divided equally and into 4 groups of 50 animals into 2 control and 2 test groups.

The control groups are:

- Vitamin sufficient group (C-100) that received *at libitum* the AIN-93 diet, an appropriate diet for use in rat safety evaluation studies (Lien et al., 2001) including
water-soluble vitamins at 100% of the suggested ratio for normal development (Table 1) and water *at libitum* for 9 months.

- Vitamin deficiency group (C-25) that received the AIN-93 diet *at libitum* and only 25% of the request ratio of water-soluble vitamins (Table 1) and water *at libitum* for 9 months.

The test groups are:

- Vitamine sufficient test group (T-100) as above (Table 1) but water *at libitum* contained a mixture of diquat, imazamox, imazethapyr, tepraloxydin, bentazone and acifluorfen in doses equivalent to ADI dose for each chemicals per day for 9 months.

- Vitamin deficiency test group (T-25) as above (Table 1) but water *at libitum* contained a mixture of diquat, imazamox, imazethapyr, tepraloxydin, bentazone and acifluorfen in doses equivalent to ADI dose for each chemicals per day for 9 months.

**Table 1.** Vitamin-mineral composition of modifying diet

<table>
<thead>
<tr>
<th>Vitamins</th>
<th>Vitamins (g/kg diet) for vitamin sufficient groups (C-100 and T-100 groups)</th>
<th>Vitamins (g/kg diet) for vitamin deficiency groups (C-25 and T-25 groups)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine (vitamin B1)</td>
<td>0.6</td>
<td>0.15</td>
</tr>
<tr>
<td>Riboflavin (vitamin B2)</td>
<td>0.6</td>
<td>0.15</td>
</tr>
<tr>
<td>Pyridoxine (vitamin B6)</td>
<td>0.7</td>
<td>0.175</td>
</tr>
<tr>
<td>Nicotinic acid (niacin or vitamin B3)</td>
<td>3</td>
<td>0.75</td>
</tr>
<tr>
<td>Calcium pantothenate (vitamin B5)</td>
<td>1.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Folic acid (vitamin B9)</td>
<td>0.2</td>
<td>0.05</td>
</tr>
<tr>
<td>Cyanocobalamin (vitamin</td>
<td>0.0025</td>
<td>0.000625</td>
</tr>
</tbody>
</table>
### Pesticides mixture

The mixture contained 6 pesticides: diquat, imazamox, imazethapyr, tepraloxydim, bentazone and acifluorfen and was administrated in doses equivalent to ADI doses for each individual chemical as follows: diquat (0.002 mg/kg bw/day) (EFSA, 2015a), imazamox (10.68 mg/kg bw/day) (WHO, 2014), imazethapyr (0.6 mg/kg bw/day) (EFSA, 2017), tepraloxydim (0.05 mg/kg bww/day) (EC, 2004; EFSA, 2014), bentazone (0.09 mg/kg bw/day) (EFSA, 2015b), acifluorfen (1.25 mg/kg bw/day) (US EPA, 1987).

### Chemicals

The pesticides: diquat, CAS Number - 6385-62-2, imazamox, CAS Number - 114311-32-9, imazethapyr, CAS Number - 81335-77-5, tepraloxydim, CAS Number - 149979-41-9, bentazone, CAS Number - 25057-89-0, acifluorfen, CAS Number - 50594-66-6 were bought from BASF SE, Germany. The vitamins: thiamine, CAS Number - 67-03-8, riboflavin, CAS Number - 83-88-5, pyridoxine, CAS Number - 65-23-6, nicotinic acid, CAS Number - 59-67-6, calcium pantothenate, CAS Number - 137-08-6, folic acid, CAS Number - 59-30-3, cyanocobalamin, CAS Number - 68-19-9, menadione, CAS Number - 58-27-5 were bought from Merck KGaA, Germany.

All rats were regularly observed, and their condition was closely monitored throughout the whole study period. Animals were examined based on appearance, movement and behavior patterns, skin and hair state, eyes and mucous membranes, excreta, and respiration. Feed and water intake was measured every two days, bodyweight of rats was registered weekly. The concentration of the pesticides in the drinking water was adjusted according to water.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>B12)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menadione (vitamin K)</td>
<td>0.075</td>
<td>0.01875</td>
</tr>
<tr>
<td>Sucrose</td>
<td>993.20</td>
<td>998.301</td>
</tr>
</tbody>
</table>
consumption in order for the animals to be exposed to the ADI doses. After 9 months of exposure the rats were evaluated for neurobehavioral modifications using the open field test, elevated plus maze test and passive avoidance test.

**Open field test**

The open-field test was performed on all animals that remained in each group after 10 animals from each group were sacrificed after 6 months of exposure for the complete blood test sand histopathological evaluation (37 rats in C-100 group, 36 rats in C-25 group, 34 rats in T-100 group and 37 rats in T-25 group) after 9 months of exposure to the deficiency diet and/or pesticides mixture. There were no significant differences regarding the bodyweight of the animals between the groups (data not showed). Several animals in each group died from various reasons not related to the experiment at different timepoints as showed in table 2.

**Table 2. The number of animals that died in each group during the experiment at different timepoints from various reasons not related to the experiment.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Days of experiment</th>
<th>Total (number of animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-182 days</td>
<td>3-365 days</td>
</tr>
<tr>
<td>C-25</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>C-100</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>T-25</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>T-100</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Animals’ behavior in the open field test is determined by the proportion between defensive and exploratory tendencies. The animal was placed in an unknown open field. The open-field box was a black square arena (90 cm × 90 cm) surrounded by grey walls (40 cm) and
divided into one central and two peripheral zones (Figure 1A). The animals were placed in the corner and allowed to explore the arena for 3 minutes. Each test was recorded and analyzed using the video-tracking option SMART 3.0 (Panlab Harvard Apparatus, Spain). For behavior evaluation, the following parameters were used: horizontal (number of transitions between zones) and vertical (number of rearings) activity; time spent in the central zone, peripheral zone 1 and peripheral zone 2 of the open field and total distance traveled. The test was conducted in the period of the animals’ minimal activity (between 10 am. and 3 pm.) (Pertsov et al., 2017).

**Figure 1.** A. Open field apparatus; B. Elevated plus-maze apparatus; C. Panlab Passive Avoidance box apparatus
Elevated plus maze test

Based on the results of the open field test that show that the groups were homogeneous and no significant differences were observed between the animals in the same group, 12 animals were randomly selected from each group and evaluated for the anxiety level after 9 months of exposure to the deficiency diet and pesticides mixture using elevated plus-maze test. The use of 12 animals per group was based on the power analysis and due to a large number of animals (a total of 200 animals), this reduction in the number of animals was necessary so as not to stretch the test for several days. The elevated plus-maze test was used to study animals’ behavior under variable stress conditions with the free choice of comfortable conditions allowing to evaluate their anxiety level. The evaluation of behaviour is based on a conflict between the animal’s desire to explore and fear of open spaces.

The elevated plus-maze consisted of two opposite arms (45 cm × 10 cm) crossed with two opposite enclosed arms of the same size with 50 cm high walls (Figure 1B). The maze was elevated 65 cm above the floor in a dimly lit room.

The rats were placed in the center of the maze facing the closed arm and were allowed to explore the maze for 5 min. A standard elevated plus-maze LE840A + video-tracking option SMART 3.0 (Panlab Harvard Apparatus, Spain) was used for the analysis of anxiety-like behavior. The number of entries, the time spent in each arm of the maze, and the total distance were recorded (Apryatin et al., 2018).

Combination of these tests precludes unexpected biases caused by differences in the intensity of fear, anxiety reactions and total exploratory activity of animals. The combined use of different methods for the assessment of behavior can enhance the verifiability and significance of the results.
Passive avoidance test

A passive avoidance test was used to assess the behavior, short-term, and long-term memory of the animals. The test was performed after 9 months of exposure to the deficiency diet and pesticides mixture on 12 animals randomly chosen from each group. The use of 12 animals per group was based on the power analysis and considering ethical considerations (electric current is used in the test). Also due to a large number of animals (a total of 200 animals), this reduction in the number of animals was necessary so as not to stretch the test for several days (which is unacceptable). The Panlab Passive Avoidance box controlled by the ShutAvoid software (LE872) (Panlab Harvard Apparatus, Spain) was used for this test. The apparatus consisted of a large white illuminated compartment and a small black dark compartment separated by a door (Figure 1C).

The experiment started with a training session, and the animals were placed in the light compartment and let explore the apparatus for five minutes. On the following day, when the animals entered the dark compartment, the door was closed, and they received an electric shock (0.4 mA, 4 sec). The latent period of staying in the light compartment was recorded. In the retention phase, 24 hours later (short-term memory) and in 3 weeks (long-term memory), the animals were placed in the light compartment, and the latency time to enter the dark compartment was recorded (Loskutova et al., 2017; Shipelin et al., 2017).

Statistical analysis

All analyses were carried out using SPSS 20 (SPSS Inc., Chicago, USA). Continuous data were expressed as mean ± standard deviation of the mean. One way ANOVA and Tukey’s post hoc tests were used for assessing differences between groups in normally distributed data, and Kruskal-Wallis and post-hoc Mann-Whitney tests with Holm-Sidak adjustment were used for non-normally distributed data. P values<0.05 were considered significant.

Results
Open field test

Significant differences have been observed in the exploratory behavior in the open field between both the control groups and test groups and also between the test groups compared to control groups. Regarding the *locomotor activity* evaluated by the number of transitions between the zones and total distance traveled by the animal, there were differences between the test groups and the control groups and these differences were associated with the vitamin deficiency. In the control groups, the locomotor activity of the vitamin deficiency group (C-25) was decreased compared to the vitamin sufficient group (C-100), however this did not reach statistical significance for the total distance traveled by the animal but the number of transitions between the zones was significantly decreased (P<0.05) (Table 3 and Figures 2A and 2B). In the test groups, a different trend dependent on the vitamin intake was observed: The pesticides mixture in the vitamin sufficient group caused a statistically significant decrease in the locomotor activity for both, the total distance traveled by the animal and for the number of transitions between the zones, compared to C-100 group (P<0.05) (Table 3 and Figures 2A and 2B). In the vitamin deficiency group, the pesticides mixture increased the locomotor activity compared to both control groups (C-25 and C-100) and compared to the T-100 group (Table 3 and Figures 2A and 2B).

The *spatial orientation activity* that was evaluated by the number of rearing showed differences between the groups. Vitamin deficiency decreased the number of rearings in the C-25 group compared to the C-100 group (P<0.05) (Table 3, Figure 2C). The pesticides mixture had no influence on the number of rearing in the T-100 group compared to C-100 group but associated with vitamin deficiency caused an increase of the spatial orientation activity of the animals compared to C-25, C-100 and T-100 groups (P<0.05) (Table 3, Figure 2C).

Differences in the anxiety behavior in the open field were observed between the groups, both between the control groups and test groups and also between the test groups compared to control groups. In the control groups, the vitamin deficiency increased the anxiety level by
decreasing the time spent in the central zone and increasing the time spent in peripheral zone 1 compared to C-100 group (P<0.05) (Table 3 and Figures 2D and F). In the vitamin sufficient test group (T-100), the pesticides mixture decrease the anxiety level of rats measured as increased time spent in the central zone and decreased time spent in the peripheral zone 1 compared to C-100 group (Table 3, Figures 2D and F). In the vitamin deficiency test group (T-25) the pesticides mixture caused a decrease of the anxiety-like behaviour compared to C-25 group associated with an increase of the time spent in the central zone and increase in the time spend in peripheral zone one and decrease time spend in peripheral zone 2 (P<0.05) (Table 3 and Figures 2D, E and F), but compared to T-100 group the anxiety level was increased (P<0.05) (Table 3 and Figures 2D, E and F).

**Table 3.** Open field test results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>C-100</th>
<th>C-25</th>
<th>T-100</th>
<th>T-25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total distance (cm)</td>
<td></td>
<td>904±56</td>
<td>902±76</td>
<td>862±61#</td>
<td>985±62*,#,$</td>
</tr>
<tr>
<td>Number of transitions between zones</td>
<td></td>
<td>5±1</td>
<td>4±1</td>
<td>4±1</td>
<td>5±1</td>
</tr>
<tr>
<td>Number of rearings</td>
<td></td>
<td>8±1</td>
<td>7±1</td>
<td>8±1</td>
<td>9±1*</td>
</tr>
<tr>
<td>Time spent in peripheral zone 1 (s)</td>
<td></td>
<td>169±2</td>
<td>171±1#</td>
<td>165±5*,#</td>
<td>173±1*,#,$</td>
</tr>
<tr>
<td>Time spent in peripheral zone 2 (s)</td>
<td></td>
<td>9±2</td>
<td>8±1</td>
<td>12±4*,#</td>
<td>6±1*,#,$</td>
</tr>
<tr>
<td>Time spent in central zone (s)</td>
<td></td>
<td>1,5±0,6</td>
<td>0,6±0,2#</td>
<td>2,3±1,0*,#</td>
<td>1,0±0,3*,#,$</td>
</tr>
</tbody>
</table>

Notes:
Values are Mean ± SEM
*P < 0.05 versus Control-25
#P < 0.05 versus Control-100
$P<0.05 versus Test-100
Figure 2. Open field test results. A. Total distance (cm). B. Number of transitions. C. Number of rearing. D. Time spent in peripheral zone 1 (s). E. Time spent in peripheral zone 2 (s). F. Time spent in center (s). *P < 0.05 versus Control-25; #P < 0.05 versus Control-100; $P<0.05 versus Test-100

Elevated plus maze test

The anxiety level evaluated by the elevated plus-maze test showed no major significant differences between the groups. In the controls group, no differences were seen between the vitamin deficiency group and vitamin sufficient group regarding time spent in open arms, time spent in closed arms and total distance traveled by the animal. Only the total number of entries to
open arms in C-25 group compared to the C-100 group was significantly decreased (Table 4 and Figure 3). In the test groups, the exposure to the pesticides mixture decreased anxiety-like behavior in the vitamin deficiency group compared to C-25 group (P<0.05), measured as decreased time spent in the closed arms, increased number of entries in the open arms and increase in the total distance traveled (Table 4 and Figure 3). Compared with the T-100 group there was a significant increase in the number of entries into the open arms and in the total distance traveled (P<0.05) (Table 3 and Figures 3C and D). The pesticide mixture in the vitamin sufficient group (T-100) decreased the number of entries into the open arms, and total distance traveled compared to the C-100 group (P<0.05) (Table 4 and Figures 3C and D).

**Table 4.** Elevated plus maze test results

<table>
<thead>
<tr>
<th>Group</th>
<th>Variables</th>
<th>Time spent in open arms (s)</th>
<th>Time spent in closed arms (s)</th>
<th>Total number of entries</th>
<th>Total distance (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-100</td>
<td></td>
<td>63±23</td>
<td>205±22</td>
<td>21±3</td>
<td>1002±96</td>
</tr>
<tr>
<td>C-25</td>
<td></td>
<td>47±15</td>
<td>227±20</td>
<td>17±2#</td>
<td>962±72</td>
</tr>
<tr>
<td>T-100</td>
<td></td>
<td>64±21</td>
<td>203±23*</td>
<td>16±3#</td>
<td>999±101</td>
</tr>
<tr>
<td>T-25</td>
<td></td>
<td>61±11</td>
<td>201±19*</td>
<td>25±4*,#,$</td>
<td>1285±116*,#,$</td>
</tr>
</tbody>
</table>

Notes:
Values are Mean ± SEM
*P < 0.05 versus Control-25
#P < 0.05 versus Control-100
$P<0.05 versus Test-100
Figure 3. Elevated plus maze test results. A. Time spend in open arms (s). B. Time spend in closed arms (s). C. Total number of entries. D. Total distance(cm). *P < 0.05 versus Control-25, #P < 0.05 versus Control-100, $P<0.05 versus Test-100

Passive avoidance test

Short-term and long-term memory and cognitive functions were assessed in the passive avoidance test. During baseline test, all the animals from control and test groups were allowed to enter the dark compartment. In the C-25 group, the latency time to enter the dark compartment was significantly decreased compared to the C-100 group (P<0.05) (Table 5, Figures 4A and 5A). The exposure to the pesticide mixture increased the entrance latency time in T-25 group compared to C-25 groups (P<0.05) and in T-100 group compared to C-100 group (P<0.05) (Table 5, Figure 4). Within the test groups, vitamin deficiency decreased the entrance latency time in T-25 group compared to the T-100 group (P<0.05) (Table 5, Figure 4). Regarding a short term memory evaluation, no alterations were observed in the control groups (Table 5 and Figure 5). The exposure to pesticides mixture slight affected short term memory test translated as an
increased percentage of entered rats to the dark compartment from 8% to 25% in T-100 group compared to C-100 group and from 0% to 25% in T-25 group compared to C-25 group without reaching statistical significance (P>0.05). Latency time to enter the dark compartment was decreased in T-25 group compared to the C-25 group and in T-100 group compared to the C-100 group (P<0.05). No differences were observed between these test groups in the short term memory test (Table 5, Figures 4, and 5). Regarding the effect of vitamin deficiency on long term memory, no differences were observed between C-25 and C-100 groups. The exposure to the pesticides mixture also showed no effect on T-100 group. The vitamin deficiency associated with exposure to pesticides mixture affected long term memory by increasing the percentage of entered rats in dark compartment from 8% in C-25 group to 50% in T-25 group (P<0.05) and decreased the entrance latency time to the dark compartment in T-25 group compared to C-25 group (P<0.05) (Table 5, Figures 4 and 5). The long term memory was significantly affected in T-25 group compared to the T-100 group (P<0.05) (Table 5, Figures 4 and 5).

Table 5. Passive avoidance test results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Short-term memory test</th>
<th>Long-term memory test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Entrance latency time (s)</td>
<td>Number (%) of entered rats to the dark compartment</td>
<td>Entrance latency time (s)</td>
</tr>
<tr>
<td>C-100</td>
<td>26±4</td>
<td>12(100%)</td>
<td>170±10</td>
</tr>
<tr>
<td>C-25</td>
<td>20±5##</td>
<td>12(100%)</td>
<td>180±0</td>
</tr>
<tr>
<td>T-100</td>
<td>33±8###</td>
<td>12(100%)</td>
<td>152±17###</td>
</tr>
<tr>
<td>T-25</td>
<td>27±4###</td>
<td>12(100%)</td>
<td>151±16###</td>
</tr>
</tbody>
</table>

Notes:
Values are Mean ± SEM
*P < 0.05 versus Control-25
##P < 0.05 versus Control-100
###P<0.05 versus Test-100
**Figure 4.** Passive avoidance tests results. A. Entrance latency (s) (baseline memory test). B. Entrance latency (s) (short-term memory test). C. Entrance latency (s) (long-term memory test). *P < 0.05 versus Control-25, #P < 0.05 versus Control-100, $P<0.05 versus Test-100.
Figure 5. Passive avoidance tests results. A. Percentage of rats that entered the dark compartment (baseline memory test). B. Percentage of rats that entered the dark compartment (short-term memory test). C. Percentage of rats that entered the dark compartment (long-term memory test). *P < 0.05 versus Control-25, #P < 0.05 versus Control-100, $P<0.05$ versus Test-100.

Discussions

Our objectives in this study were to evaluate if vitamin deficiency can potentiate the neurotoxicity of xenobiotics in long-term low-dose exposure regiments and also if the pesticide mixture in doses much below NOAEL levels can induce adverse and toxic effects to the animals after chronic exposure. It was reported previously that vitamin deficiency could modulate cytochrome P-450 enzymes expression, enzymes implicated in the metabolism of several xenobiotics including pesticides that can lead to changes of their toxicity (Docea et al., 2017; Guengerich et al., 1995). Deficiencies in B vitamins as folate, B12, and B6 are associated with neurological and psychological dysfunctions. It is speculated that in elderly, the deficiency in B vitamins is associated with an increased incidence of dementia and cognitive impairment (Lachner et al., 2012; Selhub et al., 2010). In vivo studies on rats showed that depletion of menaquinone-4 (MK-4) in brain altered the sphingolipids concentration in different brain regions and are associated with cognitive impairment (Tamadon-Nejad et al., 2018).

Nowadays a great concern represents the potentially harmful effects of chronic exposure to chemical mixtures from different sources at very low doses (Kostoff et al., 2018b; Tsatsakis et al., 2016; Tsatsakis and Lash, 2017). Pesticides are a class of chemicals to which humans are generally chronically exposed in combination via food and water consumption. It is well-known fact that residues of pesticides can be found in food products and in some cases, even in doses that are above the maximum residue limits (EFSA, 2016). Pesticides from different classes even at very low doses can interact in various ways leading to various effects as additives, synergic or
unpredicted effect to different target organ dependent of the compounds, chemical structure or 
dose (Hernandez et al., 2017). In the mixture of pesticides, used in this study, only diquat is 
known to produce neurotoxic effects at high doses much above the NOAEL levels (Dinis-
Oliveira et al., 2006; Djukic et al., 2012; EFSA, 2015). In this study, the doses used for 
individual chemicals should normally not produce any side effect on the animals as the ADIs 
levels are the NOAEL levels for animals divided by the uncertainty factor that is usually around 
100. Even so, there are studies that contradict these statements and one explanation would be that 
the setting of the regulatory limits, don’t take in account the possible interactions that could 
appear in the real-life exposure scenario. This is why this study aims to identify if there is a real 
risk at the neurological level after long-term low-dose exposure to a mixture of pesticides.

**Locomotor activity and anxiety level**

In vitamin deficiency control group, it was observed a decreased in the locomotor 
activity, spatial orientation activity, and an increase in anxiety level in open field test compared 
to the no-vitamin deficiency group. In elevated plus-maze test, the anxiety level was not 
significantly different between the control vitamin deficiency group and vitamin sufficient 
group, only slight increased anxiety in C-25 group translate by the decrease in a total number of 
entries in open arms. These findings are consistent with other studies that investigated 
deficiencies in several B vitamins and the central nervous system associated disorders. The study 
of Arora et al. (Arora et al., 2017) investigated the effect of vitamin B12 deficiency on anxiety, 
learning and memory using TCblR/CD320 knockout (KO) mouse, a model that exhibits vitamin 
B12 deficiency in the central nervous system. The results from the open field test in this model 
showed a decrease in the locomotor and spatial orientation activity and an intermediate increase 
in variables associated with anxiety that is consistent with our findings. Kuo et al. (Kuo et al., 
2007) showed that deprivation of pantothenic acid induces movement disorders in mice. Vitamin 
K deficiency was associated with decreased locomotor activity and exploratory behavior in a rat 
model that is consistent with our findings (Tamadon-Nejad et al., 2018).
In the vitamin sufficient test group, the pesticides mixture decreased locomotor activity and anxiety-like behavior evaluated by open field test compared to vitamin sufficient control group. Interestingly, in the vitamin deficiency group pesticide mixture caused a significant increase of locomotor activity compared to C-25 group and T-100 group and decreased anxiety level of rats compared to the C-25 group. The effect of the pesticides mixture on locomotor activity of the T-100 group is in agreement with previous studies that showed adverse effects at different levels after exposure to chemicals mixtures of pesticides plus food and lifestyle additives in doses around ADI (Docea et al., 2019; Tsatsakis et al., 2019b). The effects of the mixture on decreasing anxiety level can be explained as a hormetic behavior when the low doses of chemicals determine a stimulant effect that is eliminated in high doses. These findings are in accordance with other reports that evaluated neuroprotective effects of chemicals with neurotoxic potential at very low doses below the levels that can produce toxicity (Marini et al., 2007; Calabrese and Rubio-Casillas, 2018; Tsatsakis et al., 2019b). Interesting is that these hormetic effects are also preserved in the presence of vitamin deficiency. It was showed that hormetic stress responses could appear in animal studies where dietary restrictions can have anti-aging and life-prolonging effects and reduction of neurodegenerative disorders (Hayes, 2007).

**Cognitive function, short-term and long-term memory**

In the passive avoidance test, no significant differences were observed between the vitamin deficiency control group and vitamin sufficient control group either at baseline test, short term memory, or long term memory evaluation. This is somehow correlated with the studies that showed that dietary folic acid or other B vitamins deficiency that lead to increasing levels of homocysteine is not enough for memory impairments. Usually, these impairments appear only in combination with other neurological risks (Jadavji et al., 2015).

The exposure to the pesticides mixture showed a low effect on short memory in vitamin sufficient test group compared to control failing to reach the statistical significance and no effect on long term memory. Lahouel et al. (2016) showed that chronic exposure to low doses of
persistent organic pollutants mixture form of endosulfan, chlorpyrifos, naphthalene, and benzopyran could induce various disturbances of memory.

At baseline, the exposure to chemical mixture associated with vitamin deficiency increase the entrance latency compared to C-25 group. Interesting is the fact that compared to T-100 group vitamin deficiency associated with pesticide exposure increase the exploratory behavior at baseline test showed a decrease of the entrance latency time in T-25 group compared to the T-100 group. The vitamin deficiency had an additive effect with pesticide exposure on long term memory compared to vitamin deficiency only and exposure to mixture. The mechanism of these interactions is complex and not well documented. It is known that undernutrition can influence the metabolic processes and can increase the susceptibility of the organism to chemicals with neurotoxic effects, but it is not clear yet if these chemicals have an increased access to central nervous system only due to changes of the regulatory mechanisms between blood, brain, nerve and cerebrospinal fluid or through other complex changes (Spencer and Palmer, 2012).

**Conclusions**

Chronic vitamin deficiency decreased locomotor and spacial orientation activity and increased anxiety-like behavior in rats compared to vitamin sufficient group. Exposure to very low doses of a mixture of 6 pesticides affected the central nervous system, manifested as decreased locomotor activity, and stimulation in term of anxiety levels. No significant effects were seen in short- and long-term memory tests. It was interesting that the combination of vitamin deficiency and low-dose chronic pesticides mixture exposure produced stimulation of the central nervous system rather than an inhibition translating into increased locomotor activity and decreased anxiety-like behavior. This effect of stimulation of the central nervous system might be explained by chemical-induced hormesis. Long-term memory was affected by the combination of vitamin deficiency and exposure to pesticides mixture.
This study demonstrates that the chronic exposure to chemicals, even in doses considered safe can produce unpredicted or hormetic effects at the neurological level, especially when combined with vitamin deficiencies. The current approach of risk assessment that is based on a single-chemical approach for setting the safety limits cannot take into account all the real-life combination risks. The enormous effort to study such combination and mixture effects does not allow animal studies as shown as proof-of-principle here. This calls for screening experiments, for example, in this case with human brain organoids (Pamies et al., 2017). The inclusion of mixture effects into the assessment warrant such new approaches to increase the protection of the consumers.

**Declaration of interests – Article: The effect of chronic vitamin deficiency and long term very low dose exposure to 6 pesticides mixture on neurological outcomes – a Real-Life Risk Simulation approach**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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