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Lesions in the Hippocampus and Substantia Nigra of Wistar Rats' Brains Induced by Organophosphate Insecticide

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ABSTRACT

As little is known about the neurotoxicity of the histological structure of the brain, this study focuses on the histological side of four- to six-month-old adult Wistar rat brains, which were examined after 0.1 mg\g organophosphate had been administered orally. In this study, the lesions were mainly localized at the hippocampus and substantia nigra (compacta pars) region. Distinct areas of necrotic and apoptotic tissues were detected in the CA1, CA2, and dentate gyrus of the hippocampus and compacta pars of the substantia nigra. Programmed cell death in the dentate gyrus was observed as early as 72 hours after treatment and necrosis of some brain regions. Moreover, Lewy bodies were noticed in the compacta pars of the substantia nigra. The most important symptoms of parkinsonism were observed in the substantia nigra (compacta pars). These were decreased neurons, increased neuronal melanin in the neurons, and increased glial cells. The degeneration of some neurons was reported in the polymorphic and pyramidal layers. The data showed an increase in the density of the axon membrane and several changes to the axis structure, such as the disappearance of the myelin sheath in some areas along the axis.

KEYWORDS

CA1, CA2, cell degeneration, compacta pars, dentate gyrus, Lewy bodies

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1. Introduction

Organophosphorus (OP) compounds are commonly used as insecticides, plasticizers, and fuel additives. These compounds potently inhibit acetylcholinesterase (AChE), which is the enzyme that inactivates acetylcholine in neuronal synapses. Acute exposure to high OP levels can cause a cholinergic crisis in both humans and animals. The acute nervous system toxicity of OP pesticides and its results have been well documented. The inhibition of AChE leads to the accumulation of acetylcholine, which acts as a neurotransmitter in all ganglia in the autonomic nervous system, as well as many synapses in the brain, skeletal neuromuscular junctions, some postganglionic nerve endings of the sympathetic nervous system, and adrenal medulla. Organophosphate affects the long nerves or tracts in the nervous system and causes a symmetrical weakness in the peripheral muscles in the hands and feet, along with sensory impairment (Kamanyire and Karalliedde, 2004). Exposure to pesticides is considered a global health problem. An increased risk of neurodegenerative disorders has been strongly associated with chronic, repeated exposure to organophosphates. Moreover, the chronic neurotoxicity mechanism remains unclear, although it differs from cholinergic affection in acute toxicity. Although chronic exposure usually causes symptoms that are not accompanied by acute cholinergic overstimulation, it is associated with neuropsychiatric conditions, such as anxiety and depression. Furthermore, many reports found a positive correlation between different types of OPs and an increased risk of cognitive deficits and neurodegenerative disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (Narayan *et al.,* 2013; Voorhees *et al.,* 2017; Wang *et al.,* 2014). The mechanism behind OP-induced neurodegeneration remains unclear and differs from AChE inhibition, which is involved in acute toxicity. One suggested mechanism for OP-induced neurotoxicity is neuro-inflammation (Banks and Lein, 2012; Diz-Chaves and Garcia-Segura, 2013). This study aims to verify the degenerative effects of long-term exposure to OP on the dopaminergic system in rats and identify the possible contributing mechanisms. PD and AD are the two most common neurodegenerative diseases. PD is clinically depicted by severe motor symptoms, including rigidity, postural instability, a resting tremor, and bradykinesia (Sharma *et al.,* 2013). PD pathology is characterized by progressive degeneration and the loss of dopaminergic (DA) neurons in the substantia nigra (SN) pars compacta. Moreover, the deposition of α-synuclein as insoluble and toxic aggregates are characteristic hallmarks of PD. Regarding AD, patients suffer from an irreversible memory loss, progressive cognitive impairment, language disorder, and impairment in their visuospatial skills due to the degeneration of the hippocampal and cortical neurons and extracellular amyloid plaques. The earliest understanding of the disease pathology of PD and AD focuses on neuronal degeneration and consecutively observed inflammation, which is likely to be activated by the damaged neurons (Blaszczyk, 2018; Maiti et *al.,* 2017). In previous studies, lesions were consistently found in the CAP1 and CAP2 of the hippocampus, amygdala, SN, and neocortex (Nobakht, 2011). Neurodegenerative diseases, including PD and AD, have common features, including the following: protein accumulation, cell death with mitochondrial involvement, oxidative stress, and degeneration of neuromelanin, which is an organic polymer that is produced by the dopamine metabolism (Dugger and Dickson, 2016; Kouli *et al.,* 2020; Martinez et *al.,* 2019). The neurons of the SN contain large amounts of neuromelanin, and the concentration increases with age. However, this was markedly decreased in PD patients. However, PD-like pathologies are characterized by a significant neuronal loss and the emergence of Lewy bodies that are composed of highly phosphorylated a-synuclein, which were observed in the treatment animals after three months (Carbaja *et al.,* 2019; García *et al.,* 2021; Vila, 2019).

2. Materials and Methods

Thirty male Wistar rats were maintained on a 12-hour light/12-hour dark schedule and given unlimited access to rat food and tap water in the Animal Department, Faculty of Science at the University of Aleppo. The animals were divided into two groups. The first group was orally given an aqueous solution of organophosphate (0.1 mg\g body weight)

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for four months, and the second group was given water without organophosphate. Their brains were then removed from their skulls and stored for at least three days in the fixative (formaldehyde 4%). Next, each brain was cut using a microtome into a series of sections that were 10 µm–20 µm thick. The serial sections (10 µm–20 µm thick) were then stained using different stains. The first stain was hematoxylin-eosin (H&E), which is distinguished by its good staining cell structures, such as cytoplasm and nuclei. The second was Cresyl violet, which is used for the central nervous system, including the nerve cell contents and Nissl particles. In addition, Colgi and Belcshowsky stains were used for the dendrites of neurons. The stain method was carried out according to the following stages: 1) dissolving paraffin from textile preparations using an electric heater at a temperature of 50° C–52° C; 2) transferring the slides to two baths of xylol for five minutes for each bath; 3) transferring the slides to a series of graduated concentrations of alcohol (100%, 90%, and 70%); and 4) washing them in distilled water to return water to the samples for five minutes per bath. In the H&E staining method, the hematoxylin was used first for five minutes, and then the slides were washed in distilled water and placed in the eosin stain for one minute. Later, the samples were transferred from the colorant to the distilled water and then to a series of gradual concentrations of alcohol (70%, 90%, and 100%) to dehydrate. They were kept in each concentration for one minute, and then they were transferred to two baths of xylol for two minutes each. The tissue preparations were covered in glass screens after an adhesive substance (Canada balsam or DPX) was applied to preserve them. Then, they were placed in a special dryer at a temperature of 37° C until the adhesive dried out.

2.1. Colgi and Beilcshowsky Stain Methods:

If the samples were large, they were divided into small pieces of 4 ml to 5 ml. Once the samples had been washed after the fixer and their tissues had been dried using blotting paper, they were placed in the potassium dichromate 2% solution for two days in the dark. After drying, the samples were transferred to the second solution (silver nitrate 2%) for two days. In this stain method, the double impregnation or Ramon Cajal's staining technique was used. Afterward, the samples were transferred to 1% oxalic acid and washed in 5% sodium thiosulfate. The samples passed an alcohol chain after being dried with blotting paper (70-90-100-Xylol). Later, the sample passed through the paraffin chain, and they were cut into 20 µm–50 µm-thick sections. The slides passed a chain of alcohol xylol (100-90-70-water-70-90-100-xylol). Finally, there was a microscopic examination of the slides.

2.2. Congo Red Stain Method:

In this stain, the sections were deparaffinized, and the water was removed. Then, they were stained in Congo red for 30–60 minutes, rinsed in distilled water, and passed through alkali alcohol for 5–10 seconds. The sections were then washed in distilled water for five minutes, counterstained in hematoxylin for 30 seconds, and washed in distilled water for one minute. Later, the sections were dipped in ammonia water for 30 seconds to turn them blue (the ammonia water was prepared by adding a few drops of ammonium hydroxide to tap water and mixing well). The sections were then rinsed in distilled water for five minutes and dehydrated using 95% alcohol, 100% alcohol, and Xylol.

3. Results

The present histological study of the hippocampus and SN of laboratory rats who were administered organophosphates showed the following results:

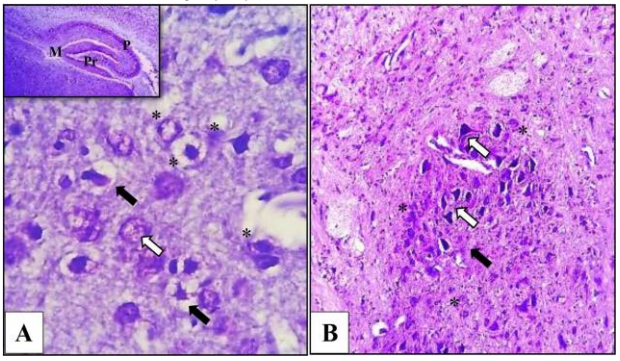
3.1. Hippocampus:

Programmed cell death was observed in some small pyramidal cells in the dentate gyrus (DG), and there were many gaps surrounding the neurons.

3.2. Substantia Nigra:

The most important symptoms of parkinsonism were observed in this area, such as reduced neurons, increased neuronal melanin in the neurons, and increased glial cells (Figure 1).

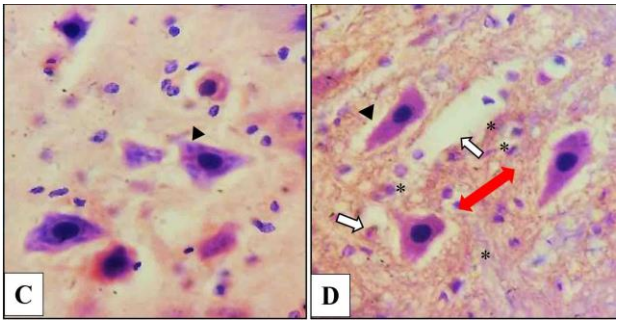
Figure 1: Cresyl violet-stained sections of the rats' hippocampus and SN (one dose of 0.1 mg/g with organophosphate for four successive weeks).



(A): The following three layers of the CA1 region of the hippocampus: the proper molecular layer (M), pyramidal layer (P), and polymorphic layer (Pr). Some small pyramidal cells appeared (arrow), and apoptotic cells appeared in the DG (black arrow). The neurons showed more vacuolation, and the cytoplasm was particularly rich in the Nissl substance of the glial cells (white arrow) x600. **(B):** In SN in PD rats, the major degenerative parkinsonian disorders showed a neuronal loss (black arrow), extra neuronal neuromelanin pigment (white arrow) x400, and gliosis in both sections (*) **(A and B).**

The histological study revealed slight damage to some neurons in the SN with the emergence of gaps around these cells and severe dendritic damage. Moreover, there was a decrease in the Nissl particles within the cytoplasm of the nervous cells and an increase of the amyloid fibers that were stained in Congo red in the space among the neurons. Additionally, as noted, the number of glial cells increased greatly within the sections (Figure 2).

Figure 2: Congo red-stained sections of the SN (a dose of 0.1 mg/day with organophosphate for four successive weeks).

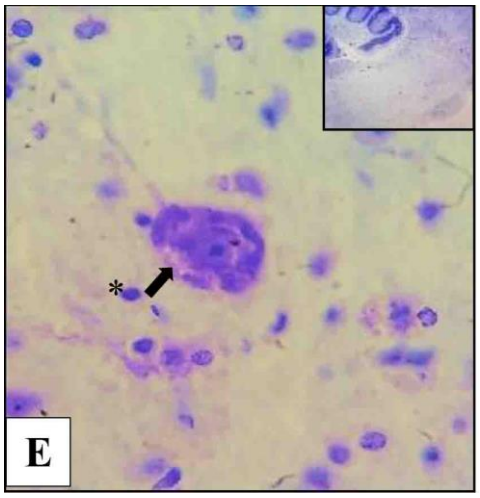


(D): Slight damage to the neuronal cells (black arrow). Microglial cell nuclei were observed in relatively large numbers (*) compared to the control, and necrotic cells were found in healthy ones. Throughout the compacta pars, some cells were also slightly affected. In these cases, the affected cells became vacuolated (white arrow), and the dendrites and cell somites were more severely damaged than the axons. Likewise, the Nissl substance decreased (arrowhead), and there was an increase in the amyloid fibers among the cells (red left-right arrow) compared to the control **(C)** x1000.

Other changes were observed in the compacta pars of the SN, such as

the separation of the components of the cytoplasm, huge gaps around the nuclei of some neurons in this region, and increased glial cells (Figure 3).

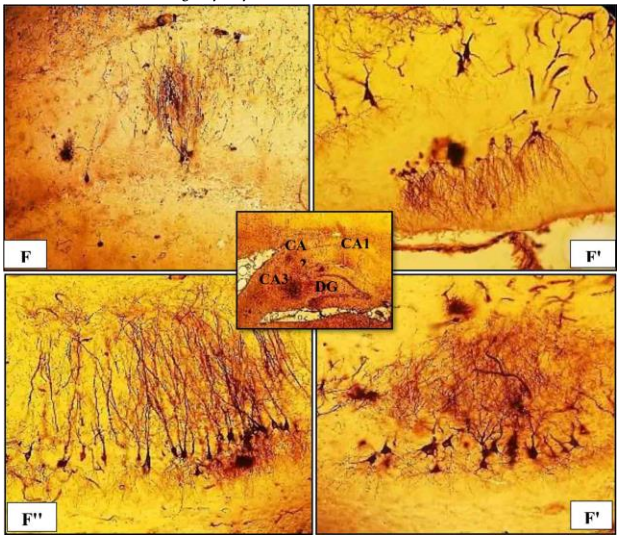
Figure 3: The Cresyl violet-stained section of the SN (a dose of 0.1 mg/day with organophosphate for four successive weeks) at the level of the cerebellum (brain stem).



(E): Neuron with segregated cytoplasmic components. Larger vacuolated organelles surround the nucleus (black arrow) x600. The neuron is segregated from its surroundings, and there are increased dark cells, which are probably glia (*).

The Golgi stain in different regions of the hippocampus (CA1, CA2, and CA3) showed a degradation of some neurons in the polymorphic and pyramidal layers after being stained and saturated in silver nitrate (Figure 4).

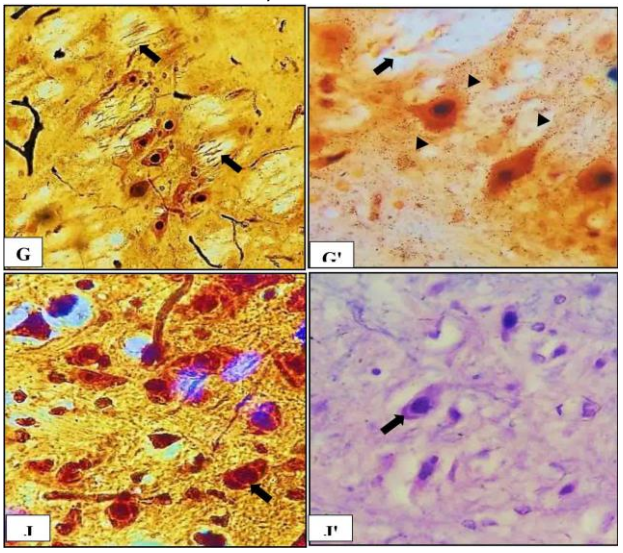
Figure 4: Colgi cox-stained sections of the rats' hippocampus (a dose of 0.1 mg/day with organophosphate for two successive months).



(F) and (F') The CA1 and CA2 regions of the hippocampus proper (molecular layer [M]). **(F")** The CA2 region of the hippocampus proper (polymorphic layer [Pr]). **(F'")** The CA3 region of the hippocampus proper (pyramidal layer [P]) and the cells that were impregnated by silver nitrate in CA1, CA2, and CA3. This was probably degenerated x600.

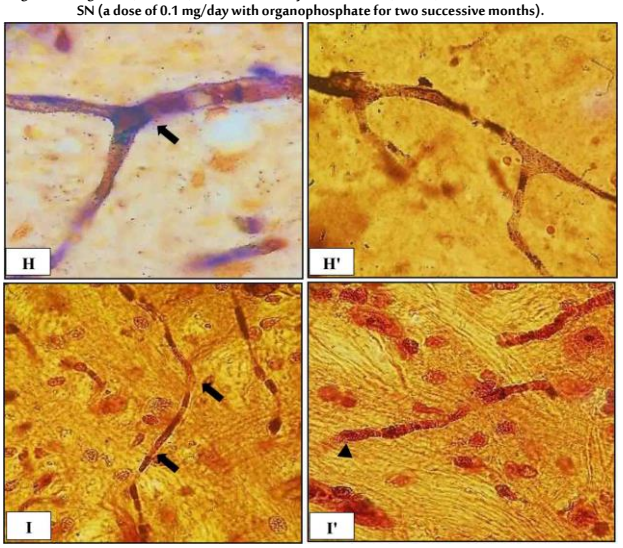
The Beilschowsky silver nitrate stain was distinguished by the double impregnation in the SN region in the compacta pars. As necrosis emerged in multiple spaces among the neurons with an increased phosphorylation of the proteins in most of the neurons' membranes, these neurons' membranes were saturated with silver nitrate and stained in black dots. Both this and the hematoxylin stain showed visible Lewy bodies in the cytoplasm of neurons as clusters (Figure 5).

Figure 5: Beilschowsky silver nitrate-stained sections of the rats' SN (a dose of 0.1 mg/day with organophosphate for two successive months) at the midbrain level (brain stem) in the compacta pars of the SN.



(G) Necrosis in many spaces among the neurons (black arrow) x600. **(G')** The membrane of most neurons stained in silver nitrate concerning the hyperphosphorylation of proteins (arrowhead) x1000. Beilschowsky silver nitrate- and H&E-stained sections of the rats' SNs (a dose of 0.1 mg/day with organophosphate for three successive months). **(J and J')** Lewy bodies (black arrow) in the pigmented cell of the compacta pars x1000 and the increased density of the axon membrane with changes to the structure of the axis, such as the disappearance of the myelin sheath in some areas along the axis. However, Schwann cells were not observed (Figure 6).

Figure 6: Golgi cox- (H and H') and Beilschowsky silver nitrate (I and I')-stained sections of the rats' SN (a dose of 0.1 mg/day with organophosphate for two successive months).



(H) The normal axon with increased membrane density compared to the control **(H')** x1000. **(I)** The axons' structure changed as the myelin sheath disappeared in some areas along the axis (black arrow), while Schwann cells were not observed (arrowhead) compared to the control **(I')** x600.

4. Discussion

Despite the increasing amount of research in this field, the effects of repeated or prolonged organophosphate exposure at low to moderate levels in both humans and animal remains unclear. This

study used lower doses and longer durations of exposure, which caused a barely detectable cholinesterase inhibition, followed by cellular damage in the brain.

Moreover, the study investigated the effects of OP on some regions in the central nervous system that are associated with PD and AD. In brief, the histological effects on the hippocampus and SN of rat brains that were treated with 0.1 mg\g of OP were confirmed.

In conjunction with previous studies, this study found apoptosis, which is programmed cell death, in several small pyramidal cells in the DG of the hippocampus, neuronal loss in the compacta pars of the SN, and dopaminergic degeneration due to chronic treatment with other types of organophosphates (Dorri *et al.,* 2015; Salyha, 2013). Moreover, idiopathic PD was characterized by the progressive loss of dopaminergic neurons in the SN (pars compacta), which leads to dopamine depletion (Dirnberger and Jahanshahi, 2013). This improved after the administration of these compounds, as demonstrated in the current research. Several studies reported a possible correlation between exposure to pesticides and the development of neurodegenerative disorders (Hernández *et al.,* 2016; Sánchez-Santed *et al.,* 2016). Since OPs are the most used pesticides, the previously mentioned studies focused on a link between OPs and the most common neurodegenerative diseases, PD and AD. Interestingly, some types of organophosphates have been proved to induce degeneration in the dopaminergic system (Astiz *et al.,* 2013; Binukumar and Gill, 2011).

Inflammation by insecticides, such as organophosphate, is thought to be the primary pathophysiological reason for neuronal degeneration, which occurs when the neutral amino acid transporter mediates the Na+ dependent entry of organophosphate into dopaminergic neurons. Consequently, organophosphate impairs redox recycling and induces oxidative stress, which leads to neuronal death (Banks and Lein, 2012; Farkhondeh *et al.,* 2020; Pearson and Pate, 2016). The increased permeability of the blood-brain barrier (BBB) and neurovascular dysfunction have been associated with severe conditions of PD. This effect could be linked to the infiltration of inflammation molecules that cause microglia activation and dopaminergic neurons death (Martinez *et al.,* 2019).

Numerous studies have demonstrated that exposure to a variety of OPs at lower and prolonged levels induces major changes in the central nervous system, such as changes to the gene expression, cell signaling pathways, and cellular ultrastructure. Moreover, exposure to various OPs increases the strict inclusion of many glial cells (Voorhees *et al.,* 2017).

It is worth mentioning that microglia are the resident immune cells (macrophages) of the brain, which can be triggered and activated in response to pro-inflammatory triggers or neuronal death. In this case, several reactive oxygen species and pro-inflammatory factors (e.g., tumor necrosis factor α and interleukin-1β) are produced, which contribute to neurotoxicity and degeneration. Similarly, OPs that include malathion may cause an inflammatory response, which could lead to the activation of microglia, as found by several studies (Ahmed et *al.,* 2017).

Since the metabolism of astrocytes is an indissociable link between neuronal health and synaptic functions, it is important to be mindful of how environmental toxicants can impact human health. Furthermore, environmental toxicant exposure can result in glucose dysfunction, including the involvement of the GLUT1 transporter, especially the astrocyte-specific transporter. Within the CNS, there is an overlap between the peripheral and astrocytic mechanisms. The engagement of the CYP detoxification system, which is robustly expressed in astrocytes as part of its role as the primary defense against xenobiotic penetrance into the CNS, suggests that astrocytes are crucial for a system-wide response to toxicants (Jiang *et al.,* 2017; Zhang *et al.,* 2019).

Lewy bodies were visible in the cytoplasm of neurons as clusters, which could be due to the presence of cytoplasmic protein aggregates in dopaminergic neurons. Although certain studies suggested that the progressive rostral spread of the Lewy body pathology reflected the clinical course of PD, other studies indicated that Lewy bodies are not the toxic species that are responsible for cell death. Hence, neuronal death may occur prior to the formation of Lewy bodies in some neurons (Saha *et al.,* 2000)

Additionally, PD patients with a longer disease duration are expected to have a wider regional distribution and greater density of Lewy body pathology combined with nigral cell loss in compacta pars. However, further research suggested that 15% of surviving nigral neurons contain Lewy bodies, and the age-adjusted proportion of Lewy body-bearing neurons is relatively stable throughout the disease duration.

Other studies suggested that the etiology of PD is a genetic basis that interacts with environmental factors.

The current study shows an increase of the amyloid fibers that were stained in Congo red in the spaces between neurons. The deposition of Aβ (mainly Aβ 40 and 42) caused them to form amyloid plaques, which are associated with reactive gliosis (Selkoe, 1994). In these areas, the activated microglia are recruited, and the reactive astrocytes exhibit a morph-functional remodeling, which modifies their interactions with neurons. Despite the reasonably close correlation between the increased phosphorylation and neuron degeneration, this study concludes that exposure to OP compounds degenerates some neurons in the polymorphic and pyramidal layers. This was proven by various staining methods. According to the hypothetical basis of binding silver to disintegrate products of proteins in neurons, this study demonstrates the emergence of necrosis in multiple spaces among the neurons, with an increased phosphorylation of the proteins in most of the neurons' membranes.

Silver-forming complexes with individual amino acids and progressive fragmentation of proteins (proteolysis) in disintegrating neurons leads to expansive sites for silver to form complexes (Anthony et *al.,* 2019).

Biographies

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