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Research Article
Assessment of Pesticide Residues in Human Blood and Effects of Occupational Exposure on Hematological and Hormonal Qualities

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Abstract
Pesticides are the first choice by farmers for use against plant pathogens, nevertheless their adverse effects to the environment. Current study was designed to measure pesticides residues in blood of spray farmers and to assess their possible effects. Blood indices and thyroid and reproductive hormones were evaluated in blood of adult male volunteers (20-48 years old). Volunteers were divided to three groups; spray-workers (directly-exposed), farmers who live in the country area (indirectly-exposed) and city inhabitants (not exposed). Spray workers had significantly decreased platelet number (PLT, 33%), ratio of large platelet (P-LCR%, 42%), average platelet volume (MPV, 70%), relative width of the distribution of erythrocytes (PDW, 56%), relative content of monocytes, basophils and eosinophils (MXD, 100%) compared to control group. In addition, blood samples of the exposed group showed significantly decreased PLT (30%), P-LCR (40%), MPV (65%) and PDW (50%) compared to the farmers. Furthermore, levels of testosterone, triiodothyronine and thyroxine hormones of spray workers were significantly low compared with the country residents. Then results were further subjected to canonical discriminant analysis to visualize the interrelationships among variables. Results highlighted the critical need for enforced official interventions that reduce overexposure of spray workers throughout Egypt.

Key words: Pesticide exposure, endocrinology, blood indices, canonical discriminant analysis, platelets

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Agricultural workers are exposed to pesticides (occupational exposure) in the open fields and greenhouses (Panemangalore *et al*., 2007; Damalas and Eleftherohorinos, 2011). Exposure to low-amounts of pesticides produced variety of adverse biochemical changes in human (Gupta *et al*., 1998; Banerjee *et al*., 1999). Detection of pesticides in biological fluids and tissues is critical for the assessment of the occupational toxicity. Levels of chlorpyrifos and diazinon pesticides were in the range of 0-1726 and 0-0.5 ng mL^{-1}, respectively in the maternal cord plasma of women living in an agricultural area (Huen *et al*., 2012). Moreover, residues of p, p-dichlorodiphenyl dichloroethylene (DDE), heptachlor epoxide, γ-HCH and dieldrin were detected in the blood samples of residents living adjacent to cotton and sugar cane farms in Sudan (Elbashir *et al*., 2015).

Blood indices including hemoglobin (HB), mean red blood cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), total leukocyte count (TLC), red blood cell (RBC) counts, monocytes, lymphocytes and neutrophil were decreased and platelet count was increased after exposure to pesticides (Azmi *et al*., 2009). Emam *et al*. (2012) reported elevated values of HB, haematocrit (HCT), RBC and platelet of farm workers who were engaged in the pesticide application. They stated that RBC, HB and platelet counts were suitable indices as warning indicators for quick diagnosis of poisoning of pesticides. Also, Varol *et al*. (2014) found that platelet number, mean volume of platelet (MPV) and platelet distribution width (PDW) were decreased significantly in blood samples of farm workers compared with control. Interestingly, adverse hormonal effects were reported at extremely low doses of pesticides but not observed at higher doses (Vandenberg *et al*., 2012). Approximately 37 pesticides were detected among 134 contaminants that were found in food samples in the EU. Of these 37, 23 pesticides were categorized as anti-androgenic and 7 others as androgenic substances (Orton *et al*., 2011). A decrease in the level of thyroid stimulating hormone (TSH) and an increase in the level of triiodothyronine (T3) hormone in blood samples of people who were exposed to pesticides compared to the control (Quraishi *et al*., 2015).

In developing countries, farmers are exposed to pesticides due to the incorrect application techniques, poor or inappropriate spraying equipment, inadequate storage practices, lack of personal protective equipment and the reuse of old pesticide containers for food and water storage (Ecobichon, 2001; Damalas and Eleftherohorinos, 2011). In 2012, approximately 14,000 t of pesticides (4,808, 2,809 and 6,374 t of insecticides, fungicides and herbicides, respectively) were used in Egypt (FAO., 2015). Additionally, the application of pesticides on crops was done by non-certified applicators (farmers) using hand held, back held or motors-drawn sprayers, which put farmers under a huge exposure stress. Therefore, the objectives of current study were to (1) Determine the pesticide residues in blood samples of volunteers, (2) Assess the haematological and endocrine effects of pesticide exposure in occupational and residential settings and (3) Use the statistical discriminant analysis to interpret and predict variables that might be used for fast detection of toxicity in order to improve safety tactics offered to pesticide workers.

MATERIALS AND METHODS

Chemicals and pesticides: Primary Secondary Amine (PSA), octadecylsilane (C18), magnesium sulfate were purchased from Agilent Technologies, Cairo, Egypt. Acetonitrile solvent, testosterone, triiodothyronine (T3) and thyroxine (T4) hormones kits were provided from local chemical company. Standards of pesticides (Table 1) were purchased from Sigma Chemicals Company, USA. A stock solution of 100 μg mL^{-1} of each pesticide was obtained from Central Agricultural Pesticide Laboratory, Egypt and stored for a maximum of 15 days at -18°C until analyzed.

Collection of blood samples: Blood samples were collected from 48 adult male subjects of 20-48 years old at Albeheira Governorate, Egypt during September, 2013. Experiment objectives were described carefully to volunteers and they signed an informed consent before the collection of blood samples. Subjects were classified into 3 groups of people: Group 1 (spray workers, occupationally exposed) was of 15 farmers who were extensively engaged in the pesticide spray process (at least once a week). Group 2 (farmers, in-directly exposed) was of 18 rural who were not involved in the pesticide application but they were living in the rural area. Group 3 (control, not exposed to pesticides) was of 15 individuals from the city who were not involved in the pesticide application or indirectly exposed to pesticides. About 12-15 mL of blood samples were aseptically collected with the help of a professional nurse into K_3EDTA tubes (for the blood picture analysis) and non-heparinized tubes for serum separation for the analysis of pesticide residues and the determination of the hormonal concentration. Blood samples were kept at 4°C until analysis that was done within 3 h. The study protocol was approved by the human and animal ethics committee of Damanhour University, Egypt.

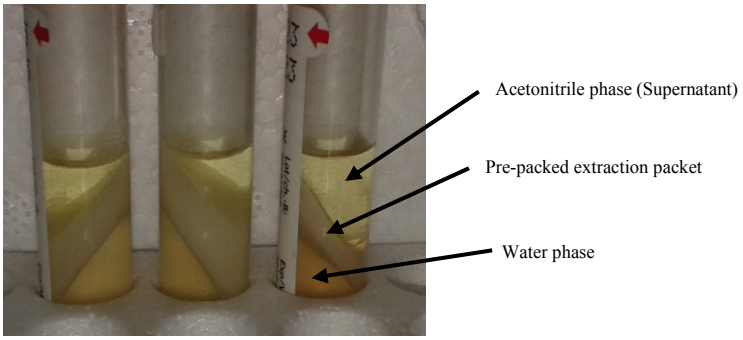

Fig. 1: Extraction of pesticide residues using the pre-packed extraction packet (6 g of magnesium sulfate and 1.5 g of sodium acetate) and 1% acetic acid in acetonitrile (v/v)

Determination of pesticide residues in the blood serum samples: Modified QuEChERS (quick, easy, cheap, effective, rugged and safe) extraction method followed by gas chromatography-mass spectrometry (GC-MS) (Usui *et al*., 2012) was used for the determination of pesticide residues. About 0.5 mL of blood serum samples was diluted threefold with distilled water (1.5 mL). Samples were placed in 5 mL plastic centrifuge tubes with 0.5 g of a pre-packed extraction packet (6 g of magnesium sulfate and 1.5 g of sodium acetate (Agilent Technologies, Cairo, Egypt)) and 1 mL of 1% acetic acid in acetonitrile (v/v). The mixture was vigorously vortexed for 30 sec, shaken up-down for 5 min, vortexed again for 30 sec and then centrifuged at 4400 rpm for 15 min at 4°C (Universal 32R, Hettich Zentrifugen model D-78532, Germany). For samples clean-up, a 600 µL of supernatant (Fig. 1) of each sample was transferred into a 1.5 mL eppendorf tube containing the solid-phase extraction sorbent (25 mg of primary secondary amine, 25 mg of end-capped octadecylsilane (C18) and 150 mg of magnesium sulfate). The tube was mixed by hand (up and down) for 10 min, vortexed for 30 sec and centrifuged at 4400 rpm for 10 min at 4°C. Supernatants were collected and filtered through 0.22 µm PTFE filters (Millipore, USA) into 2 mL clear HPLC vials. About 1 µL of each sample was injected directly into the GC-MS/MS system.

Determination of pesticide residues by GC-MS: Gas Chromatography-Mass Spectrometry (GC-MS) measurements were completed using an Agilent 6890N GC system coupled with an Agilent 5973 mass-selective detector. Samples were separated on a DB-17 ms capillary column of 0.25 mm i.d., 30 m length and 0.25 µm film thickness. The chromatographic conditions were; helium gas constant flow of 1.3 mL min^{-1}, inlet temperature of 230°C, pulsed split (pulsed pressure 250 kPa for 1 min), injection volume of 1 µL and MS transfer line temperature of 250°C. The column temperature program was as the following: 60°C for 1.5 min, increased by 30°C min^{-1} to 120°C, increased by 10°C min^{-1} to 200°C, then increased by 20°C min^{-1} to 230°C and run for 10 min at this temperature and finally increased by 30°C min^{-1} to reach 300°C and run for 7 min. The total run time was 45 min. Full scan analysis was from 40-450 m/z and was used in the experiments to determine the chromatographic and MS traits of different pesticides. Quality control was performed with selected ion monitoring (SIM) mode with one target and two or three qualifier ions. The SIM mode was selected because it allows the increased peak response by concentrating ions specific to the compounds under investigation. Analyte identification was performed by comparing both the retention time and the MS spectrum of the sample peaks with those of the standard solutions.

Quality control parameters: For the estimation of the accuracy and repeatability of the analytical method, six replicates of serum sample were spiked with 0.1 mg L^{-1} of the analytes and were processed through the whole analytical procedure. The accuracy of the analytical method was calculated from the areas obtained from the analysis of spiked samples as a percentage of those obtained from the analysis of the standard solution with an equivalent concentration. Limits of detection (LODs) were defined as the concentration of a compound giving a signal-to-noise ratio (S/N) of 3. Limits of quantification (LOQs) were calculated from S/N ratios of 1:10, which were obtained from the measurement of the samples with the lowest concentration level where peaks of studied pesticides were detected.

Blood indices analysis: The complete blood picture was analyzed using an automated blood analyzer (Sysmex KX 21, Japan) in a certified professional clinical laboratory that is routinely engaged in the analysis of health-related parameters. Blood indices were white blood cell and its parameters including: white blood cell (WBC) counts ($X \times 10^3$ µL), the ratio (%) of lymphocytes (small cells) to whole WBC (LYM%), the ratio (%) of basophils, eosinophils and monocytes (middle cells) to whole WBC (MXD%), the ratio (%) of neutrophils (large cells) to whole WBC (NEUT%), the absolute count of lymphocytes (small cells) in 1 µL of whole blood (LYM #) ($X \times 10^3$ µL), the absolute count of the basophils, eosinophils and monocytes (middle cells) in 1 µL of whole blood (MXD #) ($X \times 10^3$ µL) and the absolute number of neutrophils (large cells) in 1 µL of whole blood (NEUT #) ($X \times 10^3$ µL). Platelet count (PLT) ($X \times 10^3$ µL), platelet distribution width (PDW) (fL), mean volume of platelet (MPV) (fL) and large platelet ratio (P-LCR%). Red blood cell and its differentials including red blood cell counts (RBC) ($X \times 10^6$ µL), hemoglobin (HGB) (g dL^{-1}), hematocrit value (HCT%), mean RBC volume (MCV) (fL), mean RBC hemoglobin (MCH) (pg), mean RBC hemoglobin concentration (MCHC) (g dL^{-1}), RBC distribution width-CV (RDW-CV) (%) and RBC distribution width-SD (RDW-SD) (fL).

***In vitro* assay of acetylcholine esterase (AChE) activity:** The AChE activity was quantified by a modified method of Ellman *et al*. (1961). AChE assay was performed by incubating 20 µL of blood serum with 2.9 mL phosphate buffer (pH 8.0) and 50 µL of 5,5′-dithiobis(2-nitrobenzoic) acid (10 mM) for 10 min. Then 10 µL of acetylthiocholine iodide (75 mM) were added and the absorbance was recorded at 412 nm. The specific activity of AChE was expressed as n moles of acetylthiocholine hydrolyzed milligram protein per minute.

Determination of thyroid and reproductive hormones: The testosterone hormone determination was carried out according to the method reported by Granoff and Abraham (1979) and Tietz (1995) using the International Immuno Diagnostics Kits. The testosterone concentration in the serum samples was calculated from a standard curve and expressed as ng ml^{-1}. Triiodothyronine (T3) hormone determination was carried out according to the method reported by Burke and Eastman (1974) using the International Immuno Diagnostics. The average absorbance values (A_{450}) for each set of reference standards and samples was calculated from a standard curve and the concentration of T3 was expressed as ng mL^{-1}. Determination of the T4 hormone was carried out according to the method reported by Skelley *et al*. (1973) using the International Immuno Diagnostics Kits. The average absorbance value (A_{450}) was calculated for each set of reference standards and samples. The mean absorbance values for each specimen was used to determine the corresponding concentration of T4 in ng mL^{-1} from a standard curve.

Experimental design and statistical analysis: Results of the hematological indices and the hormonal levels were statistically analyzed using the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS) software version 9.3 (SAS., 2013) as a Completely Randomized Design (CRD). The least square means were compared using Dunnett's specific difference *post hoc* test at $p \leq 0.05$ (SAS Institute, Inc., Cary, NC). Hematological and hormonal parameters that showed significant variation among the 3 designed groups were subjected to Canonical Discriminant Analysis (CDA) and Hierarchical Cluster Analysis (HCA) to be classified across treatments using the CANDISC procedure of SAS. The canonical (CAN) scores from the analysis were used for HCA, for further unsupervised classification of treatments. The Euclidean distance between group's centers in the canonical space was used to construct a similarity measure matrix to better visualize the clustering pattern of groups of treatment and replicate combinations.

RESULTS

Pesticide residues in serum samples: Commonly used pesticides in the study area (Albeheira Governorate, Egypt) were listed in Table 1. Farmers sprayed plants with a lengthy list of acaricides, fungicides, herbicides, insecticides and nematicides to control open field and greenhouse plant pathogens. Fortunately, results in Table 1 showed that pesticides were not detected in the serum samples of individuals except for the herbicide ethofumesate. It is a selective systemic herbicide that was extensively used as pre- and/or post-emergence in controlling a wide range of grasses and broad-leaved weeds in Phaseolus beans and strawberry. It's registered in Egypt under the trade names of "Betanal maxxPro®" from Bayer CropScience Egypt and with the herbicide "Bison 40% SE" from AACO BV Netherlands (Agriculture Egypt, 2015). Ethofumesate was detected in the serum samples of only two pesticide applicators.

Table 1: List of commonly used pesticides to control plant pathogens

Common names	Uses		LOQ (ppb)	Common names	Uses		LOQ (ppb)
Bifenazate	A*	ND	50	Clodinafop	H	ND	25
Dicofol	A	ND	100	Ethofumesate	H	D	50
Hexythiazox	A	ND	25	Flucarbazon	H	ND	50
Chlorpyrifos-methyl	A,I	ND	10	Oxadiazon	H	ND	100
Dimethoate	A, I	ND	50	Pendimethalin	H	ND	50
Diazinon	A, I	ND	10	Aldrin	I	ND	10
Dichlorvos	A, I	ND	5	Azinphos-methyl	I	ND	20
Ethion	A, I	ND	5	a-BHC	I	ND	15
Endosulfan	A, I	ND	15	Bioallethrin	I	ND	50
Malathion	A, I	ND	10	Cadusafos	I	ND	50
Pirimiphos-methyl	A, I	ND	10	Chlorfluazuron	I	ND	50
Phenthoate	A, I	ND	10	Cyfluthrin	I	ND	15
Profenofos	A, I	ND	15	Delta-BHC	I	ND	10
Propetamphos	A, I	ND	15	Chlorpyrifos	I	ND	10
Quinalophos	A, I	ND	15	Gamma-cyhalothrin	I	ND	5
Triazophos	A, I, N	ND	10	Gamma-BHC	I	ND	25
Bromuconazole	F	ND	30	Lambda-Cyhalothrin	I	ND	10
Chlorothalonil	F	ND	25	Gamma-Chlordane	I	ND	25
Cyflufenamid	F	ND	50	Cypermethrin	I	ND	25
Dicloran	F	ND	20	Deltamethrin	I	ND	15
Difenoconazole	F	ND	20	Dieldrin	I	ND	5
Diniconazole	F	ND	10	Op-DDT	I	ND	5
Epoxiconazole	F	ND	20	Pp-DDD	I	ND	10
Fenarimol	F	ND	10	Pp-DDT	I	ND	10
Fluazinam	F	ND	20	Endrin	I	ND	50
Myclobutanil	F	ND	15	Esfenvalerate	I	ND	10
Propiconazole	F	ND	15	Fenamiphos	I	ND	15
Proquinazid	F	ND	20	Heptachlor	I	ND	10
Penconazole	F	ND	30	Heptachlor-epoxide	I	ND	10
Tetraconazole	F	ND	30	Methoxychlor	I	ND	25
Triticonazole	F	ND	30	Permethrin	I	ND	10
Triforine	F	ND	30	Prothiofos	I	ND	10
Triflumizole	F	ND	50	Tetramethrin	I	ND	20
Acetochlor	H	ND	30	Thiocyclam	I	ND	25
Atrazine	H	ND	10	Thiamethoxam	N, I	ND	50
Butralin	H	ND	20	Tralomethrin	N	ND	50

*A: Acaricide, F: Fungicide, H: Herbicide, I: Insecticide and N: Nematicide pesticides were examined in serum samples using GC-MS. Data were arranged alphabetically based on pesticide class and also within each class

Effects of occupational exposure to pesticides

Effects on white blood cell counts and its differentials: Results in Table 2 showed that WBC counts were decreased significantly in blood samples collected from country area (farmers), city people (smokers) and spray workers compared to the city residents (non-smokers). The non-smoking farmers had lower WBCs compared to the city inhabitants (non-smokers), which highlights the adverse effect of exposure even without being involved in the applications of pesticides, drift exposure.

The ratio of lymphocytes (small cells) to whole WBC (LYM%) values was significantly low in blood samples of pesticide applicators who smoke compared with the non-smoking farmers (rural inhabitants) (Table 2) and not different from city people who smoke. The ratio of basophils, eosinophils and monocytes (middle cells) to whole WBC (MXD%) were significantly lower in the blood of applicators compared to all other samples except it was not different from the city people (non-smokers). There were no differences in the ratio or the absolute count of neutrophils (large cells) to whole WBC (NEUT% or NEUT #) or the absolute count of lymphocytes (small cells (LYM #) in 1 μL of whole blood samples of all individuals under investigation. The absolute count of the basophils, eosinophils and monocytes (middle cells; MXD #) in 1 μL of whole blood was significantly less in blood samples of pesticide applicators and smokers either from city or country-side residents compared to the non-smoking subjects.

Effects on platelets counts and differentials: Results of platelet counts (PLT), platelet distribution width (PDW), mean volume of platelet (fL) (MPV) and large platelet ratio (P-LCR) of

Table 2: LS Means±Standard Error (SE) of white blood cell counts (WBC#) (X×10^3 µL), the ratio of lymphocytes to whole WBC (LYM%), the ratio of basophils, eosinophils and monocytes to whole WBC (MXD%), the ratio of neutrophils to whole WBC (NEUT%), the absolute count of lymphocytes in 1 µL of whole blood (LYM#) (X×10^3 µL), the absolute count of the basophils, eosinophils and monocytes in 1 µL of whole blood (MXD#) (X×10^3 µL) and the absolute count of neutrophils in 1 µL of whole blood (NEUT#) (X×10^3 µL). Platelet count (PLT) (X×10^3 µL), Platelet Distribution Width (PDW) (fL), mean volume of platelet (fL) (MPV) and large platelet ratio (P-LCR%) in serum of blood samples human volunteers

	City inhabitants (-control)		Rural inhabitants (+control)		Applicators	
Criteria	Non-smoking	Smoking	Non-smoking	Smoking	Non-smoking	Smoking
WBC# (X×10^3 µL)	9.80±0.69^{a}	7.32±0.27^{b}	9.68±0.28^{a}	6.82±0.40^{b}	7.31±0.31^{b}	6.60±0.44^{b}
LYM (%)	29.30±2.50^{ab}	29.41±0.98^{ab}	33.58±1.02^{a}	32.38±1.44^{ab}	31.89±1.11^{ab}	27.58±1.58^{b}
MXD (%)	4.95±1.17^{ab}	8.51±0.46^{a}	8.53±0.48^{a}	7.82±0.68^{a}	3.90±0.52^{b}	6.90±0.74^{a}
NEUT (%)	65.75±4.20^{a}	61.48±1.65^{a}	63.00±1.72^{a}	59.80±2.43^{a}	65.89±1.88^{a}	66.62±2.66^{a}
LYM# (X×10^3 µL)	2.00±0.69^{a}	2.48±0.27^{a}	2.53±0.28^{a}	2.50±0.40^{a}	2.52±0.31^{a}	2.40±0.44^{a}
MXD# (X×10^3 µL)	0.55±0.19^{ab}	0.76±0.08^{ab}	0.88±0.08^{a}	0.60±0.11^{ab}	0.42±0.09^{b}	0.40±0.12^{b}
NEUT# (X×10^3 µL)	5.25±1.26^{a}	3.25±0.49^{a}	4.70±0.51^{a}	2.67±0.73^{a}	2.92±0.56^{a}	4.92±0.80^{a}
PLT (X×10^3 µL)	281.00±18.80^{a}	276.00±7.37^{a}	261.25±7.68^{a}	285.17±10.86^{a}	157.10±8.41^{b}	156.00±11.89^{b}
PDW (fL)	14.10±1.02^{a}	13.53±0.40^{a}	13.63±0.42^{a}	14.68±0.59^{a}	9.77±0.46^{b}	9.42±0.65^{b}
MPV (fL)	10.80±0.55^{a}	10.73±0.22^{a}	10.53±0.23^{a}	10.52±0.32^{a}	8.31±0.25^{b}	8.38±0.35^{b}
P-LCR (%)	32.25±3.37^{a}	29.32±1.32^{a}	29.82±1.37^{a}	27.38±1.94^{ab}	18.17±1.51^{c}	19.08±2.13^{bc}

Within each row, numbers followed by the same superscript letter(s) were not significantly different based on Dunnett's *post hoc* specific comparison (p<0.05)

Table 3: LS Mean±Standard Error (SE) of red blood cell counts (RBC) (X×10^6 µL), hemoglobin (HGB) (g dL^{-1}), hematocrit value (HCT %), mean RBC volume (MCV) (fL), mean RBC hemoglobin (MCH) (pg), mean RBC hemoglobin concentration (MCHC) (g dL^{-1}), RBC distribution width-CV (RDW-CV) (%) and RBC distribution width-SD (RDW-SD) (fL) in serum samples

	City inhabitants (-control)		Rural inhabitants (+control)		Applicators	
Criteria	Non-smoking	Smoking	Non-smoking	Smoking	Non-smoking	Smoking
RBC (X×10^6 µL)	5.00±0.40^{ab}	4.78±0.13^{ab}	4.14±0.14^{b}	5.19±0.20^{a}	5.18±0.15^{a}	5.08±0.21^{a}
HGB (g dL^{-1})	14.05±0.81^{abc}	13.82±0.32^{bc}	12.32±0.33^{c}	15.10±0.47^{ab}	14.98±0.36^{ab}	15.86±0.51^{a}
HCT (%)	39.05±1.68^{bc}	41.67±0.66^{ab}	35.13±0.69^{c}	43.60±0.97^{ab}	43.69±0.75^{ab}	45.18±1.06^{a}
MCV (fL)	84.35±1.85^{a}	86.38±0.73^{a}	89.01±0.76^{a}	89.92±1.07^{a}	87.60±0.83^{a}	85.92±1.17^{a}
MCH (pg)	27.30±0.66^{b}	27.37±0.30^{b}	29.93±0.27^{a}	30.42±0.38^{a}	30.38±0.30^{a}	30.66±0.42^{a}
MCHC (g dL^{-1})	32.35±0.80^{a}	33.32±0.31^{a}	33.69±0.33^{a}	33.62±0.46^{a}	34.26±0.36^{a}	34.32±0.51^{a}
RDW-SD (fL)	44.65±2.83^{a}	44.73±1.11^{a}	45.33±1.16^{a}	46.23±1.63^{a}	41.89±1.27^{a}	44.62±1.79^{a}
RDW-CV (%)	13.65±0.86^{a}	13.70±0.40^{a}	13.71±0.35^{a}	13.75±0.50^{a}	12.93±0.39^{a}	13.86±0.55^{a}

Within each row, numbers followed by the same superscript letter(s) were not significantly different based on Dunnett's *post hoc* specific comparison (p<0.05)

blood samples were presented in Table 2. Exposure to pesticides during the spray process showed serious effects on PLT number. Pesticide applicators had significantly less PLT number compared to the rural or city inhabitants. Similar effects were found for the PDW (FL) and MPV (FL), where the pesticides applicators had significantly reduced values compared to control group. Pesticide sprayers who did not smoke had significantly less P-CLR values compared with country-side or city inhabitants, but were not different from the same group of people who smoke.

Effects on red blood cell counts and differentials: Results of red blood cell (RBC) counts, hemoglobin content (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), RBC distribution width-CV (RDW-CV) and RBC distribution width-SD (RDW-SD) were presented in Table 3. Red blood cell counts of blood samples of farmers (non-smokers) were significantly less compared to pesticide sprayers, city people and farmers (smokers). Hemoglobin and hematocrit values showed similar trends to RBCs. The MCH values were significantly less in blood samples of city inhabitants compared to the farmers either the applicators or residents. The values of MCV (fL), MCHC, RDW-CV (%) and RDW-SD (fL) values were normal in all other blood samples.

Effects on reproductive and thyroid hormones content: Results of effects of pesticide exposure on reproductive hormone (testosterone) and thyroid hormones (triiodothyronine (T3) and thyroxine (T4)) were illustrated in Table 4. Pesticide exposure significantly reduced the androgenic hormone-testosterone-content of blood samples of rural residents and spray workers who smoke compared to other groups of people. Likewise, the triiodothyronine (T3) and its prohormone, thyroxine (T4) tyrosine-based hormones produced by the thyroid gland, which function in the regulation of metabolism were affected by the exposure to pesticides. Blood samples of spray applicators (non-smokers) had a decrease in T4 content compared with the other groups and it was not different from that of country-side residents

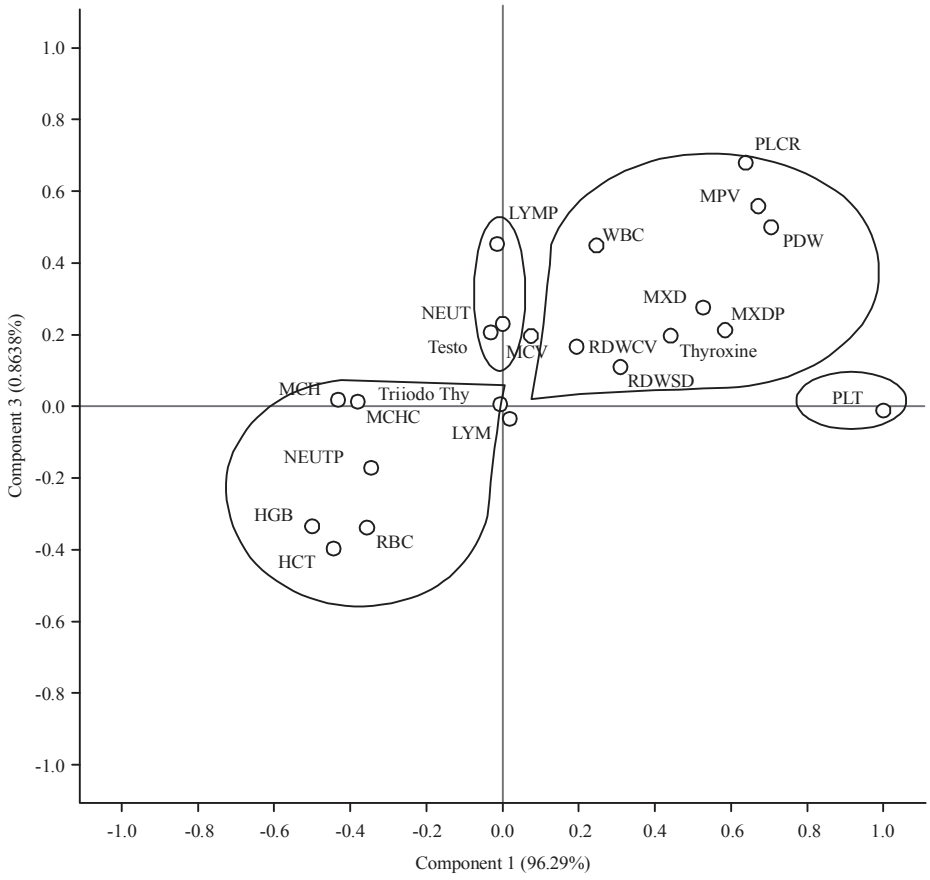

Fig. 2: Component pattern analysis plot of the PCA procedure. A visual illustration tool that shows similarity classification of the dependent variables where plotting PCA1 and PCA3 explained about 97.15% of variance of hematological and hormonal variables that used to differentiate among groups of volunteers: applicators; farmers who do the pesticide spray, farmers who don't apply the pesticides as positive control and city inhabitants as negative control

Table 4: LS Mean±Standard Error (SE) of testosterone (ng mL^{-1}), triiodothyronine (T3) (ng mL^{-1}) and L-thyroxine (T4) (ng mL^{-1}) in serum samples

	City inhabitants (-control)		Rural inhabitants (+control)		Applicators	
Criteria	Non-smoking	Smoking	Non-smoking	Smoking	Non-smoking	Smoking
Testosterone	5.57±0.67^{a}	3.29±0.26^{ab}	2.83±0.28^{bc}	1.54±0.39^{c}	2.46±0.30^{bc}	3.27±0.43^{ab}
Triiodothyronine (T3)	1.52±0.38^{a}	1.09±0.15^{a}	1.49±0.15^{a}	1.14±0.22^{a}	1.34±0.17^{a}	1.41±0.24^{a}
L-Thyroxine (T4)	9.31±0.92^{a}	6.49±0.36^{ab}	6.00±0.37^{b}	5.49±0.53^{b}	3.40±0.41^{c}	5.94±0.58^{b}

Within each row, numbers followed by the same superscript letter(s) were not significantly different based on Dunnett's *post hoc* specific comparison ($p<0.05$)

who smoke. The L-thyroxine (T4) hormone contents of blood samples of city people (smokers), farmers, spray farmers (smokers) were significantly less compared to the city people who don't smoke.

Discriminant analysis (interrelationship among independent variables): Canonical discriminant analysis was applied to plot the similarity measure matrix from the Euclidean distance between groups' centers in the canonical space to better visualize the clustering pattern of dependent variables (Fig. 2). Similarity classification of the dependent variables showed that plotting the principal component 1 (PCA1) and principal component 3 (PCA3) explained about 97.15% of the variance of hematological and hormonal variables that were used to differentiate among groups of volunteers: applicators; farmers who apply the pesticides, farmers who don't apply the pesticides as positive control and city inhabitants as negative control. It was obvious from the dimension plot similarity matrix that the variables of WBC, PDW, P-LCR, MPV, RDW, MXD%, MXD# and T4 were projected almost together in a similar sphere, which highlighted strong association between them. Also, RBC, HGB, HCT, MCH, MCHC,

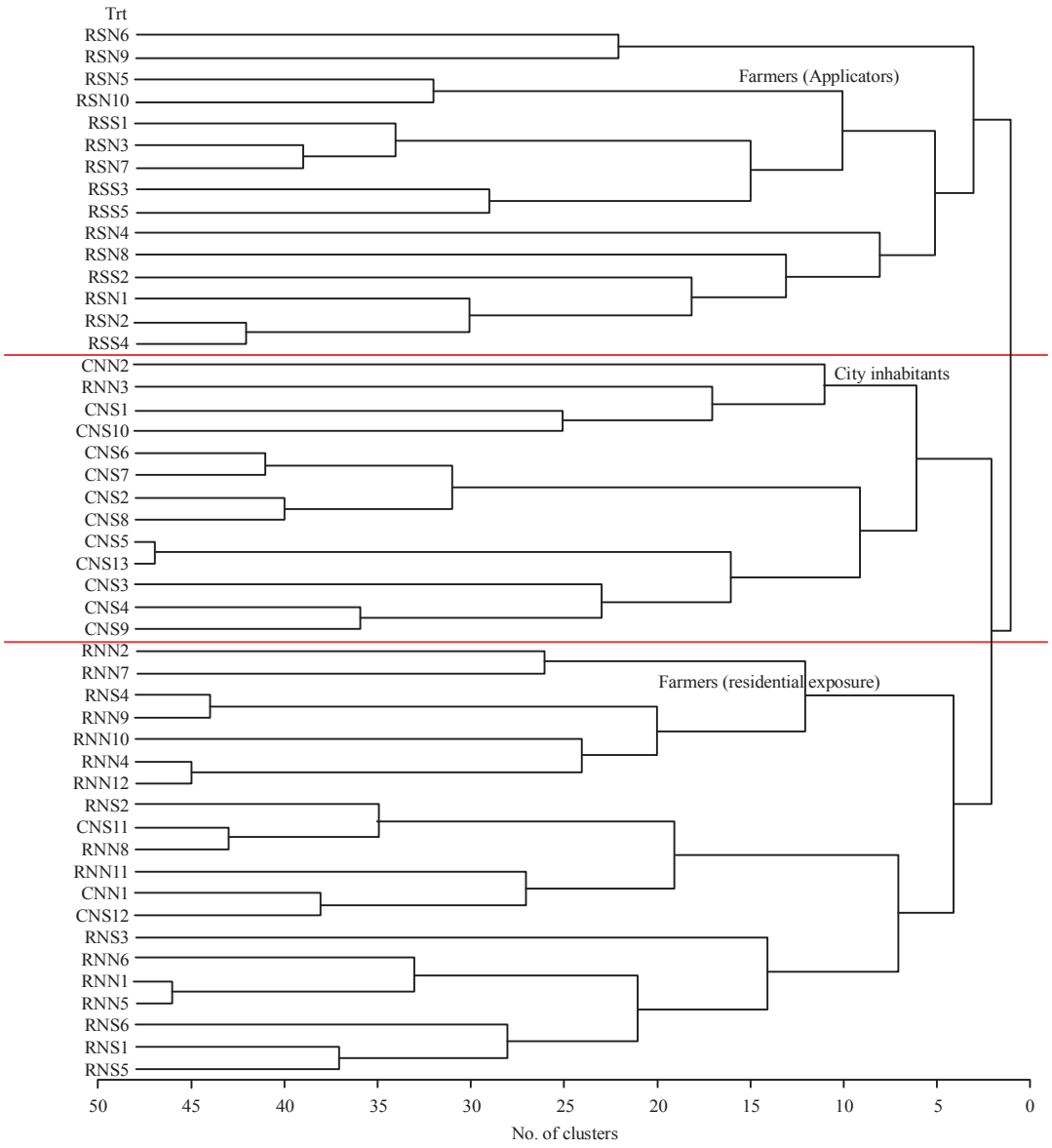

Fig. 3: Tree dendrogram of the clustering patterns and their proportion of explained variance (clusters explained 100% of the variation) using the CANDISC procedure of the hematological and hormonal variables that were used to differentiate among groups of volunteers. Participants code and number was represented by (from left to right) three letter designations; the first column: city or rural, the second column: spray or not spray and third column: smoke or not and the fourth and fifth columns were participant number

LYM%, NEUT% and T3 were plotted together, while the PLT were projected in a different position, which emphasize the importance of the platelet number as a discriminating factor in the occupational poisoning of pesticides. Testosterone, LYM# and NEUT# were highly associated. The PCA revealed that dependent variables were projected in four major principal spheres and they account for more than 97% of the variance. These principal components or representatives of each group might be used as predictor variables in subsequent analyses related to occupational exposure.

Additionally, hierarchical cluster analysis was used to classify groups; spray workers (smoking or not), farmers (residential exposure, smoking or not) and city residents (smoking or not). A tree dendrogram of the clustering patterns and their proportion of explained variance (clusters explained 100% of the variation) using the canonical discriminant (CANDISC) procedure of the hematological and hormonal variables was constructed (Fig. 3). There was a clear distinction between groups of volunteers. Three major clusters for each group of volunteers was gathered separately. The first cluster

group was further classified into un-differentiated pattern where there was no clear separation between smokers and non-smokers. The second cluster plotted the group of people who live in the city and within this cluster, it was amazing to find that smokers were clustered separately apart from the non-smokers. The third group (rural inhabitants) was similar to first group where there was no distinction between smokers or non-smokers individuals.

DISCUSSION

Pesticide exposure pose serious consequences on exposed person where the control or limiting of such issue depends mainly on the use of personal protective equipment and personal hygiene. Normally, pesticides were not detected in the serum samples may be due to the fast biotransformation and detoxification processes including increased excretion. For example, human metabolism converted the organophosphate insecticides into dimethyl and diethyl phosphorothioates when analyzed in plasma samples of humans with detection limits of 150 and 50 ng mL^{-1}, respectively (Drevenkar *et al*., 1994). Detoxification process may involves an increase in the activity of acetylcholine esterase (AChE), which current study did not report any significant differences among subjects in the relative activity of this enzyme (data not shown).

Hematological parameters were used as biomarkers of occupational toxicity (Tryphonas, 2001; Collins and Dusinska, 2009). White blood cells and its components are important constituents of the immune system, so any adverse effects on white blood cells might be reflected in the immune system. Platelets (thrombocytes) are the smallest type of blood cell and they are important in blood clotting. In case of bleeding, the platelets swell, clump together and form a sticky mass that helps end the hemorrhage. Too few platelet numbers might cause uncontrolled bleeding while too many platelets might cause blood clot in the blood vessel. Moreover, the estimation of the red blood cell distribution width parameters is helpful to show if the cells are all of the same size and shape or different. Current study highlighted the importance of blood indices chemistry in the estimation of the occupational exposure to pesticides. Low values of total WBCs, RBCs, HGB and HCT measures in the blood samples of the group with the highest serum PCB levels (Serdar *et al*., 2014). Hu *et al*. (2015) reported that the majority of selected health indicators, such as monocytes, monocyte percentage, red blood cell, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width coefficient of variation, platelet count and platelet distribution width were significantly affected after 3 days of exposure to pesticides. However, Gaikwad *et al*. (2015) reported no significant differences between RBC, platelet count and HGB values in blood samples of the study group and control groups, WBC count was significantly decreased in blood samples of pesticide sprayers.

Current study specified that pesticide exposure has altered the content of the thyroid and reproductive hormones. Similarly, the content of the thyroid and reproductive hormones of spray workers and cotton pickers were disturbed. Serum levels of testosterone were increased in serum of spray applicators and T4 was reduced in blood serum of cotton pickers (Khan *et al*., 2013). Chronic occupational exposure to organophosphate and carbamate pesticides caused damage of the sperm chromatin and decreased the semen quality but it did not affect the levels of testosterone and T4 hormones (Blanco-Munoz *et al*., 2012; Miranda-Contreras *et al*., 2013; Jamal *et al*., 2015). Multivariate analysis showed no interaction between smoking and pesticide exposure (Fig. 2). Perhaps a larger sample size was needed to ascertain the effects of occupational exposure and this will be considered in the future. On the contrary, the findings reported herein showed that farmers who smoke and were exposed to pesticides were under greater risk than non-smokers and these results were supported by De Jong *et al*. (2014a, b) and Lee *et al*. (2014).

CONCLUSION

The hierarchical cluster analysis efficiently classified groups of treatments; applicator, farmers and city residents. Tree dendrogram clustering patterns disclosed a clear distinction between groups of volunteers. Three major clusters for groups of volunteers were clustered separately. The final groupings correctly classify 100% of the sprayers, 80% of the city non-sprayers and 94% of the farmer non-sprayers, which was consistent with differential disruption of normal metabolism of several hormonal and immune system variables. Based on the results reported herein it seems that the biochemical measures refer to a chronic state. Moreover, the principal component analysis showed that the hematological and hormonal parameters might be good predictor variables in subsequent analyses related to pesticides exposure. Also, current study highlights the need for the enforcement of the official regulations to reduce overexposure of spray workers throughout Egypt. Nevertheless, sample size employed in current study was a potential limitation of current study.

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