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Neurotoxic Effects of Chronic Malathion Exposure in BALB/c Mice

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KEYWORDS
Malathion
Neurobehavioral deficits
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ABSTRACT

Malathion is one of the commonly used organophosphates pesticides (OPs) in Egypt. Chronic exposure to some OPs has been linked to many neurological disorders such as cognitive deficits, Parkinson's disease and mood disturbances. It is suggested that neuro-inflammation has an important role in mediating such diseases. The present research aims to investigate the neurotoxic effects of chronic malathion exposure in BALB/c mice and to clarify the possible role of inflammation. This study included 48 adult mice that were randomly divided into four groups (12 mice each) as follows: control group: mice did not receive any treatment and three test groups which were given malathion dissolved in distilled water once daily by gastric gavage for two months at 50, 100 and 200 mg / kg respectively. Neurotoxic effects were assessed through two behavioral tests then histopathological examination of the brains was done. The results revealed that malathion-exposed mice developed locomotor impairment in the form of increased foot slips and decreased efficient path in parallel rod floor test in addition to impaired performance of open field test. The neurobehavioral deficits were associated with histopathological changes e.g., decreased corpus striatum fiber density, increased dopaminergic neurodegeneration in substantianigra and increased microglial activation. The present findings suggest a potential role of neuro-inflammation in malathion-induced neurotoxicity.

Introduction

Organophosphate pesticides (OPs) including malathion are worldwide environmental pollutants which have been linked to neurodegenerative disorders (Jokanovi´c and Kosanovi´c, 2010;Sánchez-Santeda et al., 2016).

There is aplenty of studies concerning the neurotoxicological outcomes of acute exposure to malathion in laboratory animals. Various neuromotor, cholinergic, physiological, affective and cognitive disorders were reported at doses lower than that producing cholinesterase inhibition (Moser, 2007). However, little is known about the mechanisms involved in mediating the neurotoxic effects in case of chronic OPs exposure which may be better than acetylcholinesterase inhibition in prediction of OPs-neurotoxicity (Lein et al., 2012; Starks et al., 2012).

Inflammation is one of the suggested mechanisms because of the availability of experimentally validated inflammatory biomarkers that correlate well with neurobehavioral deficits observed with neurodegenerative disorders (Schütt et al., 2016).Hence, the aim of the present study was

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to evaluate the neurotoxic effects of chronic malathion exposure in BALB/c mice and to elucidate the possible role of neuroinflammation.

Material and Methods

This study was approved by Medical Research Ethics Committee, Faculty of Medicine, Mansoura University (code no.: MD/106). All chemicals were purchased from Sigma -Aldrich™ (Saint Louis, MO, USA) unless otherwise declared.

Animals and experimental design:

This work was conducted in Medical Experimental Research Centre, Faculty of Medicine, Mansoura University, Egypt. Animals included 48 BALB/c mice of both sexes (Albinoinbred strains), 4 month-old, 25-30 g weight. They were housed in clean cages under standard laboratory conditions including suitable temperature (22±2 ºC), good lighting and aeration. They were fed a standard laboratory diet and tap water. Mice were randomly divided into four groups (12 mice each) as follows: control group that received no treatment. Three test groups received malathion (57% commercial grade, Al Nasr Company for chemical industries) dissolved in distilled water and given orally by gastric gavage once daily for two months in three different doses; group 1 "50 mg/kg"; group 2 "100 mg/kg" (N'GO et al., 2013) and group 3 "200 mg/kg" (Selmi et al., 2012).

Assessment of the neurotoxic effects of malathion:

1- Neurobehavioral tests:

Evaluation of the locomotor activity in mice was done at the end of second month by using ANY-box ® (Stoelting Company, USA) which is a multi-configuration behavior apparatus. Two different behavioral tests were performed in a room that was completely isolated from external noises:

a. Parallel rod floor test:

Parallel rod floor test apparatus consists of a clear acrylic plastic box 20 cm x 20 cm x 30 cm (width, length, height) and a series of parallel stainless steel rods placed on stainless steel base plate that acted as a floor for the chamber. The locomotor activity was assessed using two parameters:

i. Foot slips (numbers of errors) which are measured by a touch sensor underneath the parallel rod floor.
ii. Efficient path (horizontal distance travelled by the mouse in cm).

b. Open-field test:

This apparatus is constructed of a clear acrylic plastic box 40cm x 40cm x 35cm (width, length, height) fits to ANY-box base. Two perpendicular lines were drawn on the floor with a marker and were visible through the clear wall. These lines divided the floor into four equal quadrants: north east (NE), north west (NW), south east (SE) and south west (SW). Each animal was placed individually at the center of the apparatus, allowed to explore it freely and observed for five minutes. Each mouse trial was recorded for latter analysis, using a camera that positioned above the apparatus. Locomotor activity was assessed for each mouse using numbers of mid zone cross in open field apparatus.

2- Histopathological examination of the brain:

At the end of the second month, under deep anesthesia with thiopental (100 mg/kg, intraperitoneal), the mice were perfused through the aorta with 50 ml of 10 mM phosphate-buffered saline (PBS), followed by 150 ml of 4% paraformaldehyde. After perfusion, each brain was rapidly dissected and fixed for 2 days with 10% paraformaldehyde. The brain pieces were processed into paraffin blocks and then cut by microtome at 4-5

micron on glass slides. Slides were deparaffinized and blocking of endogenous peroxidase using 30% hydrogen peroxide in methanol for 10 minutes and serum blocking solution (10% non-immune serum) for 10 minutes were used. Antigen retrieval was done by emersion of the slides in EDTA solution for 20 minutes at 90° C in water bath.

a. Immunohistochemistry for dopaminergic system (Arias-Carrión et al., 2007*):*

The dopaminergic system i.e. substantianigra (SN) and corpus striatum (CS) were stained using anti-TH antibody. Brain sections were incubated with primary mouse monoclonal anti TH antibody (diluted 1: 1000) over night at 4°C. After several washes with PBS, sections were incubated for one hour at room temperature with biotinylated secondary antibody (1: 500). The sections were then incubated with avidin-biotin- peroxidase solution for one hour at room temperature. All sections were washed several times. The reaction product was revealed by incubating the sections with diaminobenzidine. Slides were counterstained with Meyer`s hematoxylin, dehydrated and covered.

b. Immunohistochemistry for microglia activation (Astiz et al., 2013):

Ionized calcium binding adaptor molecule 1 antibody was used for microglia staining. Brain sections were incubated over night at 4 °C with a rabbit polyclonal antibody against the microglia marker anti-IBA1 (diluted 1:2000). Primary antibody was diluted in washing buffer containing 5% normal goat serum. After incubation with the primary antibody, sections were rinsed in washing buffer and incubated for two hours at room temperature with biotinylated goat anti-rabbit immunoglobulin G (diluted 1:300 in washing buffer). After several washes in buffer, sections were incubated for 90 min at room temperature with avidin–biotin peroxidase complex. The reaction product was revealed by incubating the sections with diaminobenzidine. Brain sections were dehydrated, mounted on the slides and examined with standard Olympus® light microscope (Olympus® model CX31RTSF)**.**

3- Image analysis*:*

Pictures were captured by a digital camera (Olympus® model E-420) and analyzed by using image J software version ij146-jdk6 for windows 7 (http://download.cnet.com/ImageJ-32-bit/3000-2192_4-75879102.html).

a. Image analysis of dopaminergic system:

- Mean optical density of TH-positive dopaminergic fibers in the corpus striatum was assessed using the image J software. To evaluate the entire striatal complex, the images were taken at six rostral-caudal levels corresponding to antero-posterior (AP): +1.60, +1.20, +0.20, -0.30, -0.90 and -1.40 mm from bregma. The striatum in each section was delineated and measured using the image J software. Non-specific background was correlated by subtraction of the non-specific binding as measured from the corpus callosum and for the TH-positive staining completely denervated areas of the striatum. The data represented the average of the six levels (Carlsson et al., 2006).
- The degree of neuro-degeneration in SN was assessed manually. Evaluation depended on the thickness of the ventromedial part of SN. Neuro-degeneration was ranked from one to four indicating loss of up to 25%, 50%, 75%, 100% of SN thickness respectively. In case of absent degeneration, the section was given the degree zero.

b. Image analysis of microglia: IBA1 positive cells were counted in the corpus striatum using image J software.

Statistical analysis

The statistical analysis of data was performed using the computerized statistical package for the social sciences (SPSS) version 22.0., released 2013 and excel program for figures. Quantitative data was described as means ± standard error of mean (SEM). The analysis of data was done to test statistically significant difference between groups.

For quantitative data; student t-test was used to compare between two groups. One way analysis of variance (ANOVA) test was used to compare more than two groups followed by Tukey's post hoc test. P is significant if ≤ 0.05 at confidence interval 95%.

Results

Table (1) and figure (1) illustrate the results of neurobehavioral assessment and histopathological examination of the brain inthe studied groups. The present work revealed that malathion-exposed mice developed locomotor impairment in the form of increased foot slips and decreased efficient path in parallel rod floor test which were statistically significant compared to the control group.

Open field test showed that the numbers of mid zone cross were increased significantly in group (1) while they decreased in group 2 and group (3) in comparison to control group. Additionally, north east quadrant entry frequency increased significantly while south east quadrant stay duration showed a significant decline in the three malathion-exposed groups in comparison with control mice. Regarding immobility, no significant affection was noticed among the studied groups. There were no cholinergic manifestations in any of the studied groups.

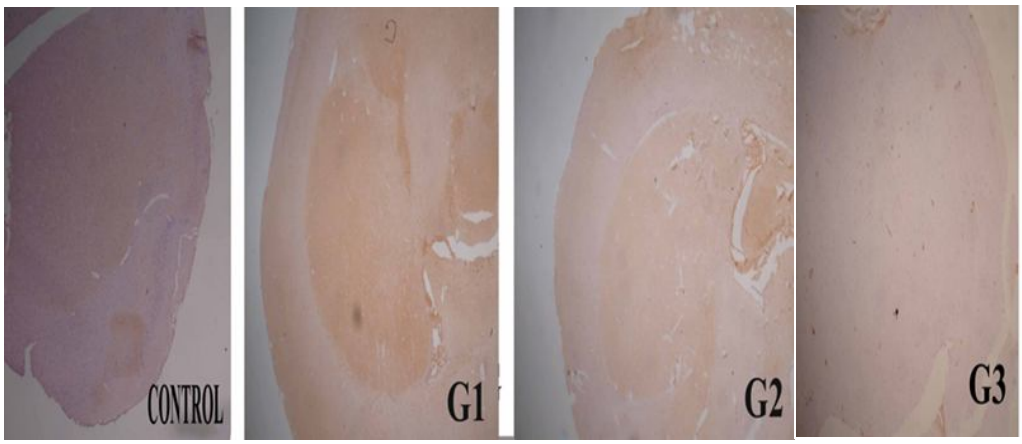
These neurobehavioral deficits were associated with histopathological changes in the brains of mice in the form of decreased CS fiber density, increased dopaminergicneuro-degeneration in SN in malathion-exposed groups which were statistically significant when compared to the control mice.

Furthermore, numbers of activated microglia in degenerated areas of the mice brains were increased in all malathion exposed mice with a statistically high significance in groups receiving malathion at doses of 100 and 200 mg/kg when compared to the control group while this increase was not significant in mice receiving malathion at a dose of 50 mg/kg (p=0.83).

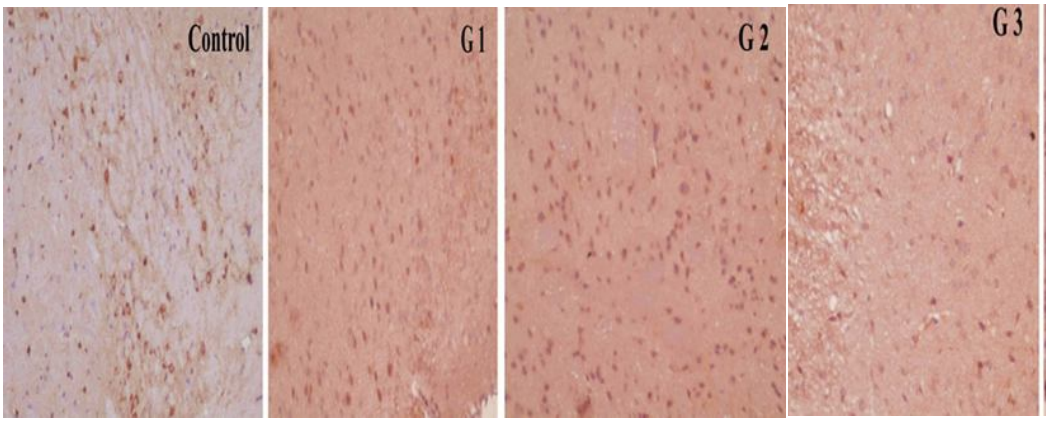
Table (1): Results of neurobehavioral assessment (parallel rod floor and open field tests) and histopathological examination of the brain inthe studied groups.

Groups (n=10 mice each)	Control group	Group 1 M (50 mg/kg)	Group 2 M (100 mg/kg)	Group 3 M (200 mg/kg)
Parameters				
A. Neurobehavioral assessment #				
1. Parallel rod floor test				
Foot slips	0.90±0.28	1.00±0.39	2.50±0.22	3.60±0.24
p value		0.08[a]	0.00[a]	0.00[a]
Efficient path	35.00±1.67	36.00±1.63	28.00±1.33	26.00±2.45
p value		0.07[a]	0.01[a]	0.00[a]
2. Open field test				
Mid zone cross	1.10±0.23	2.10±0.43	1.60±0.37	0.80±0.37
p value		0.03[a]	0.28[a]	0.59[a]
North East quadrant entry frequency	1.30±0.30	2.10±0.23	1.50±0.22	2.60±0.40
p value		0.03*[a]	0.58[a]	0.01*[a]
South East quadrant stay duration	45.60±6.38	25.90±3.45	17.80±2.26	27.60±3.72
p value		0.006*[a]	0.000*[a]	0.035*[a]
Immobility	0.30±0.15	0.70±0.33	0.1±0.1	0.60±0.24
p value		0.06		
B. Histopathological examination				
Fiber density of corpus striatum	157.92±5.19	104.28±4.38	104.07±2.69	91.52±1.79
p value		0.00*[a]	0.00*[a]	0.00*[a]
Degree of dopaminergic neuro-degeneration in substantianigra	0.00±0.00	2.75±0.13	3.50±0.15	4.00±0.00
p value		0.00*[a]	0.00*[a]	0.00*[a]
Number of microglia	121.33±1.26	124.83±6.08	176.50±9.09	252.50±19.96
p value		0.83[a]	0.00*[a]	0.00*[a]

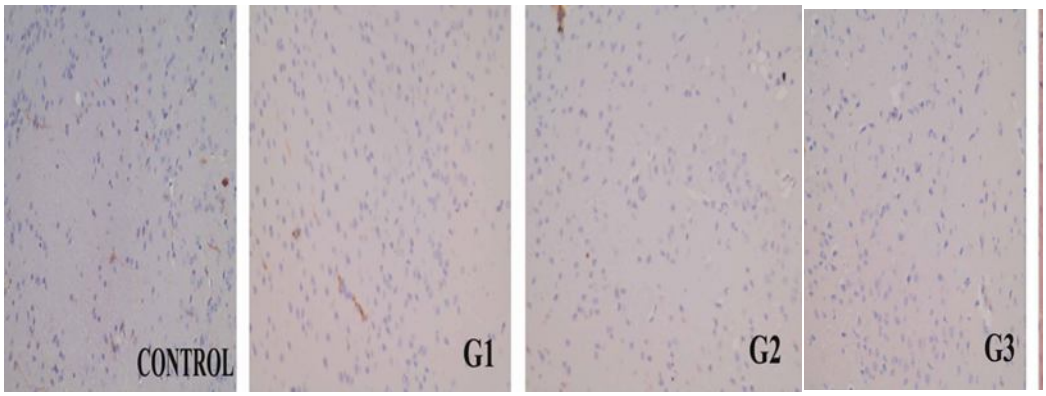
M: malathion, # N.B. Data are expressed as mean ±SEM, a: means *p* value compared to the control group, *p value is significant if ≤ 0.05.



A: Tyrosine hydroxylase (TH) immunohistochemistry of corpus striatum in the studied groups (×4 magnification).



B: Tyrosine hydroxylase (TH) immunohistochemistry of substantianigrain the studied groups (× 20 magnification).



C: Activated microgliastained with ionized calcium binding adaptor molecule 1 (IBA-1) in the studied groups (×20 magnification).

Fig. (1): Immunohistochemical examination of corpus striatum (A), substantianigra (B) and activated microglia (C) in the studied groups. Control received no treatment. G1, G2,G3: micegroups given malathion at doses of 50, 100 and 200 mg/kg respectively.

Discussion

Malathion is one of the most commonly used organophosphates in many countries including Egypt (Al Naggar et al., 2015). There is significant evidence suggesting an association between chronic malathion exposure and neurobehavioral deficits that occur without previous cholinergic symptoms. However, there is insufficient data concerning the mechanisms modulating such association (dos Santos et al., 2015). In the present study, assessment of malathion- induced neurotoxicity in BALB/c mice was done through neurobehavioral testing and immunohistochemical examination of the brain.

In order to investigate the neurotoxic effects of chronic malathion administration on locomotion and behavior in the studied mice, open field test was performed in the current work. Furthermore, parallel rod floor test was used to assess simultaneously impairment of locomotor activity and motor incoordination (Kamens and Crabbe, 2007; Li et al., 2016).

The present results demonstrated that the malathion-treated mice showed increased foot slips and decreased efficient path in parallel rod floor test in addition to impaired performance of open field test in the form of significantly reduced south east quadrant stay duration, increased north east quadrant entry frequency when compared to the control group. There was also significant increase of numbers of mid zone cross in low dose malathion administration (50mg/kg) while the other test groups did not differ significantly from control.

The present results indicated increased anxiety of malathion-treated mice in comparison to the control group. It was claimed that the reluctance of an animal to move from one place to another, or into the central area in the open field test, is indicative of increased anxiety (Jamilet al., 2016) which is in turn, a predominant clinical symptom in many neurodegenerative diseases (Baquero and Martín, 2015). To confirm these findings, brain histopathological examination demonstrated concurrent presence of decreased CS fiber density, increased dopaminergic neuro-degeneration in SN and increased microglia activation which again support the presence of neurobehavioral impairment in the present work.

Previous studies revealed that chronic exposure to OPs leads to neurobehavioral deficits (Acker et al., 2011; N'Go et al., 2013; Saravi et al., 2015; Dorri et al., 2015) and neurodegenerative disorders (Campaña et al., 2008; Torres-Altoro et al., 2011; Salyha, 2013; Salama et al., 2015) which are in line with the findings of the present workalthough some of these researches investigated various types of OPs in different doses, other animal species and different methods of administration.

There are very few studies investigating the role of neuro-inflammation as a possible mechanism for neurotoxicity occurring with exposure to different types of OPs (Astiz et al., 2013; Astiz et al., 2014; dos Santos et al., 2015; O'Callaghan et al., 2015) which support the present results and corroborating the evidence claiming that OPs-induced neurotoxicity is correlated with neuro-inflammation.

This could be explained by the following facts; microglia are the resident immune cells (macrophages) of the brain which can become activated to produce several reactive oxygen species and proinflammatory factors (e.g., tumor necrosis factor α, interleukin-1β) where many of these factors are neurotoxic and contribute to neuro-degeneration (McGeer and McGeer, 2010; Fischer and Maier, 2015).

It was stated that chronic activation of microglia (increased number and amoeboid morphology, where long thin processes extend

from the cell body into the surrounding) is implicated in the progression of many neurodegenerative disorders Such activation can trigger neurotoxic pathways leading to progressive degeneration to the nearby neurons (Kierdorf and Prinz, 2013; Amor et al., 2014).

In this context, microglia can become chronically activated by a proinflammatory trigger or in response to neuronal death to produce inflammatory cytokines (Taetzsch and Block, 2013). In other words, OPs may initiate an inflammatory response leading to the activation of microglia and astrocytes (Hanisch and Kettenmann, 2007) as noticed in the current study.

In conclusion, all the previous observations support the hypothesis that propose a potential mechanistic pathway for neuro-inflammation in mediating the neurotoxicity of chronic malathion administration in the present work. Such findings could be extrapolated to humans as they are likely similar to the neurobehavioral and cognitive deficits observed in individuals chronically exposed to malathion. Further studies are recommended to investigate the role of the anti-inflammatory drugs and if they have a beneficial protective effect against OPs-induced neurotoxicity and neurodegenerative disorders.

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الآثار السمية العصبية للملاثيون في فئران التجارب البيضاء

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و محمد سلامة و داليا أحمد السعيد

قسم الطب الشرعي والسموم الإكلينيكية - كلية الطب- جامعة المنصورة

يعد مركب الملاثيون من أكثر أنواع مبيدات الفوسفات العضوية استخداما فى مصر. وقد ثبت ارتباط التعرض المزمن لبعض هذه المبيدات بالكثير من الأمراض العصبية والنفسية مثل العجز المعرفي، مرض باركنسون ، واضطرابات المزاج. ومن المرجح أن يكون للالتهاب العصبي دورا هاما في حدوث تلك الأمراض. ويهدف هذا البحث إلى دراسة الآثار السمية على الجهاز العصبي الناتجة عن التعرض المزمن للملاثيون في الفئران البيضاء وتوضيح الدور المحتمل للالتهاب العصبي.

وقد تم إجراء الدراسة على عدد ٤٨ فأرا بالغا من فئران التجارب البيضاء، تم تقسيمها عشوائيا إلى أربع مجموعات (١٢ فأر لكل مجموعه) كالتالي: المجموعة الضابطة: فئران لم تتلقى أي علاج وثلاث مجموعات مختبرة: أعطيت الفئران الملاثيون المذاب في ماء مقطر مرة واحدة يوميا عن طريق الفم لمدة شهرين بجرعات مختلفة كالآتي: ٥٠ ،١٠٠،٢٠٠مجم/ كجم على التوالى.وتم التحقق من التأثير السمي العصبي للملاثيون عن طريق عمل بعض الاختبارات السلوكيه العصبية لتقييم مدى التدهور في الوظائف الحركية والسلوكية في الفئران ثم تم دراسة التغير المرضي الحادث في الأنسجة و الخلايا العصبية في جهاز الدوبامين العصبي في مخ الفئران.

وقد تبين حدوث خلل في النشاط الحركي في الفئران جراء التعرض المزمن لمادة الملاثيون والذي تتناسب طرديا مع مقدار الزيادة في الجرعة. كما ارتبط الخلل الحركي الناتج بحدوث تغيرات نسيجية مرضية في الخلايا العصبية و تضمنت: تلف خلايا الدوبامين العصبية في المادة السوداء (substantianigra) ونقص في كثافة الألياف العصبية في الجسم المخطط (corpus striatum) و اللذان تناسبا طرديا مع الزيادة في جرعة الملاثيون. كما تبين أيضا أن هناك ازدياد في أعداد الخلايا الدبقية الصغيرة (microglia) في المناطق التي ظهرت بها التغيرات المرضية في مخ الفئران مما يؤكد دور الالتهاب العصبي كأحد الآليات الهامة التي يمكنها تفسير التأثير السمي العصبي الناتج عن التعرض المزمن للملاثيون.