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Original Article

Garlic and alpha lipoic supplementation enhance the immune system of albino rats and alleviate implications of pesticides mixtures

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Abstract: This study aimed to investigate age dependent immune-system response versus exposure to different doses of mixture of (chlorpyrifos, profenofose, and fenitrothion) and/or combined with 60 and 250 mg kg^{-1} alpha lipoic acid and garlic, respectively. 120 males of albino rats were divided to two groups according to age; weaning group (2 months age and 60-80 gm.), adult (6 months and 180-200 gm). Each age was divided into 6 subgroups treated orally for 3 months , G1 (control), G2 high dose (HDPM) CPF10 mg kg^{-1}, PRO 3 mg kg^{-1}, FEN 6 mg kg^{-1}, G3 low dose (LDPM) CPF 1 mg kg^{-1}, PFN 0.3 mg kg^{-1} and FEN 0.6 mg kg^{-1}, G4 AOX (alpha lipoic + Garlic), G5 HDPM + AOX and G6 LDPM + AOX. Results showed significant inhibition in serum acetylcholinesterase (AChE), elevation in malondialdehyde (MDA) concurrent with reduction in total reduced glutathione (GSH) in both ages was recorded as well as, decrease in IGG, IGM, Lymphocyte transformation and Phagocytosis humeral and cellular immunity confirmed by alteration in lymph nodes architecture. This study was concluded that the supplementation with alpha lipoic acid and garlic improved previous alternations slightly to be more or less near the control level in both adult and weaning rats. It seems that, immune-responses of both adult and weaning rats were slightly similar.

Keywords: Organophosphorus pesticides, alpha lipoic acid, garlic, immunotoxicity, weaning

Introduction

Due to the pervasive use of pesticides, we are exposed to them on a daily basis whether we work with them directly or not. The most common route of pesticide exposure is through the ingestion of contaminated food and/or water. It has been hypothesized that altered immune function may be an early indicator of immunetoxicity, eventually having an effect on immunologically based diseases, such as cancer, hypersensitivity, and autoimmunity Luster and Rosenthal 1993 [1]. Immune system alterations can include a decrease in neutrophil and macrophage function, a decrease in the number of thymocytes, a decrease/increase in mitogen-induced proliferation, a decrease in antibody-dependent cell cytotoxicity and a decrease/increase in cytokine secretion two groups of individuals are at a greater risk [2-5], agricultural workers and children [6, 7]. Exposure to some pesticides, including phenoxy herbicides, organochlorine and organophosphorus insecticides, has been associated with an increased risk for non-Hodgkin's lymphoma [8]. Furthermore, maternal and perinatal exposure to pesticides has been associated with an increased risk for lymphoma [9]. On the other hand, pesticides applicators exposed to mixture of Ops, carbamates, phenoxy herbicides and pyrethroids have increase in blood tumor necrosis factor and decrease in blood IgM [10] indicating enhanced macrophage activation and impaired humoral defense. chlordane, hexachlorobenzene, chlorpyrifos induced decrease in cell-mediated immunity that was

associated with increased allergic reactions and autoimmune diseases, suggesting that pesticide-induced immunosuppression may have an impact on individual's ability to evoke an allergic response [11]. Garlic (Allium sativum) has been used medicinally since before the time of the Sumerian civilization (2600-2100 BC) [12]. Healthful properties of garlic are legion and over a thousand scientific reports enumerated its functional activities which include free radical scavenging activities, immune stimulation, curing cardiovascular diseases, anti-cancer, and anti-infectious properties [13-15]. Most biological effects of garlic are attributed to its characteristic organosulfur compounds. The major sulfur-containing compounds in intact garlic are δ-glutamyl-S-allyl-L-cysteines and S-allyl-L-cysteine sulfoxides (allin). When garlic is extracted with an aqueous solution, the δ-Glutamyl-S-allyl-L-cysteines are converted into S-allylcysteines (SAC) through an enzymatic transformation with δ-glutamyl-transpeptidase [16, 17]. Garlic works as an immune stimulant.

The immunostimulating effects of garlic and its components/preparations include increase in the total white blood cell (WBC) count and enhanced bone-marrow cellularity (Kuttan 2000). Lipoic acid (ALA), a disulphide derivative of octonic acid, and its reduced form dihydrolipoicacid (DHLA) are natural compounds widely distributed in plants and animals. They are synthesized through a reaction catalyzed by lipoic acid synthase within the mitochondria. ALA are unique antioxidant ALA/DHLA system. Thus DHLA is able to reduce not only reactive oxygen species (ROS) but also oxidized forms of other antioxidants. ALA regenerates other antioxidants and for this reason it is called an antioxidant of antioxidants an increase in intracellular glutathione, which is vital to the immune system functioning [18] dietary lipoic acid is effective in attenuating oxidative stress induced by drugs [19].

In the present study, we investigated the response of immune system of weaning and adult albino rats versus intoxication with low and high doses of mixture of organophosphorus pesticides, and how combined supplementation of garlic and alpha lipoic acid counteract this effect.

Materials and methods

Experimental animals

120 males of Albino rats were divided into two main groups according to age; weaning group (2 months age) weighed (60-80 gm) and Adult group (6 months age) weighed (180-200 gm). They were obtained from the breading animal's house of Central pesticide laboratory, Agriculture Research Center Dokki, Cairo. The animals were acclimatized to the experimental conditions for two weeks before start of experiment to exclude any diseased animals. The animals were housed in metallic cages in air conditioned room at 25°C with 12 h light/dark cycle. The animals were fed on balanced ratio and had free access tap water throughout the experimental period. All animals were treated according to the standard procedures laid down by OECD guidelines 2009 [20] for combined chronic toxicity.

Pesticides

All required information of tested pesticides (chlorpyrifos, profenofose and fenitrothion) were mentioned in **Table 1**.

Antioxidants

Alpha lipoic acid (thiotic acid), supplied by EVA Pharma Co., treated at 60 mg kg^{-1} [21]. Garlic, about 30 g peeled garlic was crushed and extracted with 60 ml distilled water (500 mg ml^{-1}) [22]. The extract was used at 250 mg kg^{-1}.

Experimental design

After two weeks of acclimatization period, animals were divided into two main groups according to age, weaning group (age 2 months with weighed 60-80 gm) (Group 1) and adult group (age 6 months with weighed 180-200 gm) (Group 2). Each age group of rats were divided into 6 subgroups (10 rats in each) treated as follows:

(Group 1) served as control (without treatment).

(Group 2) rats were orally treated with high dose of pesticides mixture (HDPM) via gastric intubation 5 days per week for 3 months.

(Group 3) rats were orally treated with low dose of mixture pesticides (LDPM) via gastric intubation 5 days per week for 3 months.

(Group 4) rats were orally treated with antioxidants as positive control (garlic 250 mg kg^{-1} and alpha lipoic acid 60mg kg^{-1}), consecutively given by gastric intubation 5 day per week for 3 months

(Group 5) the rates were orally treated with antioxidants (garlic 250 mg kg^{-1} and alpha lipoic acid 60 mg kg^{-1}) 5 day per week for 3 months 1 h after administration of mixture of high dose pesticides mixture given by gastric intubation

(Group 6) rats were orally treated with antioxidants (garlic 250 mg kg^{-1} and alpha lipoic acid 60 mg kg^{-1}) 5 day per week for 3 months 1 h after administration of mixture of low dose pesticides mixture given by gastric intubations.

Sampling

Blood samples were collected from the retro-orbital venous plexus at the end of experimental period according to Schermer [23]. The blood samples were divided to two parts, first part as whole blood on heparin to investigate lymphocyte transformation and Phagocytosis. While second part as non-heparinized blood was centrifuged at 3500 rpm for 15 min. in a refrigerated centrifuge to separate serum, which was kept frozen at -20°C, for subsequent biochemical analysis. Animals were sacrificed and their submandibular and sub-axillary lymph nodes were quickly dissected and washed in normal saline then they were fixed in 10% formalin solution for histopathological studies.

Biochemical assays

Cholinesterase (ChE) was assayed in serum by the method of [24]. Malondialdehyde (MDA) occurs in lipid peroxidation and was measured according to [25] in the serum after incubation at 95°C with thiobarbituric acid in aerobic conditions (pH 3.4). The pink color produced by these reactions was measured spectrophotometrically at 532 nmto measure malondialdehyde levels. Total reduced glutathione (GSH) was determined in erythrocytes by the method of [26] based on the development of a yellow color when DTNB is added to the supernatant of the precipitated RBCs containing sulfhydryl groups.

Immune response evaluation

Immunoglobulins IgM and IgG were determined using a rapid cassette test kits were supplied by Pan Bio, Ltd., Windsor, Queensland, Australia.

Lymphocyte transformation test

Separation of Lymphocyte: Using sterile Pasteur pipettes, the heparinized blood was layered carefully on the surfaces of Lymphocyte Separation medium. Ficoll hypaque (1:1) in a 50 ml sterile polysterine centrifuge tube. The tube was centrifuged for 40 minutes at 2400 rpm in a cooling centrifuge (+ 4°C). The mononuclear leukocyte forming a band at the ficoll plasma interface were carefully aspirated by a sterile Pasteur pipette and dispensed in a clean sterile centrifuge tube containing cold RPMI-1640. The separated buffy coat was washed with RPMI-1640 medium 3 times each for 10 min. at 2400 rpm, 2000 rpm, and 1500 rpm respectively. The last washing was applied to remove any remnants of thrombocytes. After the last washing, the sediment washed lymphocytes were re-suspended in 1 ml RPMI-1640 medium containing 10% of foetal calf serum.

B- Total lymphocyte count was carried out according Hudson and Hay (1980).

To 100 µl of lymphocyte suspension, 100 µL of 0.4% trypan blue were added then mixed well and immediately transferred to the haemocytometer. Cells were then left for 2 minutes to settle down using a × 40 objective lens. The non-stained (living) bright cells that have oval, ovoid, or rounded nuclei in the central triple ruled squares were counted. Dead cells took the stain and appeared blue. At least 100 viable lymphocyte were counted. The number of viable lymphocyte per ml RPMI medium was calculated according to the following equation:

$$\text{RPMI} = \frac{\text{Number of counted lymphocyte} \times 25 \times 104 \times \text{Dilution factor}}{\text{Number of triple ruled squares}}$$

Standardization of lymphocyte concentration: According to viability cell count, the viable lymphocyte were adjusted at a final concentration of 2X 10^6 cells/ml and suspended in RPMI medium containing 10% foetal calf serum (FCS).

Preparation of mitogens (non-specific mitogen): Phytohemagglutinin (PHA) was obtained as a

Table 1. Common, trade, chemical names and toxicity of tested pesticides

Common name	Trade name	Formulation	Chemical name (IUPAC)	LD_{50} (mg/kg)	LDPM (mg/kg)	HDPM (mg/kg)
Chlorpyrifos	Dursban	48% EC	O, O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate	111.6	1.0	10
Profenofose	Curacron	72% EC	O-(4-bromo-2-chlorophenyl) O-ethyl S-propyl phosphorothioate	445	0.3	3.0
Fenitrothion	Sumithion	50% EC	O, O-dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate	584	0.6	6.0

LD50: Lethal Dose, 50%; LDPM: Low dose pesticides mixture; HDPM: High dose pesticides mixture.

Table 2. Effect of Chronic intoxication with high (HDPM)and low(LDPM) doses of pesticides mixture in presence or absence of combined antioxidants on Acetylcholinesterase (AchE), oxidative stress marker (MDA) and total Reduced Glutathione (GSH) in serum of weaning and adult albino rats

Groups / Parameters	Weaning			Adult		
	AchE (UIL)	MDA (µmol/ml)	GSH (mg/dl)	AChE (UIL)	MDA (µmol/ml)	GSH (mg/dl)
Control	483.02±112.46	165.05±20.58	21.83±1.09	565.80±95.70	99.86±9.52	20.66±1.83
HDPM	152.62±47.64^{a}	332.54±33.38^{a}	16.97±10.53^{a}	211.28±75.77^{a}	127.60±38.01^{a}	13.41±1.23^{a}
LDPM	235.75±50.31^{a}	319.32±43.02^{a}	18.46±4.26^{a}	328.52±54.12^{a,b}	117.25±18.76	15.41±1.23^{a}
+ ve control	414.66±172.66^{b,c}	185.52±19.48^{b,c}	24.61±2.10^{a}	655.20±145.45^{b,c}	83.05±12.43^{b,c}	23.62±1.44^{a,b,c}
HDPM + AOM	171.01±82.58^{a,d}	325.93±39.28^{a,d}	22.59±1.72^{c,d}	237.11±22.77^{a,d}	112.48±12.62^{d}	19.99±1.55^{d}
LDPM + AOM	199.56±50.44^{a,d}	312.97±59.11^{a,d}	22.77±4.05^{d}	204.15±50.73^{a,c,d}	105.69±18.42	21.47±1.48^{b,c,d}

All data were expressed as mean ± SD (Standard Deviation) of 10 albino rats. a: significant differences versus control at P<0.05; b: significant differences versus HDPM treated groups at P<0.05; c: significant differences versus LDPM treated groups at P<0.05; d: significant differences versus antioxidant treated groups (+ ve Cont.) at P<0.05; e: significant differences versus HDPM + AOM treated groups at P<0.05.

powder and reconstituted in 5 ml RPMI medium. The required concentration could be made to 15 µl ml^{-1} [27]. This concentration was tested to be the optimum for use.

Setting up of lymphocyte culture

Flat bottom sterile microtiter tissue culture plates with 96 well, were used for cultivation of lymphocyte. Three wells were used and each contained 100 µL of suspended lymphocytes (2×10^6 cells) in 50 µl growth media (RPMI + 10% FCS) served as cell control. Three wells containing 100 µl of suspended lymphocytes + 50 µl PHA (non-specific mitogen) (15 µg ml^{-1}). Three wells containing 150 µl of RPMI-1640 medium (medium control) the total volume per each cell well was adjusted to 150 µl. The plates were incubated at 37°C in a CO_2 incubator, Lymphocytic transformation and 5% blastogenesis were assayed after 48-72 hours using tetrazolium dye (MTT) assays according method was carried out by [28].

Phagocytosis assay

Phagocytosis was carried out according [29], blood was collected in heparin-coated universal bottles was used to prepare leucocytes for bacterial Phagocytosis. The mixtures of bacteria (S. aureus) and leucocytes were incubated at 37°C for 2 hours with regular stirring. The viability of bacteria before incubation and after incubation was estimated by the method of [30]. Then the mixtures were centrifuged at 2000 g for 5 min. at 4°C. The supernatants were used to estimate the percentage of bacteria phagocytosis using the formula:

$$\text{Phagocytosis } \% = \frac{(\text{CUF before incubation I CUF after incubation})}{\text{CUF before incubation}}$$

Histopathological studies

Histopathological examination was carried out according [31]. The submandibular and auxiliary lymph nodes were dissected and washed in normal saline then they were fixed in 10% formalin solution for 14-18 h, processed in a series of graded ethanol and embedded in paraffin. Paraffin sections were cut with at 5 µm thickness and stained with hematoxylin and eosin (H&E) for light microscopic examination. The sections were examined and photographed by Olympus light microscope (Olympus BX51, Tokyo, Japan) with attachment photograph machine (Olympus C-5050, Olympus Optical Co. Ltd., Japan).

Statistical analysis

Data were checked for unequal variance (Bartlett's test). Statistical analysis was based

Table 3. Effect of Chronic intoxication with high (HDPM) and low (LDPM) doses of pesticides mixture in presence or absence of combined antioxidants on (IGG, IGM) in serum of weaning and adult albino rats

Groups / Parameters	Weaning		Adult	
	IGG (mg/dl)	IGM (mg/dl)	IGG (mg/dl)	IGM (mg/dl)
Control	1201.32±41.25	192.26± 13.20	1193.30±49.04	163.21±5.79
HDPM	1112.99±92.35[a]	100.02±3.63[a]	933.40±18.47[a]	109.77±7.06[a]
LDPM	1143.69±50.41	119.43±8.13[a,b]	1027.81±57.918[a,b]	146.03±9.29[a,b]
+ ve control	1361.43±46.26[a,b,c]	225.67± 10.54[a,b,c]	1503.86±60.47[a,b,c]	217.57±11.97[a,b,c]
HDPM + AOM	1197.45±27.22[b,d]	147.09±22.22[a,b,c,d]	1037.18±38.84[a,b,d]	152.87±8.39[b,d]
LDPM + AOM	1223.84±38.83[b,c,d]	177.37±7.32[b,c,d,e]	1200.12±45.57[b,c,d,e]	155.89±15.66[b,d]

All data were expressed as mean ± SD (Standard Deviation) of 10 albino rats. a: significant differences versus control at P<0.05; b: significant differences versus HDPM treated groups at P<0.05; c: significant differences versus LDPM treated groups at P<0.05; d: significant differences versus antioxidant treated groups (+ ve Cont.) at P<0.05; e: significant differences versus HDPM + AOM treated groups at P<0.05.

Table 4. Effect of Chronic intoxication with high (HDPM) and low (LDPM) doses of pesticides mixture in presence or absence of combined antioxidants on (Lymphocyte transformation, Phagocytosis) in heparinized blood of a weaning and Adult albino rats

Groups / Parameters	Weaning		Adult	
	Lymphocyte Transformation index	Phagocytosis (%)	Lymphocyte Transformation Index	Phagocytosis (%)
Control	1.23±0.08	77.80±3.34	1.29±0.05	76.20±3.27
HDPM	0.96±0.08 [a]	74.00±2.54[a]	0.98±0.09[a]	69.80±1.48[a]
LDPM	1.23±0.07[b]	73.60±1.14[a]	1.18±0.01[a,b]	71.80±1.30[a]
+ ve control	1.67±0.03[a,b,c]	87.00±1.58[a,b,c]	1.70±0.06[a,b,c]	86.60±1.14[a,b,c]
HDPM + AOM	1.17±0.05[b,d]	76.60±1.81[c,d]	1.20±0.03[a,b,d]	74.20±1.30[b,d]
LDPM + AOM	1.31±0.01[a,b,c,d,e]	78.60±1.14[b,c,d]	1.31±0.01[b,c,d,e]	76.80±2.16[b,c,d,e]

All data were expressed as mean ± SD (Standard Deviation) of 10 albino rats. a: significant differences versus control at P<0.05; b: significant differences versus HDPM treated groups at P<0.05; c: significant differences versus LDPM treated groups at P<0.05; d: significant differences versus antioxidant treated groups (+ ve Cont.) at P<0.05; e: significant differences versus HDPM + AOM treated groups at P<0.05.

on comparing the values between the untreated control group with the pesticide mixture treated groups (HDPM, LDPM), antioxidant treated group (+ ve control) and pesticide mixture & antioxidant supplemented groups (HDPM + AOX, LDPM + AOX). The results are expressed as means ± SD of 10 animals/group. The statistical significance of the data has been determined using one way analysis of variance (ANOVA-LSD) using SPSS statistical software package version 13. The confidence level of significance was set at P<0.05.

Results

Biochemical assay results

As depicted in (**Table 1**) weaning and adult rats intoxicated with both doses of pesticides mixture HDPM and LDPM induced remarkable inhibition in serum acetylcholinestrase (AChE) enzyme, the inhibition was significant versus control group at P<0.05. Supplementation with mixture of antioxidants (garlic aqueous extract and alpha lipoic acid) cannot counteract this inhibition. Elevation in malondialdehyde (MDA), oxidative stress biomarker, was concomitant to the effect of pesticides mixture on AChE enzyme, all through the treated groups pronounced in weaning treated groups. This elevation is slightly counteracted in adult more than weaning groups in antioxidants supplemented groups as compared to the intoxicated ones. It should be noted here that the induction in oxidative stress was due to the reduction in total glutathione content (GSH) in all intoxicated groups significant versus control at P<0.05. on the other hand supplementation with combined

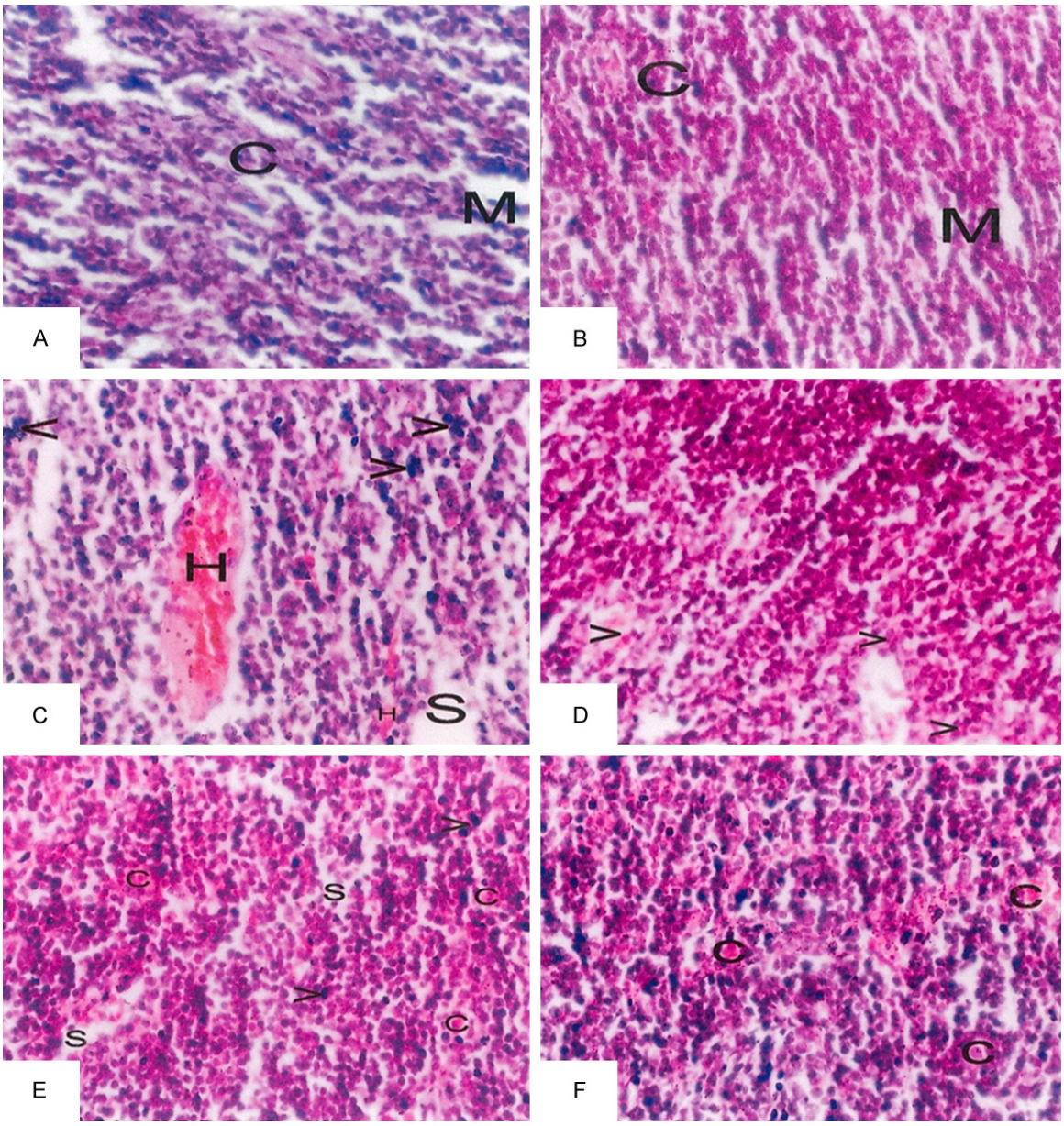

Figure 1. H&E staining of lymph node section from weaning rats. A: Control group–; (H&E × 40). B: Positive control groups C: Group III of the HD pesticides treated; D: Group IV of the HD pesticides and antioxidant treated; E: Group V of the LD pesticides treated; F: Group VI of the LD pesticides and antioxidant treated medullary cords (m); medullary sinuses (C); hemorrhagic spots (H), sinuses (S).

antioxidants counteract this reduction in all treated groups to be more or less nearly to the control groups significant versus control and non-supplemented groups at $P<0.05$. (**Table 2**) Express the effect of HDPM and LDPM on some markers for humoral immunity, IGG and IGM in serum of weaning and adult rats. Data revealed that intoxication with HDPM induced significant decrease in IGG level versus control in serum of weaning and adult rats with percentage changes from control (-7.35%) in weaning and (-2.18%) in adult. Meanwhile, IGM recorded significant decrease in both intoxicated groups with percent changes (-4.8%) in weaning rats and (-3.27%) in adult rats. LDMP intoxication induced the same effect where significant reduction in IGG was remarkable in adult treated animals versus control and HDMP groups with % changes -9.03% from control. Moreover, IGM recorded significant reduction in both

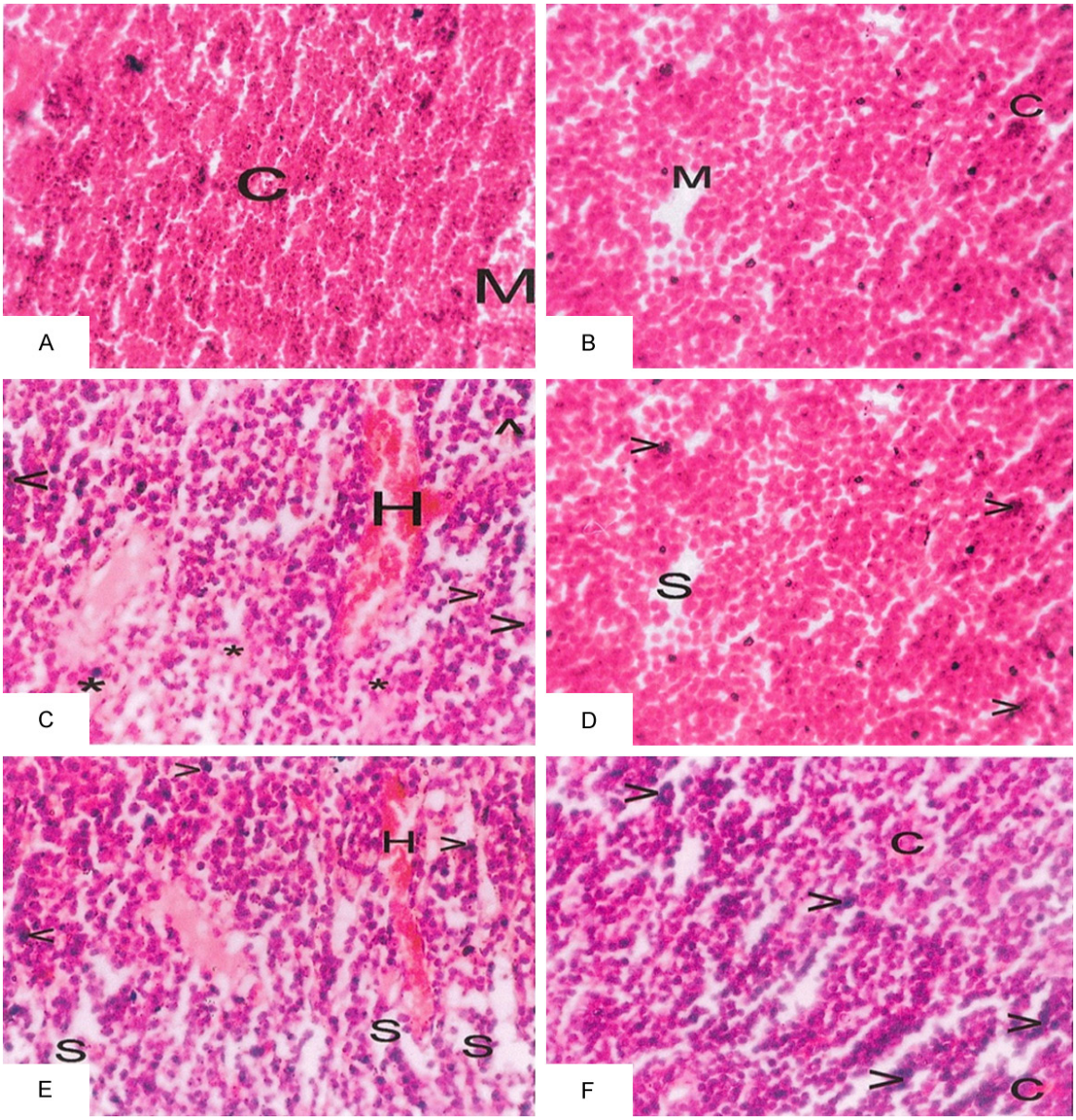

Figure 2. H&E staining of lymph node section from adult rats. A: Control group containing the follicles with their germinal and the (H&E × 20). B: A positive control group (H&E × 20). C: Group III of the HD pesticides treated (H&E × 40). D: Group IV of the HD pesticides and antioxidant treated adult rats (H&E × 40). E: Group V of the LD pesticides treated adult rats (H&E × 40). F: Group VI of the LD pesticides and antioxidant treated adult rats (H&E × 40). The Cortex (C); Medulla (M); hemorrhagic spots (H), sinuses (S).

groups versus control and HDMP groups with -5.6% and -10.5% from control in weaning and adult groups respectively. On the other hand, supplementation with mixture of antioxidants (aqueous garlic extract and alpha lipoic acid) to + ve control as well as pesticides mixture intoxicated groups induced remarkable enhancement in humeral immune parameters IGG and IGM significant versus-ve control and versus intoxicated groups as depicted in **Table 2**.

Demonstrated data in (**Tables 3**, **4**) declared that weaning rats intoxicated with high dose mixture (HDPM) recorded significant decrease in lymphocyte transformation index (LTI) with percentage change from control (-21.95%). However, rats intoxicated with low dose mixture has no change from control. In adult rats pronounced decrease was obvious in both intoxicated groups, the percentage of decrease from control was -24.03% and -8.53% in HDPM and

LDPM, respectively. It should be noted here that great enhancement in (LTI) was observed in both + ve control groups supplemented with combined antioxidant per se that was reflected on all intoxicated groups pre supplemented with ALA and garlic aqueous extract. Phagocytosis is a vital function of the immune system against any xenobiotic. Intoxication with HDPM and LDPM induced significant inhibition in phagocytosis process in both weaning and adult intoxicated groups at $P<0.05$. This inhibition was counteracted by supplementation with combined antioxidants to reach nearly to the control level (**Table 3**).

Histopathological results

The control Group of the weaning and adult rats showed normal architecture of the lymph node showing the cortex and the medulla; The cortex contain the densely packed lymphoid follicles with less dense germinal centre (**Figure 1A**, plate 1) and the medulla shows the medullary cords and the medullary sinuses (**Figure 2A**, plate 2). + ve control group of the weaning and adult rats given antioxidants alone, showed normal architecture of the lymph node showing the cortex and the medulla; The cortex contain the densely packed lymphoid follicles with less dense germinal center (**Figure 1B**, plate1) and the medulla shows the medullary cords and the medullary sinuses (**Figure 2B**, plate 2).

Intoxication with HDPM showed a hemorrhagic spots in weaning group, disturbance of the normal architecture and decreased cellularity all over the lymph node, some cells appeared with condensed chromatin among the lymphocytes as demonstrated in (plate 1, **Figure 1C**). However, adult rats showed many histological alterations; these alterations included hemorrhage and necrosis between the cortical follicles distortion of the follicular architecture, few macrophages were noticed and decreased cellularity in both the cortex and the medullary cords (**Figure 2C**, plate 2). On the other hand, supplementation with antioxidant mixture to HDPM showed mild changes in the form of widening of the medullary sinuses, few macrophages were seen but cellularity was close to normal in weaning group as demonstrated in (plate 1, **Figure 1D**).

In case of the adult (plate 2, **Figure 2D**) no hemorrhage was noted, lymph node architecture was still preserved, decreased cellularity was also noticed but to a much lesser degree than the intoxicated one. Rats that were given the Low dose pesticides mixture (LDPM) showed mild congestion of the lymph node parenchyma and condensed chromatin was noted in few cells of the cortex in the weaning rats (**Figure 1E**, plate1).

The adult showed hemorrhage in the lymph node parenchyma, widening of the medullary sinusoids. Some cells appeared with condensed chromatin, densely stained that may be due to apoptotic changes (**Figure 2E**, plate 2). Histological alterations decreased or disappeared; the Low dose group with antioxidants appeared absolutely normal apart from mild congestion in weaning group (**Figure 1F**, plate 1). While the adult rats appeared almost normal in architecture and cellularity, only mild congestion and few cells showed condensed chromatin (**Figure 2F**, plate2).

Discussion

Organophosphorus pesticides are heavily employed over the world for their broad insecticidal effect either in the field or in the commercial greenhouses, to suppress the population of several common insect pests, which infest plants and cause serious economic damage [32]. The primary mechanism of action of organophosphate pesticides is inhibition of carboxyl ester hydrolases, particularly acetylcholinesterase (AChE) [33]. The inhibition of AChE is the most plausible explanation for much of the symptomatology following OP intoxication [34]. These findings support our results of adult and weaning rats intoxicated with low and high doses of mixture of profenofose, fenitrothion and chlorpyrifos had significant reduction in serum acetyl choline esterase (AChE) enzyme. Recent studies indicate that pesticide intoxication produce oxidative stress by the generation of free radicals and alternation in antioxidant or oxygen free radical scavenging enzyme system Causing tissue lipid peroxidation in mammals and other organisms [35-37] induction of lipid peroxidation biomarker (MDA) in all intoxicated groups, LDPM and HDPM. Significant increase in the lipid peroxidation is one of the primary effects induced by oxidative stress. It may also be correlated with the reduction in the antioxidative defense enzyme systems. Intoxication with pesticides has high affinity for glutathione

[38]. So, pesticides-glutathione conjugation formed depletes the glutathione from the cell and thus decreasing antioxidant potential. Because of the wide use of pesticides for domestic and industrial purposes, the evaluation of their immunotoxic effects is of major concern for public health [39]. In the present study, we selected IgG and IgM as markers of humoral immunity, and lymphocyte transformation and leukocyte phagocytosis as markers of cell-mediated immunity. The results showed that pesticides mixture groups induced decrease in each of IgG and IgM, the rate of lymphocyte transformation and the rate of leucocyte phagocytosis also decrease in both age groups. These finding run in parallel, with those obtained by Rodgers 1997 [40] who reported treatment with low doses of malathion for 90 days reduction in immune functions. Main while, an increase occurrence of asthma and atopic reactions in parallel with decrease acetylcholinesterase in individuals exposed to low doses of fenthion and dichlorvos [41]. Also, decrease in IgM and IgG in blood of 304 pesticides applicators exposed to a mixture of Ops and carbamates and pyrethroids indicating enhanced macrophage activation and impaired humoral defense. Applicators exposed to a mixture of Ops, carbamates, phenoxy herbicides and pyrethroids associated with increased immunological parameters neopterin and soluble tumor necrosis factor, and decreased IgM, suggesting enhanced macrophage activation and impaired humoral defense [42, 43].

Administration of immunosuppressive agents induce depletion of lymphocytes or reduction in cellularity that can occur in a diffuse manner or be limited to either the cortex or medulla [44] This supports our study in which decreased cellularity was obvious with the administration of high dose of the pesticides mixture in both the adult and the weaning rats, and it was observed mainly in the cortical zone of the lymph node. Macrophages were noticed in the adult high dose toxicity suggesting increase in the phagocytic activity in cases of poisoning with alpha-cypermethrin [45]. In the present study the weaning rats also exhibited low cellularity and some cells showed condensed chromatin that may be explained as apoptotic changes in the lymphocytes, on the other hand the adult group showed this type of cells in the group of low dose toxicity of the Pesticide mixture. Pesticide-induced immunosuppression may be a consequence of a direct effect of these pesticides or its metabolites on the immune system [46], an increase in tissue acetylcholine concentration and interaction of this neurotransmitter with lymphocytes and/or accessory cells [47]. It has been suggested that cholinergic stimulation leads to suppression of plaque forming cells (PFC) response during organophosphate (Malathion) exposure and may be mediated by a direct effect of acetylcholine upon specific subcomponents of the immune system, which are critical to the PFC response. The cholinergic receptors have been identified on lymphocytes and macrophages [46].

Interaction of organophosphate with lymphoid cells is likely to activate free radical mechanism which may be responsible for immunosuppression. Lymphocyte dysfunction may be an integral part of pesticide-induced immunosuppression and presents an approach which may serve to delineate the possible mode of action. Increased oxygen free radical (OFR) generation by pesticide exposure was found to exert deleterious effects on different components of immune system [48, 49]. OFRs have many molecular and cellular targets in the immune system and selective depletion of T-lymphocytes, decreased blast transformation with phytohemagglutinin (PHA), which may be the ground for decreased cellularity after 3 months of the pesticides mixture intake [46]. However the ability to identify histopathological changes in lymphoid tissues was highly dependent on the severity of the specific lesion and the tissue compartment measured in these studies. Overall, histopathological changes were most frequently and most consistently reported in the thymus cortex and medulla and in the spleen and lymph node follicles (cellularity and germinal center development) [44]. The present study declared that the natural antioxidants (ALA and garlic extracts) had a considerable ability to counteract the reducing effect of pesticide mixture-intoxication on the level of (IGG & IGM) and the rate of (lymphocyte transformation & leucocyte phagocytosis), as a result of improving the level of GSH and decreasing the level of MDA. It is worth to mention here that antioxidants supplementation alone (+ ve control group) exhibited an increase on the level of (IGG & IGM) and the rate of (lymphocyte transformation & leucocyte phagocy-

tosis) in both age groups. These findings were not recorded before and point to the possible role of oxidative stress in including immunomodulation secondary to pesticide mixture administration. ALA induced elevation of GSH was proposed to be through its role in enhancing the intracellular cysteine, the rate limiting amino acid in GSH synthesis [50]. ALA was found to be highly reactive against variety of [51, 52]. The improving role of garlic extracts that seen in the present study could be attributed initially to the antioxidant properties of garlic, garlic is not a single compound, and actually it is a mixture of organosulfur compounds and allylcysteine derivatives [53-55]. allylcysteine derivatives of Garlic also has the ability to inhibit enzymes involved in lipid synthesis, prevent lipid peroxidation of oxidized erythrocytes and LDL, increase antioxidant status [56]. Garlic stimulates the proliferation of lymphocytes and macrophage phagocytosis, induces the infiltration of macrophages and lymphocytes in transplanted tumors, induces splenic hypertrophy, stimulates and modulates cytokines production, and brings and enhances activities of the natural killer cell and the lymphokine-activated killer cell [57].

Conclusion

In conclusion, both adult and weaning rats intoxicated with LDPM and HDPM for three months had slightly similar responses, as they have significant reduction in AChE enzyme and reduction in defense system represented by GSH that induced significant induction in oxidative stress biomarker (MDA). Decrease in Humeral and cellular immunosuppression were also confirmed by histopathological examination in lymph nodes. On the other hand, simultaneous supplementation with alpha lipoic acid and garlic aqueous extract improved previous alternations slightly to be more or less near the control level in both adult and weaning.

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Disclosure of conflict of interest

None.

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