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Inflammatory Cytokines, Oxidative Stress Biomarkers and Clinical Manifestations of Organophosphorus Pesticides-Exposed Researchers

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Abstract

Organophosphorus pesticides (OPs) exposure may be associated with an increased risk of immunotoxicity in the occupational workplace. The study was designed to investigate and compare the correlation between clinical symptoms, oxidative stress, and the immunological effects of OPs exposure among occupationally exposed researchers. Clinical manifestations associated with pesticide exposure were collected from 49 researchers who were occupationally exposed to OPs (exposed group), and 51 non-exposed researchers (control group). The levels of cholinesterase enzymes (Acetylcholinesterase (AChE) and Butrylcholinesterase (BuChE)) have been estimated as an exposure biomarker for pesticide exposure. The oxidative stress status was evaluated by malondialdehyde (MDA) assessment, while total antioxidant capacity (TAC) could be an indicator of the antioxidant mechanism. We used Interleukin –6(IL-6) and Interferon- gamma(IFN-γ) as immune biomarkers as well as Tumor Necrosis factor – Alpha (TNF-α) as a biomarker of cellular immune function. Our results revealed a significant decline in AChE, BuChE, and TAC levels, while there was a significant elevation in MDA in occupationally exposed researchers versus control group. In addition, the levels of IL-6, IFN –γ, and TNF-α have been increased in exposed researchers. From clinical examination, the chief morbidity was pertained to neurological disorders and bronchial asthma as a result of occupational exposure to OPs pesticides. Muscular weakness was significantly syndicated with the decline in AChE and BuChE levels. Bronchial asthma was closely associated with MDA, IL 6, and TNF-α. Furthermore, in numbness, MDA, IL-6, IFN-γ, and TNF-α, were significantly higher. This study also indicated that MDA, IL-6, and TNF-α levels were significantly boosted up in exposed researchers who were not wearing a mask. This could be a risk factor for some adverse health hazards.

Keywords: Organophosphorus Pesticides, AChE , BuChE, Cytokines, oxidative stress, MDA, TAC, Personal Protective Equipment (PPE)

Introduction

Organophosphorus are compounds that most commonly used in agriculture as pesticides [1]. Despite its popularity and widespread prevalence, the pesticides raise serious concerns about health risks arising from prolonged exposure[2]. Occupational exposure to OPs may cause immunotoxicity, cardiovascular, neurological, reproductive and carcinogenic disorders [3]. There are two types of cholinesterase enzymes; acetylcholinesterase (AChE) that present in neural tissues and the erythrocyte membrane [4] and plasma cholinesterase (BuChE) that is synthesized primarily in the liver and released at a high rate into plasma[5]. Prolonged exposure to OPs ceases cholinesterase activities. However, cholinesterase inhibition alone cannot explain the wide range of clinical disorders[6]. In addition, Oxidative stress and disturbances of cytokine balance are other factors that contribute to pesticides health hazards[6]. Oxidative stress-induced by pesticides enhances reactive oxygen species (ROS) that alter the oxidative balance within cells [7]. Antioxidants considered as scavenging enzymes act as defense mechanisms in the detoxification of free radicals[7]. Severity of oxidative damage can be estimated by measuring malondialdehyde (MDA, the end product of lipid peroxidation), while the

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total antioxidant capacity (TAC) is one of the antioxidant defense mechanisms[8]. As the balance between pro-inflammatory and antiinflammatory mediators regulate the inflammatory response [9] and OPs induce low-grade inflammation, so, chronic exposure to OPs cause disturbances between the release of pro-inflammatory mediators[10]. Cytokines such as Tumor Necrosis factor – Alpha (TNF-α), interleukin (IL-1, IL-6 and IL-10), or type 1 interferon (IFN) secretion increased by activated macrophages that leads to up-regulation of inflammatory reactions [10], and antiinflammatory cytokines, that inhibit pro-inflammatory cytokine synthesis and adhesion molecule expression while increasing the levels of specific cytokine inhibitors [9]. Therefore, it was useful to investigate the disturbance of the cytokine balance from exposure to pesticides. Occupational exposures to pesticides are mainly due to inhalation and skin absorption[11]. Respiratory inhalation occurs when OPs are sprayed in "an inhalable form". Dermal absorption occurs through skin contact with pesticides or through clothing and tools contaminated with pesticide residues[11].

The goal of this study was therefore to assess the association between the inflammatory cytokines and the oxidative stress biomarkers with clinical manifestations and their correlation with the use of PPE in researchers who are occupationally exposed to pesticides.

2. Subjects and Methods and sampling

2.1. Study Design and Population

A cross-sectional study was conducted in 49 researchers occupationally exposed to pesticides and 51 non-occupationally exposed controls. Both groups were recruited from different Egyptian governmental research centers, and matched their socioeconomic status, age, and smoking habits. The approval of the Ethics Committee was received from the Ethical Committee at the National Research Centre, Egypt. Written consent was obtained from all the selected subjects.

2.2. Questionnaire & Clinical examination

The questionnaire included details of the socio-demographic aspects, smoking habits, and history of health problems for any system of the body. Also, clinical examination of all systems was performed with special concern of respiratory and neurological systems.

2.3 Sample collection

About 5 ml of the venous blood was collected from each subject and divided into two separate tubes. Two ml of blood was left in a dry tube for 30 minutes at room temperature for coagulation and then centrifuged for 10 minutes at 3000 rpm for serum separation for AChE, TAC, MDA, IL6, IFN-γ and TNF-α determination. A further three ml of blood was collected in an EDTA tube, centrifuged for 3000 rpm for 10 minutes to separate plasma for determination of BuChE. All samples were stored at -20^{o} C until determination by colorimetric method.

2.4 Determination of cholinesterase activities

2.4.1 Acetylcholinesterase

The immunoassay kit was used to measure the activity of human acetylcholinesterase in serum in vitro by (Sandwich-ELISA, Sunredbio, Shanghai).

2.4.2 Butyrylcholinesterase

The activity of butyrylcholinesterase was measured in plasma using a colorimetric method according to Knedel and Bottger[12].

2.5 Determination of oxidative stress markers

2.5.1 Malondialdehyde

Serum MDA activity was colorimetrically determined in the serum [13].

2.5.2 Total antioxidant capacity

This assay based on the ability of the enzyme to inhibit the phenazine methosulphate -mediated reduction of nitroblue tetrazolium dye.

2.6 Immunological Profile

2.5.3 Serum Cytokines

Cytokines (IL-6, TNF-α and IFN-ϒ) were quantitatively analyzed using ELISA kit purchased from SinoGene Biotech Co.,Ltd, China.

3- Statistical analysis

Statistical analysis of the collected data and the laboratory results was performed with SPSS version 24. The comparisons of quantitative variable between two groups were performed using independent t-test and expressed as mean and standard deviation (Mean ±SD). Qualitative results were expressed as number (no.) and percent (%).Qualitative results were analyzed using

Pearson's Chisquare (χ2) and likelihood ratio will be used in cases where more than 25% of the cells had expected count < 5. Pearson correlation coefficient was used to study the relationship between the different quantitative variables.

4-Results

The exposed researchers were occupationally exposed to OPs for about 6.3±1.9 years. There was no significant difference in age (*P*-value=0.38) between workers and control groups (39.4±1.42 and 37.02±1.79 respectively). The exposed researchers consisted of 38(77.6%) males and 11(22.4%) females while the control group contained 35 (68.6%) males and 16 (31.4%) females. There was no significant difference in male and female percentage between two groups (*P*-value =0.371). About 7 (14.3%) of the exposed workers were smokers while 42 (85.7%) were not smokers. In contrast, 11 (21.6%) of the controls were smokers while 40 (78.4%) were not. There was no significant difference in the smoking habits (*P*-value =0.289) between the two groups.

By means of a questionnaire and clinical examination, several clinical risks were detected in exposed populations . Fig. (1) represents different clinical manifestations; neurological disorders (muscle weakness (53.06% and numbness 59.18%), respiratory dysfunction (bronchial asthma 51.02%), eye irritation (20.41%), and dermatitis (30.61%).

Concerning estimated biochemical parameters; these results indicated a significant decline in biomarkers of exposure (AChE and BuChE) in exposed researchers relative to the control group. There was a significant elevation in oxidative stress biomarker (MDA), inflammatory cytokines (IL- 6, IFN- γ, and TNF-α) while there was a significant decline in the TAC level.

Fig.2 represents a significant negative correlation between duration of exposure and BuChE (r =0.104, p value <0.05)while there was a significant positive correlation between duration of exposure with MDA (r =0.072, p value <0.05), IL-6 (r = 0.678, p value <0.01) and TNF-α (r = 0.544 , p value <0.01).

Table (2) showed that in exposed researchers, AChE was positively correlated with BuChE and TAC but negatively correlated with IL-6 while BuChE was negatively correlated with IL-6 and IFN-γ. Additionally, TAC was negatively correlated IL-6 and IFN-γ. There was appositive correlation between MDA with IL-6 and TNF-α. Also, IL-6 was positively correlated with IFN-γ and TNF-α.

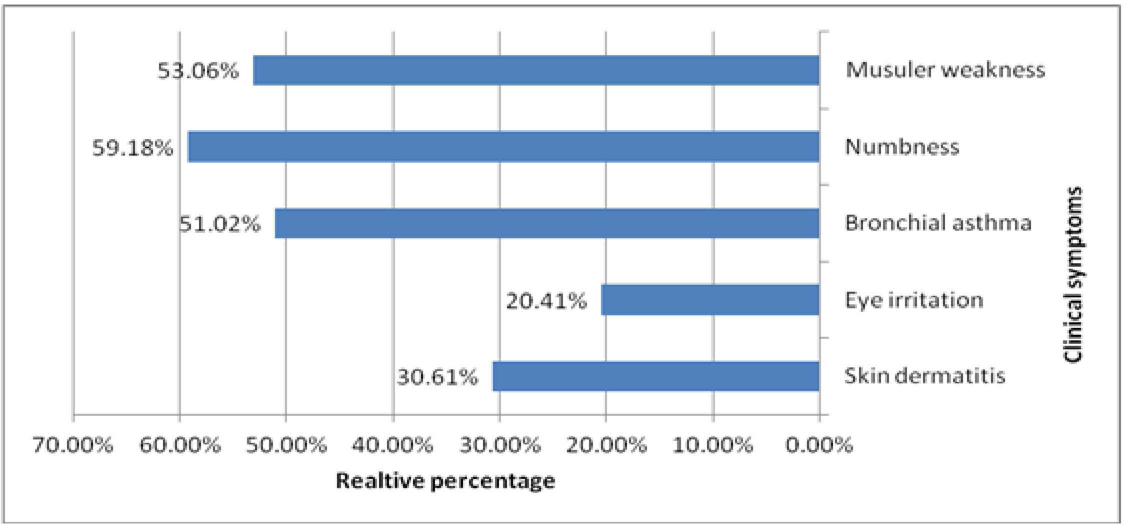

Figure1. Prevalence of clinical Symptoms among the exposed group.

Table 1: Comparison of different estimated biochemical parameters between researchers and their controls

Variable	Control (51)	Exposed (49)	*P*-value
AChE (ng /mL)	352.45±23.35	184.59±13.15	<0.001
BuChE (U/ L)	3018.06±78.33	2011.59±270.25	<0.005
TAC (mM/L)	1.23±0.05	0.70±0.02	<0.001
MDA (nmol/ml)	6.71±0.39	9.29±0.65	<0.005
IL- 6 (ng/L)	1.05±0.14	3.09±0.25	<0.001
IFN-γ (pg/ml)	663.48±84.25	1380.15±79.14	<0.001
TNFα (ng/l)	98.00±9.64	127.63±11.35	<0.005

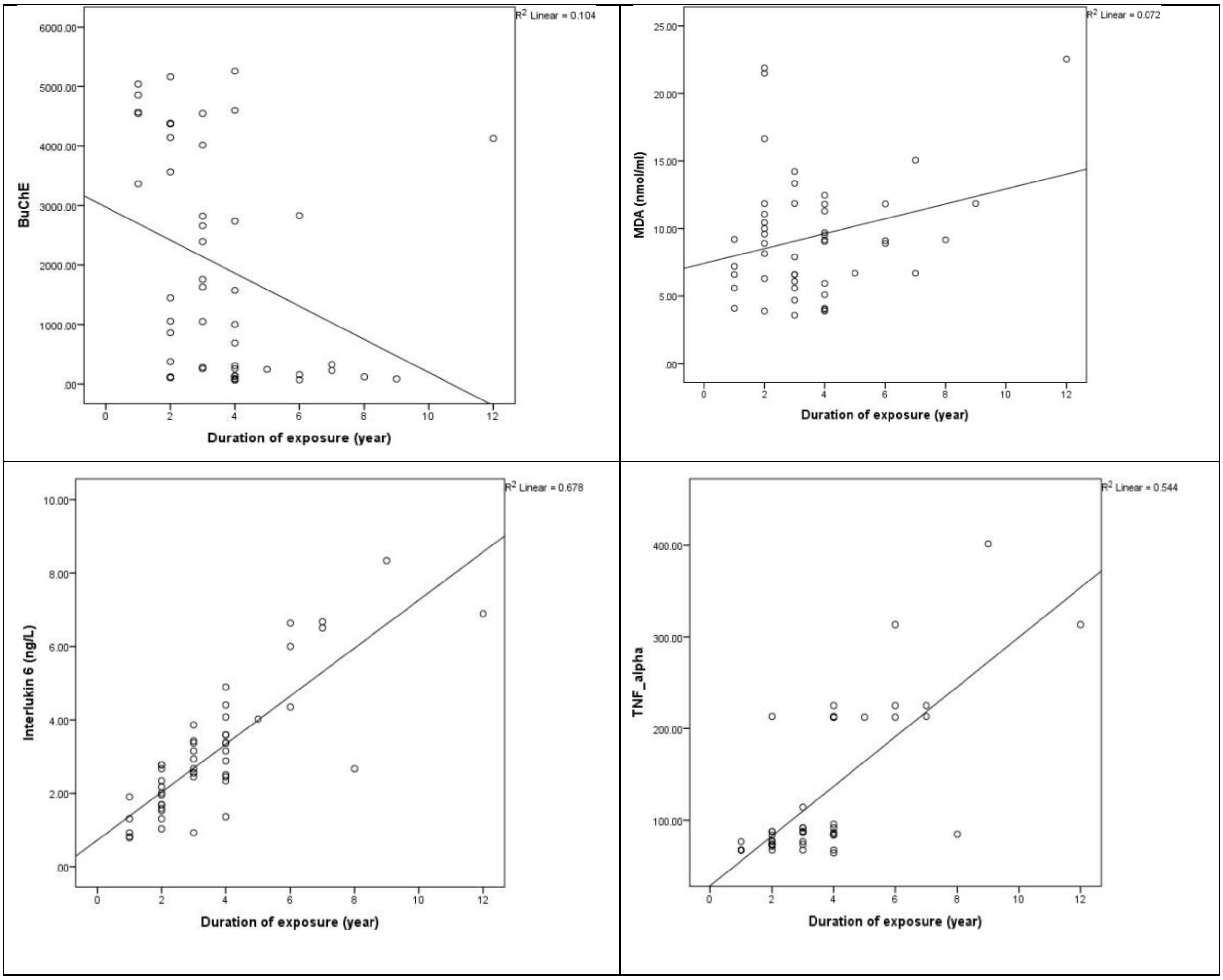

Figure2: The Correlation between BuChE (ng/L), MDA (nmol/ml), IL-6 (ng/L) and TNF-α (ng/l) with duration of exposure (year) in the exposed group

Tab.2 correlation analysis between estimated biomarkers among pesticides-exposed researchers

	AChE (ng /mL)	BuChE (U/ L)	TAC (mM/L)	MDA (nmol/ml)	IL-6 (ng/L)	IFN-γ (pg/ml)
BuChE (U/ L)	0.12*	-------------				
TAC (mM/L)	0.35**	0.14	________			
MDA (nmol/ml)	-0.16	-0.13	-0.31	_______		
IL-6 (ng/L)	-0.37*	-0.32*	-0.46	0.49*	--------------	
IFN-γ (pg/ml)	-0.36	-0.08*	-0.61	0.68	0.23*	-------------
TNF-α (ng/l)	-0.19	-0.11	-0.28*	0.18*	0.83**	0.04

*Correlation is significant at the 0.05 level (2-tailed).

**Correlation is significant at the 0.01 level (2-tailed).

Table (3): Comparison of pesticides exposure biomarkers with clinical symptoms in the exposed group

Clinical symptoms		TAC (mM /L)	MDA (nmol/ml)	IL-6 (ng/L)	IFN-γ (pg/ml)	TNF-α (ng/l)
Muscular-weakness	with	0.51±0.02*	9.8±1.1	1532.0±90.0*	3.52±0.6	134.5±21.4
	without	0.98±0.04	8.8±0.8	1208.5±127.3	3.09±0.39	129.0±18.6
Numbness	with	0.71±0.02	11.1±1.1*	1585.6±63.0**	3.91±1.8*	134.5±16.7*
	without	0.79±0.1	8.0±0.8	1082.2±149.6	2.39±0.67	93.8±24.4
Bronchial asthma	with	0.69±0.04	17.2±5.4*	1395.9±81.4	3.60±0.63	226.12±14.1*
	without	0.73±0.01	9.0±0.6	1009.8±249.8	2.19±0.32	116.75±28.9
Eye-irritation	with	0.72±0.01	12.2±0.3*	1395.9±81.4	3.22±0.71*	131.9±16.6
	without	0.74±0.02	9.3±1.5	1391.5±87.0	1.89±0.19	115.3±20.0
Skin-dermatitis	with	0.70±0.03	8.7±0.6	1321.4±159.4*	3.31±0.41	134.5±22.0
	without	0.73±0.03	7.4±0.7	1009.8±249.8	3.09±0.41	127.9±19.9

Table (3) illustrated the association between estimated cytokines and oxidative stress biomarkers with clinical symptoms in exposed researches. Muscular weakness was combined with elevation of IL-6 levels and decline in TAC level. The levels of MDA, IL-6, IFN-γ, and TNF-α was significantly raised in researchers who represents the symptoms of numbness. In exposed subjects with bronchial asthma, there was a significant boost in MDA, IFN-γ, and TNF-α. In addition, eye-irritation was significantly related to higher levels of MDA, and IFN-γ. Skin-dermatitis was significantly linked to a heightened IL-6 level.

Regarding, the use of PPE among exposed researchers, 61.20% were wearing gloves, 71.4% were wearing mask and 63.3% cared for wearing the apron. Our results revealed that the exposed group who was not interested in wearing gloves showed a significant decrease in BuChE and increase in TNF-α level. Respecting those who did not wear a worn mask, there was significant decrease in BuChE and significant increase in MDA, IL-6, and TNF-α levels. Exposed group who were not wearing apron demonstrated significant elevation in MDA levels.

Table (4): Comparison of biochemical estimated parameters with usage of different PPE.

parameters	PPE-gloves		PPE-mask		PPE- apron	
	Yes (71.4%)	**No (28.6%)**	**Yes (61.2%)**	**No (28.8%)**	**Yes (63.3%)**	**No (36.7%)**
AChE (ng /mL)	204.3±26.6	188.3±16.5	204.5±22.1	185.0±18.2	201.6±23.3	188.0±17.6
BuChE (U/ L)	3049.9±453.2	1579.3±297.01*	2682.8±386.4	1542.1±336.3*	2030.1±342.2	1981.0±422.4
TAC (mM/L)	0.72±0.02	0.70±0.02	0.71±0.02	0.68±0.03	0.73±0.04	0.71±0.02
MDA (nmol/ml)	8.11±1.34	9.52±0.69	6.21±1.21	9.01±0.84*	7.21±0.62	10.21±0.91*
IL-6 (ng/L)	2.62±0.34	3.21±0.33	2.22±0.2	3.61±0.43**	3.01±0.34	3.09±0.34
INF- γ (pg/ml)	1364.9±133.4	1313.7±106.6	1204.1±121.3	1416.1±114.2	1371.5±152.3	1303.4±100.9
TNF-α (ng/l)	77.0±2.5	146.1±14.2**	78.9±2.1	158.7±16.1**	121.8±15.0	128.2±15.2

* P-value <0.05
** P-value <0.001

5-Discussion

Although the use of pesticides has great benefits, it has several health risks associated with humans who are occupationally or environmentally exposed to these compounds [14]. The Occupational pesticides exposure has been previously proved to induce immune dysfunction increasing risk of several immune-associated diseases [14 ,15]. The mechanism by which pesticides alter immune system homeostasis is still under discussion especially at workplace. As shown in Figure (1) there was an increase in wheezy chest and asthma in some cases of OPs exposed researchers (51.02%), and this result was in agreement with Fareed et al. [16], who reported that occupational exposure to OPs is associated with an increased risk of respiratory symptoms, asthma and chronic bronchitis [16]. Sapbamrer et al. [15], also found difficulty in breathing with chronic pesticides exposure among rice farmers occupationally exposed to OPs [15]. Many factors may be involved in the development of asthma related to OPs exposure such as inhibition of cholinesterase enzymes [17]. Drake et al. [18] reported that pesticides exposure interact with AChE increasing the amount and residence time of acetylcholine at muscarinic and nicotinic receptors in target tissues, resulting in bronchoconstriction[18].

Our results prevailed also the persistence of some neurological disorders in pesticides exposed researcher; muscle weakness (53.06%) and numbness (59.19%). In consistent with our results, Sapbamrer et al. [15] detected a neurological dysfunction among occupationally pesticides exposed farmers demonstrated by significant muscle weakness and numbness [15]. Additionally, Hongsibsong et al.[19], reported muscle weakness and numbness in OPs exposed cases [19]**.** Conversely, some studies have reported no strong associations between occupational exposure and neurobehavioral performance or neurodegeneration[20]. According to our study, Eye irritation also showed a significant increase in exposed compared to the control group (20.41%). Also, dermatitis was detected in (30.61%) of exposed researchers that was mostly in the forearm and hands with relapses and remissions. Our results agreed with Farahat et al.[21], who reported that pesticides caused dermatitis (32.5%) in women exposed to OPs [21]. In consistent with our clinical manifestation's results, Hongsibsong et al. [19], reported a significant health symptom, such as breathlessness, chest pain, dry throat, numbness, muscle weakness, cramping, dizziness, eye irritation, white/red rash, and white/red pimple among exposed farmers[19].

The inhibition in activities of AChE and BuChE is commonly used as biomarkers for occupational and environmental exposure to OPs [22]. The advantages of these biomarkers for prediction of pesticide exposure may be attributed to their easy measurement, high sensitivity, and depending on the level of exposure [22].We found a significant decrease in the levels of AChE and BuChE in researchers occupationally exposed to OPs in comparison to the unexposed control group**.** Former studies have also reported decline in AChE and BuChE levels in pesticides exposed subjects relative to unexposed group [23, 4].

In addition to inhibitory effect of pesticides on cholinesterase activity, pesticides act as a powerful promoters of many cellular pathways. The long-lasting exposure to OPs leading to the generation of reactive oxygen species that exhaust and consume antioxidant agents present in the body [24,25]. Disturbance of cellular oxidative balance related to pesticides exposure can be detected by measurement of lipid peroxidation by-product (MDA) and TAC level [26]. In this study, there was a significant increase in MDA and a significant depletion in TAC between exposed researchers and controls **Table (1)**. The increase in MDA levels among pesticides exposed subjects was also reported in many former studies [4, 27]. Other studies detected a significant decrease of TAC among exposed farmers to OPs [28, 29]. In contrast, Ahmadi et al.[30], reported a significant increase in TAC in the pesticide-exposed group that could be due to the adaptive response to the generated free radicals [30].

In the present study, we detected the levels of inflammatory cytokines (IL-6, TNF-α and IFN-γ) that showed a significant increase in the exposed group **Table (1)**. Zaw et al., [31] reported that agricultural workers who were exposed to organophosphate pesticides have significantly higher serum levels of IL-6 in the exposed group than the non-exposed group [31]. On the other side, Taghavian et al. [32] didn't find a significant increase of IL-6 among exposed group [32]. Massawe et al., [33] reported an increase in levels of TNF-α, and IFN-γ in pesticides exposed group [33]. Schaper et al., [34] and Costa et al.[35] explained that OPs cause induction, production and the release of pro-inflammatory mediators, including cytokines such as TNF-α, interleukin (IL)-1, IL-6 and IL-10, or type 1 interferon (IFN) [35]. The release of pro-inflammatory cytokines will lead to activation of immune cells and release of further cytokines (cytokine storm) [34]. The Increased level of proinflammatory cytokines together with increased biomarkers of oxidative stress are indicators of immunotoxicity [35].

The present study indicated a positive correlation between MDA, IL6, and TNF levels and duration of pesticides exposure **as illustrated in Figure (2)**. Our results reported a significant rise in MDA, IL6, and TNF levels with increasing duration of exposure. The BuChE level was significantly declined with more duration of exposure.

The correlation between measured biochemical biomarkers was represented in **Table (2)**. Regarding our results, there was a negative correlation between IL6 and both AChE and BuChE. Zaw [31] et al., was in constant with our result that showing significant negative correlation between erythrocyte acetylcholinesterase activity and serum IL-6 level in pesticides-exposed group [31]. Our study indicated a significant positive correlation between MDA with IL-6 and TNF-α**,** while TAC was negatively correlated with TNF-α. **table (2)**. Mohammadzadeh. et al.[36] also reported significant increase in MDA level with IL-6 and TNF in rats treated by malathion [36]. This correlation may be attributed to, depletion of antioxidant mechanisms that stimulate more production of inflammatory cytokines with increased risk of exposure hazards. In addition a positive correlation was noticed in our result between IL-6 and IFN-γ and between IL-6 and TNF-α **table (2)**. Cauvi et al.[37], has reported that in particular IFN-γ and IL-6 were required for the expression of each other in immunotoxicity [37]. Both IL6 and TNF-α are important proinflammatory cytokines that are prominent in chronic inflammation [38].

From our study, Occupationally exposed researchers who complained from bronchial asthma was characterized with increased levels of MDA , IFN-γ, TNF-α **Table (3)**. Proskocil et al.[39], also were in line with us that OPs induce airway hyperactivity associated with increase of TNF-α [39]. Uysal and Karaman [40], reported that poisoning with OPs increases lipid peroxidation, disrupts the antioxidant system, induces histopathological changes, and increases apoptotic cell death in lung tissue [40]. Zaw et al.[31], found a significant negative correlation between serum IL-6 level and respiratory function parameters in OPs exposed group [31].

According to our study, Neurological symptoms (muscular weakness and numbness) was associated with increased level of MDA, IL6, IFN-γ, TNF-α, and with decreased TAC level-**table (3)**. Our result agreed with Fareed et al.[16], who reported a decreased level of TAC associated with muscular weakness in OPs exposed workers[16]. Caro-Gamboa et al [22], reported that Malathion (organophosphorus pesticides) can activate microglia to release inflammatory mediators (IL-6, TNF-α) and induce neuroinflammation[22]. Kulkarni et al.[41], mentioned that the association of interferon-gamma with neurotoxicity[41].

Exposed researchers with eye irritation have increased levels of MDA and IFN-γ **Table (3)**. General toxic effects of pesticides on the eyes are symptomatically classified as mainly external, resulting from direct ocular exposure. Subsequent absorption of OPs pesticides in the ocular tissue causes toxic effects, and/or internally through oxidative stress damage of ocular cells [42]. Ishikawa's epidemiological study revealed that 64 out of 7,435 (0.86%) farmers had impairment in ocular movements and abnormal contraction of the pupil in response to light, which improved after administration with antidotes of OPs (e.g atropine) [43].

In addition, we revealed exposed researchers with dermatitis related to significant rise in IL-6 **Table (3)**. Similarly, to the eye, OPs could affect skin mostly externally through direct contact and internally through disturbance in the immune system. Oxidative stress was recently involved in atopic dermatitis which was also accompanied by increased levels of proinflammatory cytokines [44].

Besides, incomplete or lack of use of personal protection equipment (PPE) by pesticides workers may potentiate the deleterious health effects in humans increasing the concentration of active chemicals in skin, mouth, eye, and respiratory tract, leading to an increase of pesticides absorption [45,46].

The use of PPE is considered the key to prevent adverse health symptoms of OPs exposure **Table (4)**. Our data suggested that OPs exposed researchers not constantly wearing a face masks during work showed a lower level of BuChE but higher levels of MDA, IL6, TNF-α than those regularly use face masks. Another study revealed a significant increase in adverse respiratory symptoms (shortness of breath and asthma) in OPs exposed workers not wearing respiratory PPE [47]. We detected more BuChE inhibition level in exposed researchers not constantly wearing gloves than those regularly wearing gloves **Table (4)**. As the skin is an important route of absorption of OPs so, bare skin increases absorption which was detected by BuChE inhibition level in the exposed population. In addition, researchers not regularly wore an apron had an increased level of MDA as a result of increased OPs exposure with subsequent increased oxidative stress. Hofmann et al.[48] reported that , not wearing chemical-resistant boots was strongly associated with BuChE inhibition level in OPs farmers[48].

Conclusion

From the current study can conclude that the severity of exposure to OPs was associated with consumption rate of antioxidant mechanisms and increased disturbance rate of inflammatory cytokines especially IL-6 and IFN-γ. There was a strong correlation between oxidative stress and alteration in cytokines levels within exposed researchers. This disturbance could trigger to a variety of immnuo-disorder associated diseases. In this study bronchial asthma and neurological disorders was the most common clinical symptoms across exposed subjects. Furthermore, the use of PPE was observed to play an important in modulating immunotoxicity. So it's much recommended for researchers to maintain the required precautions and PPEs while handling with pesticides in their labs.

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