Egypt. Acad. J. Biolog. Sci., 10(1): 49- 61 (2018)

Egyptian Academic Journal of Biological Sciences
D. Histology & Histochemistry

2090 – 0775
www.eajbs.eg.net

Ultrastructural Effects of Chlorpyrifos and Phenthoate on the Midgut of *Chrysomya albiceps* Larvae (Diptera: Calliphoridae)

Khater, K.S
Department of Zoology, Faculty of Science, Zagazig University, Zagazig-44519, Sharkia, Egypt.
E.Mail.: k_s_kamel@hotmail.com

ARTICLE INFO
Article History
Received: 5/10/2018
Accepted: 22/11//2018

Keywords:
Chlorpyrifos, Phenthoate, Midgut, Ultrastructure, *Chrysomya albiceps*, Larvae

ABSTRACT

The Ultrastructural effects of chlorpyrifos and phenthoate on midgut of third larval instar of *Chrysomya albiceps* was illustrated with the aid of transmission electron microscope. It was clear that chlorpyrifos considered to be most potent than phenthoate. The number of pupae and emerged adults were reduced after treatments with all concentrations of the two insecticides. There was a predominance of males over female (ratio, 4.5:1), respectively after treatment with 15 ppm chlorpyrifos. Based on ultrastructural changes, after treatment with phenthoate, densely compressed cells with irregular shrank nucleus that has fragmented nucleoli and lacked condensed chromatin were observed in the midgut cells. The rough endoplasmic reticulum becomes swallowed. The microvilli clumped and shrunk. After treatment with chlorpyrifos, Numerous number of large vacuoles observed at the periphery of the midgut cells. The mitochondria show different degrees of deformation from clumping of cristae to rupture of mitochondrial membrane. The cells of midgut possessing short, shrunk or atrophied microvilli. Lysis of most cellular organelles is the most characteristic features after chlorpyrifos treatments.

INTRODUCTION

Chrysomya albiceps is a species belonging to the blowfly family, Calliphoridae (Queiroz, 1991& 1996; Povolny, 2002). It is of great medical and sanitary importance, being associated with cutaneous myiasis in both man and animals (Hall and Farkas, 2000; Verves, 2004). It causes economic damage to cattle breeding by causing primary myiasis (Queiroz, 1996). This blowfly species considered being an important vector in spreading of the carbuncle which is caused by *Bacillus anthracis* in South Africa (Braack and Retief, 1986). In Africa, it plays a significant role as a predator of other dipteran larvae. They may seriously alter the qualitative and quantitative species composition on a corpse (Grassberger et al., 2003). In addition to medical and veterinary importance, this species can be formative in forensic Entomology because it is the first insect to visit the carrion. Estimation of the post-mortem period achieved by examination of developmental stages of the fly on the corpse (Kotrba et al., 2012). Chlorpyrifos and phenthoate are belong to organophosphorus pesticides and they have low mammalian toxicity. Chlorpyrifos [0, O-diethyl 0-(3,5,6-trichloro-2-pyridyl) phosphorothioate] is a broad-spectrum organophosphorothioate insecticide which shows activity against many insects**.** It has contact and stomach action. It is used primarily to kill mosquito larvae. Phenthoate is

[S-alpha-ethoxycarbonylbenzyl O,O-methyl phosphoro dithioate], an organothiophosphate insecticide. It is used in controlling mosquitoes, blowflies and houseflies. Phenthoate is described as a cholinesterase inhibitor with contact and stomach action. It has low mammalian toxicity with neglectable residual activity. The strong pungent odor of phenthoate repelled adults and egg laying can be prevented.

MATERIALS AND METHODS

Insect Colony:

The colony of *Chrysomya albiceps* were maintained under laboratory conditions (25±2°C and 54-73 RH). The larvae were collected from rabbit carcasses used in the experiment. The larvae were reared in plastic jars (10.5x7 cm) containing approximately 50 gm fresh beef meat. About 50 larvae were kept in each jar to avoid competition. The pupae were transferred to new clean plastic jars containing wheat bran as a medium for pupation. The pupae were transferred to rearing cages (30x30x30 cm) for adult emergence. Emerging adults were supplied with 10% sucrose solution and offered fresh meat as a source of protein and medium for egg deposition.

Testing Technique:

Early third instar larvae of *C. albiceps* were exposed to five different concentrations of the two selected compounds under investigation 5, 10, 15, 20, 25 ppm for chlorpyrifos and 50, 100, 150, 200, 250 ppm for phenthoate. The procedures were replicated five times for each concentration. Twenty larvae were used for each replicate. Two milliliters from each concentration was added to 20 gm of meat in the glass jar (6x9 cm). In the control experiments, water only added to the meat. Experiments were carried out under laboratory conditions at 27±2° C, 80±5 %R H and 16:18 light: dark cycle (Smith et al., 2000). Larval mortality was recorded 24 h after treatments.

Histopathological and Ultrastructural Techniques:

The effect of median lethal concentrations of LC_{50} of the two selected compounds on midgut of larvae was investigated. The midgut specimens of both normal and treated larvae were placed in fresh 5% glutaraldehyde for 24 hours. The midgut was washed by phosphate buffer for 1 hour. The samples were then put for 1-2 hours in 1% osmium tetroxide. They were subjected to ascending series of ethanol to propylene oxide infiltration with acetone and embedding in Araldite carried out (Khattab et al., 2004). Semi-thin sections were stained with 0.25% toluidine blue (Davis, 1971) and examined using light microscopy. Ultrathin sections were then cut, stained with uranyl acetate and lead citrate and photographed in a JEOL 1200 EXIL Transmission Electron Microscope at the Electron Microscope Unit, Faculty of Pharmacy, Al Azhar University.

Statistical Analysis:

Average larval mortality was subjected to probit analysis for calculating LC50, LC90, LC95 and other statistics at 95% confidence limits of the upper confidence limit (U.C.L.) and lower confidence limit (L.C.L.), and chi-square values were calculated by using the software developed by Reddy et al. (1992). All experiments contained 5 replicates. The results were analyzed by one – way analysis of variance (ANOVA) using CoStat statistical software (cohort software, Berkeley). Means were compared by the LSD test ($P<0.05$).

RESULTS

Toxicological Studies:

The LC_{50}, LC_{90} and LC_{95} values resulted from treatment of third larval instar of *Chrysomya albiceps* with chlorpyrifos and phenthoate insecticides were reported in the table (1). The LC_{50} values were 18.4 and 110.7 ppm for chlorpyrifos and phenthoate, respectively. It was clear that chlorpyrifos considered to be most potent than phenthoate.

The number of pupae is reduced after treatment of third larval instar with the two selected compounds; it reaches 4.6±0.67 and 0.0 ±0.0 after treatment with 25 ppm chlorpyrifos and 250 ppm phenthoate, respectively compared with 19.8±0.2 in control (Table 2). The number of emerged adults reduced after treatments with all concentrations of both tested compounds and completely ceased following treatments of larvae with 200 and 250 ppm of phenthoate (Table 2). There was a predominance of males over females (ratio, 4.5:1), respectively after treatment with 15 ppm chlorpyrifos.

Table (1): LC_{50}, LC_{90}, LC_{95}, slope function and Chi-square of *Chrysomya albiceps* larvae after treatment with chlorpyrifos and phenthoate.

Compound	LC_{50} (L.C.L- U.C.L)	LC_{90} (L.C.L–U.C.L)	LC_{95} (L.C.L-U.C.L)	Slope ± SE (df = 4)	Chi (X^2) (F)
Chlorpyrifos	18.4 (16.5 -21.0)	42.9 (34.3 -61.9)	54.6 (41.6- 85.1)	3.4±0.3	6.7 (531.16)
Phenthoate	110.7 (79.7 – 139.6)	250.1 (220.5–419.4)	315.1 (288.2-585.0)	3.6±0.2	18.8 (109.07)

L.C.L, Lower confidence limits; U.C.L., Upper confidence limits; df, degree of freedom

Table (2): Effect of chlorpyrifos and phenthoate on number of pupae, pupation percentage, number of adults, adult emergence percentage, and sex ratio of *Chrysomya albiceps*.

Treatments	Concentrations (ppm)	Pupal stage		Adult stage		
		Number of pupae (Mean ± SE)	Pupation (%)	Number of emerged adults (Mean± SE)	Adult emergence (%)	Sex Ratio (M: F)
Chlorpyrifos	5	16.6±0.55^a	83	12.8±4.38^b	64	1.75:1
	10	11.2±0.48^b	56	9.0±1.22^b	45	1.5:1
	15	10.4±0.67^b	52	8.6±0.24^b	43	4.5: 1
	20	7.4±0.60^c	37	3.8±0.48^c	19	1.4: 1
	25	4.6±0.69^d	23	3.0±0.94^c	15	0.8: 1
$LSD_{0.05}$	-	1.64	-	2.18	-	-
Phenthoate	50	5.0±0.10^c	25	2.0±0.20^c	10	0.4: 1
	100	6.8±0.20^b	34	3.4±0.24^b	17	2.4: 1
	150	2.4±0.24^d	12	1.2±0.48^c	6	1: 1
	200	1.2±0.73^e	6	0.0±0.0^d	0	-
	250	0.0±0.0^e	0	0.0±0.0^e	0	-
$LSD_{0.05}$	-	1.16	-	0.87	-	-
Control	-	19.8±0.20^a	99^a	19.8±0.20^a	99^a	1.3:1

Each datum represents the mean of five replicates

Means within a column followed by the same superscript letter are not significantly different (LSD test, $P<0.05$), (p value= 0.000***).

Histopathological Studies:

The alimentary canal of *C. albicpes* larvae consists of foregut, midgut, and hindgut which strongly convoluted in the body cavity. The larval midgut is the longest and most efficient portion of the alimentary canal where digestion and absorption take place. The midgut consisted of a single layer of cuboidal epithelial cells rested on a basement membrane (Fig. 1a). The lumen of midgut contains peritrophic membrane enclosing food particles (Fig. 1a&2c). Based on ultrastructure, the midgut epithelium contains a slightly regular nucleus with clumped chromatin and has prominent nucleoli, (Fig.1a&d). The cuboidal cells have free microvilli, and numerous secretory granules (Fig. 1d). There are numerous fat globules scattered in the cytoplasm (Fig. 2a&b).The most abundant organelles are mitochondria both elongated and rounded types and small dense lysosomes that are scattered in between mitochondria (Fig. 1c,e&f).

The cytoplasm containing small elements of rough endoplasmic reticulum and the Golgi system observed as sacs parallel to each other (Fig. 1e & f). There is well-developed muscle layer under basement membrane indicate good feeding on protein diet (Fig. 1b&c).

The most characteristic changes in the midgut of third larval instar after 24h of feeding a treated diet with chlorpyrifos and phenthoate were recorded. After treatment with LC_{50} phenthoate dense compressed cells with an irregular shrunk nucleus that has fragmented nucleoli and no clumped chromatin. Vacuoles also appeared in the cytoplasm (Fig. 2d). Blebbing in the cell membrane (Fig.3a) and presence of phagolysosomes in the cytoplasm are diagnostic ultrastructural changes (Fig. 2f). The rough endoplasmic reticulum becomes swallowed (Fig. 3c). The microvilli show different degrees of deformations, swallowing (Fig. 3f), clumped and shrunk (Fig. 3d&e) and lysosomes are increased (Fig. 3b, c& f). Mitochondria show different degrees of deformation from the destruction of cristae, clumping of internal constituents and rupture of membrane (Fig. 3b &d) and lysis of the peritrophic membrane also observed (Fig.3e). Shrinkage of cytoplasm and accumulation of most cellular organelles, lack of condensed chromatin and deformed mitochondria are observed (Fig.2e). Following treatment with LC_{50} chlorpyrifos the cells have clear signs of apoptosis. There is a number of large vacuoles at the periphery of the cells (Fig.4c&d). Most cellular organelles were degenerated and disappeared (Fig.4a&b). The peritrophic membrane completely disappeared. The cells possessing short, shrunk or atrophied microvilli (Fig. 4c&d). Lysis of most cellular organelles and cells rupture is the most characteristic features after chlorpyrifos treatments (Fig. 4a, b, c& d).

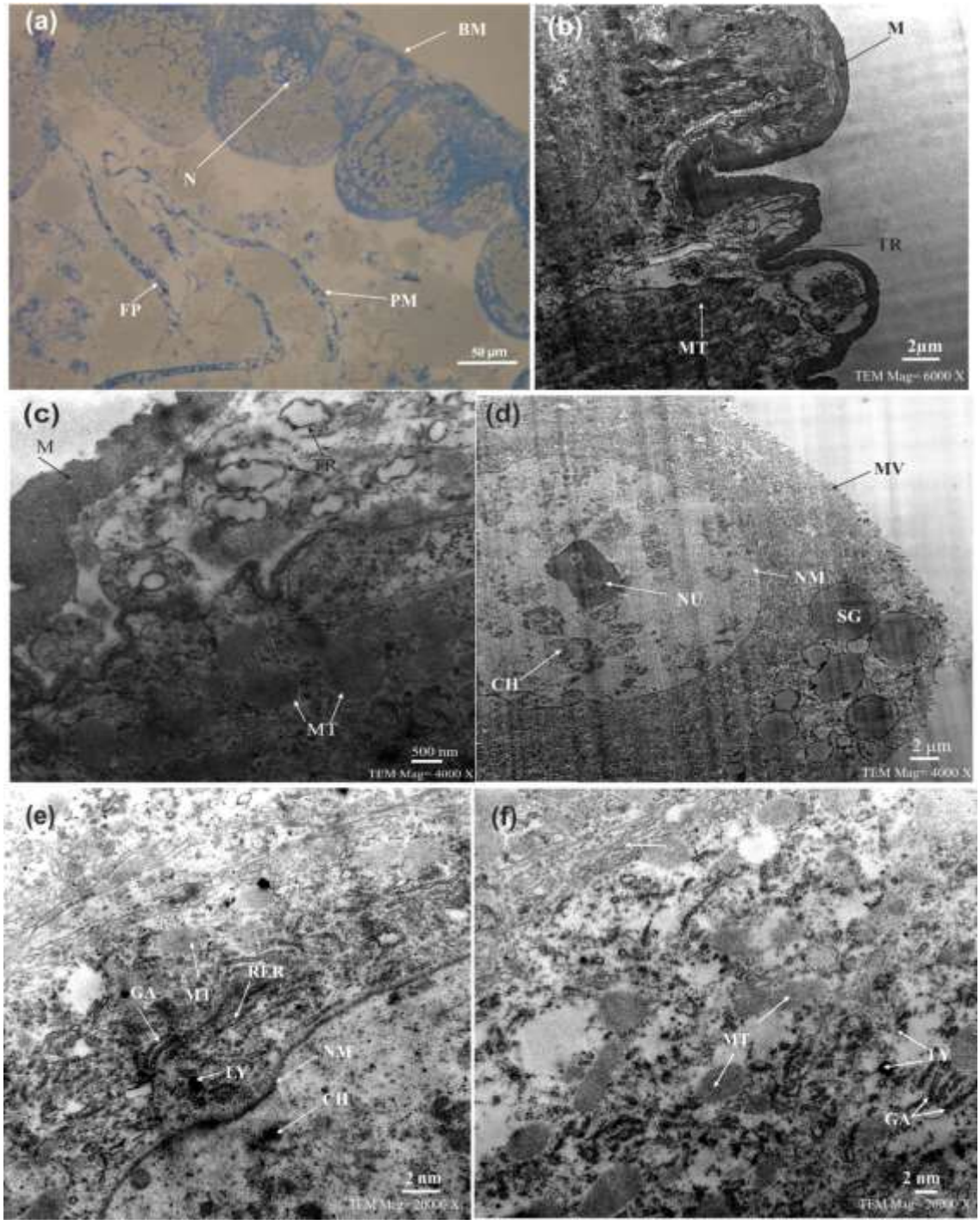

Fig. (1): a) Semi-thin section in the midgut of third larval instar of *Chrysomya albiceps* showing normal single layer of cuboidal cells rest on basement membrane, and peritrophic membrane containing food particles; b- f) TEM photograph in midgut of normal third larval instar of *Chrysomya albiceps* showing b) Muscle layer enclosing the epithelial cells and numerous mitochondria and trachea. C) Higher magnification of b; d) Epithelial cell has rounded nucleus with chromatin and prominent nucleolus, the cytoplasm has numerous secretory granules and microvilli at free border; e) Nucleus with double nuclear membrane, rough endoplasmic reticulum with ribosomes, Golgi apparatus and lysosomes; f) Elongated and rounded mitochondria, Golgi apparatus and lysosomes. (BM, basement membrane; CH, chromatin; FP, food particles; GA, Golgi apparatus; LY, lysosomes; M, muscles; MV, microvilli; N, nucleus; NM, nuclear membrane; NU, nucleolus; PM, peritrophic membrane; TR, trachea).

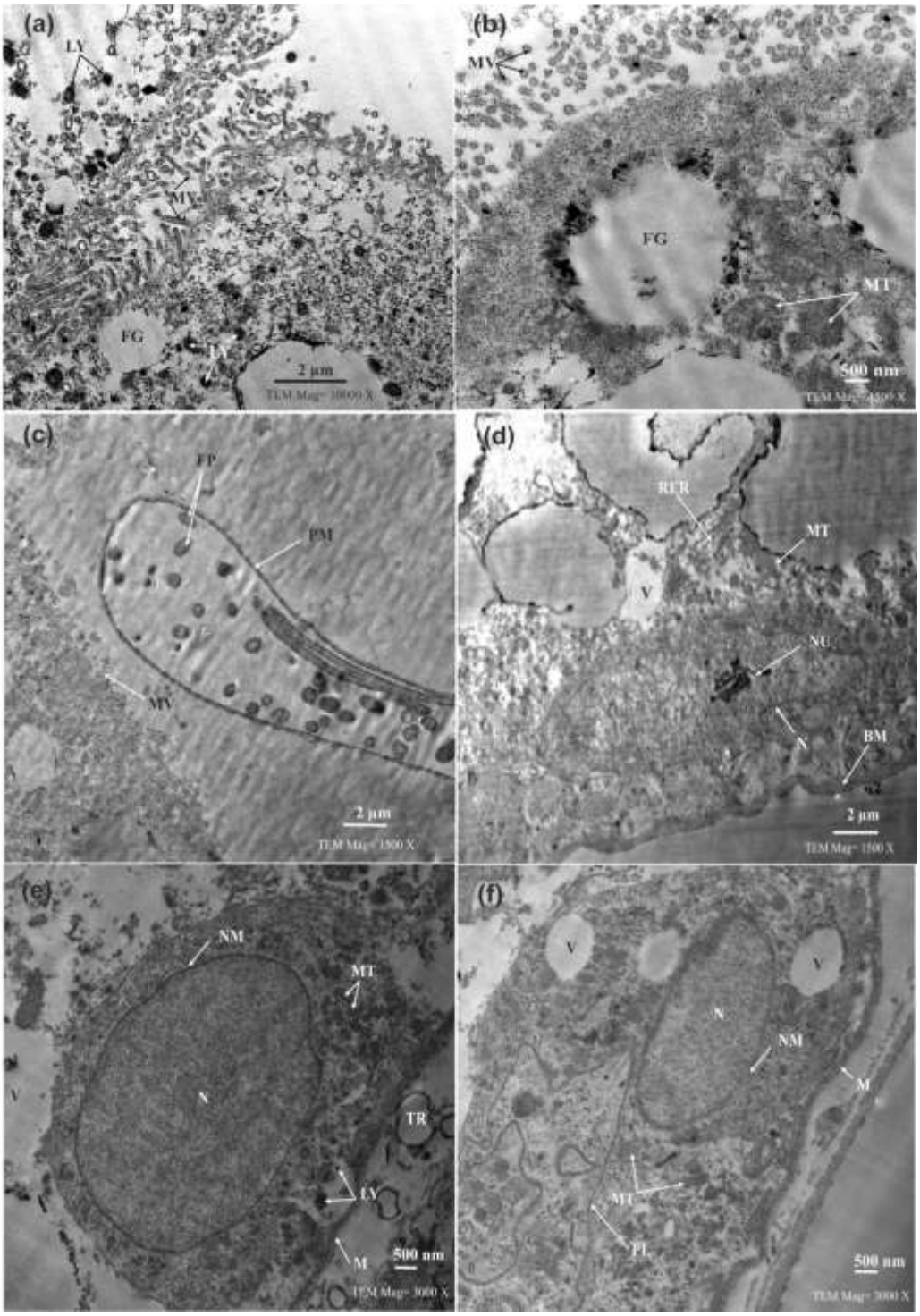

Fig. (2): a-c) TEM photograph of normal midgut cell showing, a) Free border with numerous elongated microvilli, fat vacuoles, and lysosomes; b) Mitochondria concentrated at the periphery of the cell in between the fat globule ; c) Double wall peritrophic membrane containing food particles; d-f)TEM photograph of midgut cell after treatment with LC_{50} of phenthoate showing; d) Irregular nucleus, fragmented nucleolus and lacked condensed chromatin and appearance of vacuole in cytoplasm; e) Nucleus lacked condensed chromatin, shrinkage of the cytoplasm and accumulations of most cellular organelles; f) Shrunken nucleus with lacked condensed chromatin, vacuoles in the cytoplasm and presence of phagolysosomes. (BM, basement membrane; FG, fat globule; FP, food particles; LY, lysosomes; M, muscles; MT, mitochondria; MV, microvilli; N, nucleus; NM, nuclear membrane; NU, nucleolus; PL, phagolysosome; PM, peritrophic membrane; RER, rough endoplasmic reticulum ,TR, trachea; vacuole).

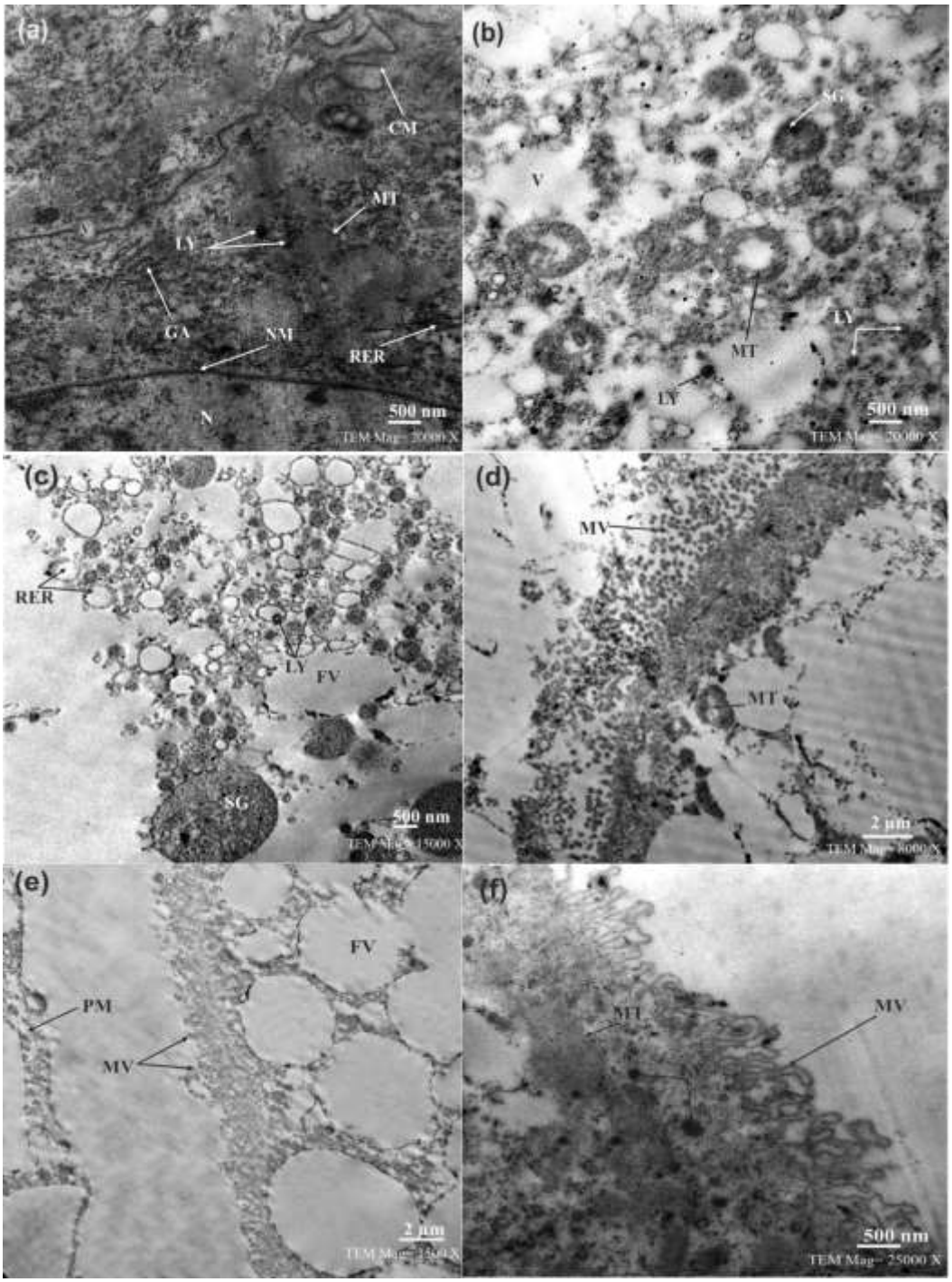

Fig. (3): a-f) TEM photograph of midgut cell after treatment with LC_{50} of phenthoate showing, a) Blebbing of cell membrane, deformation in Golgi sacs and lysosomes; b) Deformation in mitochondria and numerous secretary granules and lysosomes; c) Swallowed endoplasmic reticulum and increased number of lysosomes; d) accumulation of vacuoles at the periphery and the microvilli become clumped; e) Destructed peritrophic membrane and clumped microvilli; f) Shrunken microvilli and numerous lysosomes between mitochondria. (CM, cell membrane; FV, fat vacuole; GA, Golgi apparatus; LY, lysosomes; MT, mitochondria, MV, microvilli; N, nucleus; NM, nuclear membrane us; PM, peritrophic membrane, RER, rough endoplasmic reticulum; SG, secretory granules; V, vacuole).

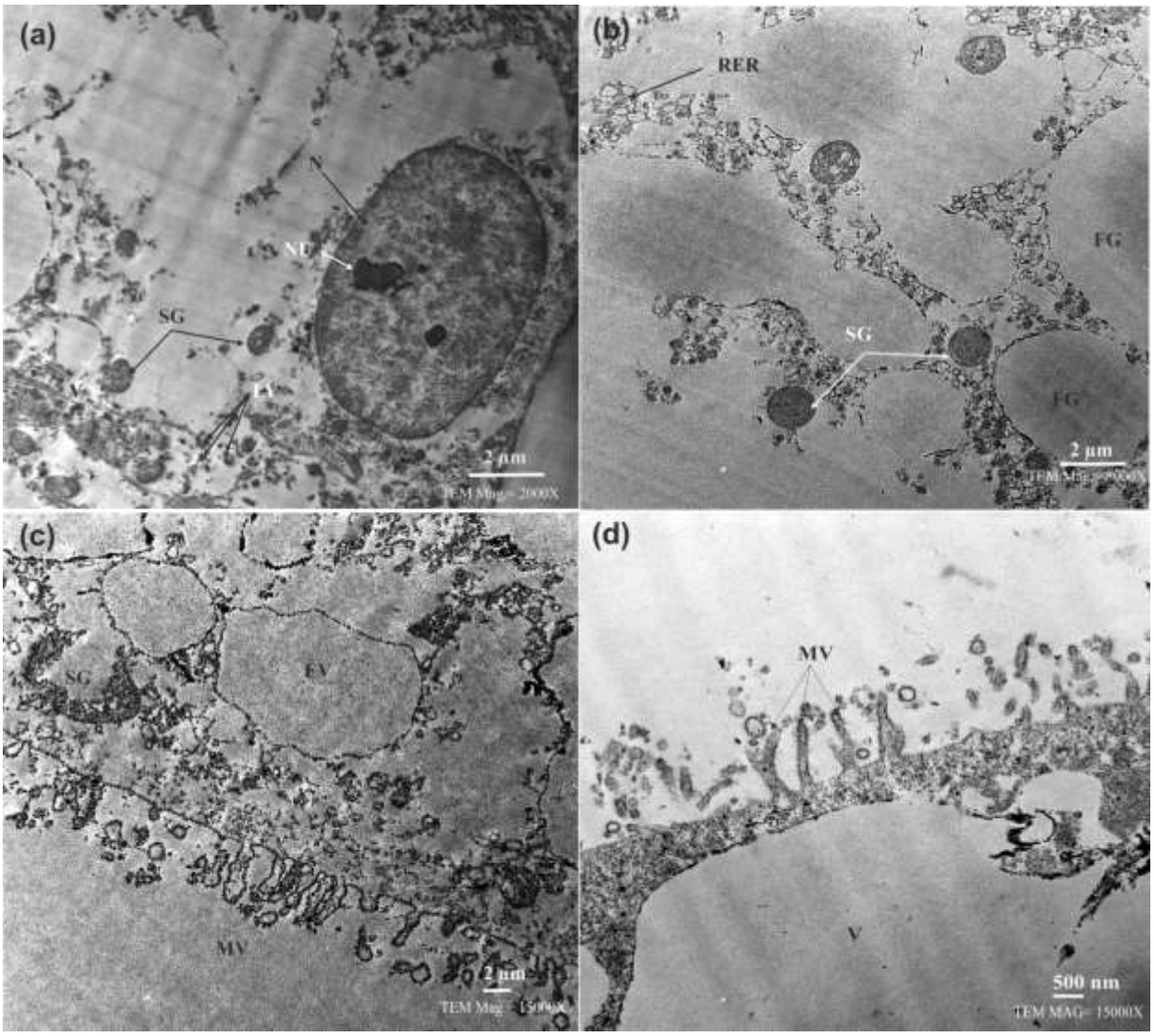

Fig. (4): a-d) TEM photograph of midgut cell after treatment with LC_{50} of chlorpyrifos showing, a) Nucleus with fragmented nucleolus and lysis of most cellular organelles; b) large ruptured fat vacuole and swallowed endoplasmic reticulum; c) swallowed and shrunken microvilli and ruptured secretary granule; d) Higher magnification of figure c showing swallowed microvilli. (FV, fat vacuole; MV, microvilli; N, nucleus; NU, nucleolus; RER, rough endoplasmic reticulum; V, vacuole).

DISCUSSION

It was clear that, chlorpyrifos considered to be more effective against *c. albiceps* larvae than phenthoate. High significant reduction in the number of pupae after treatment of third larval instar of *c. albiceps* with the two selected compounds was observed. The number of emerged adults is greatly reduced following treatments with all concentrations and completely ceased following treatments of larvae with 200 and 250 ppm of phenthoate. There was a predominance of males over females (ratio, 4.5:1) after treatment with 15 ppm chlorpyrifos.

Previous studies investigated the effect of organophosphorus and other compounds on different insect species. Dahm (1971) reported greater toxicity to house fly by a diisopropyl homologue of parathion than the honey bee. Abd Rashid et al. (2008) reported that, the larvae of *C. megacephala* show retarded development after treatment with malathion. Liu et al. (2009) studied the effect of malathion on *C. megacephala* fed on meat treated with malathion. They found that, treatment cause delaying effect on development compared with control. Yan-Wei et al. (2010) indicated that treatment of *C. megacephala* with malathion prolong larval and pupal duration. Ahmed and Vogel (2015) investigated the effect of some insecticides against *Aedes aegypti*. They added that, diafenthiuron, diflubenzuron, novaluron, and Lufenuron were the least toxic while Chlorfenapyr insecticide shows the highest toxicity against fourth instar larvae. Askari- Saryazdi et al. (2015) observed high toxicity on second instar larvae of *Liriomyza sativa* after treatment with chlorpyrifos. Asid et al. (2017) suggested that, diazinon and pirimiphos-methyl when mixed together showed higher toxicity to house fly than when applied separately. Glavan et al. (2018) elucidated that the toxic effect of diazinon on the honey bee is related to the effect on acetylcholine esterase. Zhao et al. (2018) said that, treatment of *Bradysia odoriphaga* by the median lethal concentration of chlorfenapyer reduce the population parameters. They added that consumption of food by larvae and pupal weight were significantly decreased compared with control.

The midgut of third larval instar of *C. albiceps* is the most important part of the digestive system where digestion and absorption of nutrients take place as in another insect (Dow, 1986). The midgut tissue is lined with simple cuboidal epithelium which are possessed microvilli and has numerous secretory granules. A peritrophic membrane is located within the lumen of the midgut (Boonsriwong et al., 2007). The microvilli increased surface area for efficient nutrients absorption (Chapman, 1998). This structure observed in many insect species, Secundino et al. (2005) in sand fly, *Lutzomyia longipalpis* and *Anopheles darlingi* (Okuda et al., 2005).

The basement membrane facilitates the transport of materials between midgut and hemolymph (Houk et al., 1980; Taha et al., 2010 and Baker et al., 2012). Secretory granules are the most prominent structure of the midgut cells near the free border, they are present with large numbers, suggests a secretory role of these cells (Baker et al., 2012). The cytoplasm of the midgut cells of *C. albiceps* has numerous vesicles of rough endoplasmic reticulum, these structures are concentrated around the nucleus. This may indicate the synthesis of proteases for protein digestion as recorded by Staubli et al.

(1966) and Taha et al. (2010). A large number of secretory granules and vesicles of rough endoplasmic reticulum may be responsible for peritrophic membrane production (Filimonova, 2005). Mitochondria are prominent in the apical pole of the cells than the basal region, which illustrate the transport role of the apical pole as described by (Hecker, 1977; Houk, 1977 and Baker et al., 2012).

The most pronounced ultrastructural effects of LC50 of phenthoate and chlorpyrifos on midgut cells of *C. albiceps* were irregular shrunk nucleus that has fragmented nucleoli. The rough endoplasmic reticulum becomes swallowed. The cells possessing short, shrunk or atrophied microvilli. Lysis of most cellular components is the most characteristic features after chlorpyrifos treatments. These observations are in accordance with those finding previously reported. Dahi et al. (2011) stated that, epithelial cells completely ruptured after treatment of *Spodoptera littoralis* with pyriadlyl and the peritrophic membrane lack the property of lying the epithelial cells of the midgut. Distortion in the epithelial cells of the midgut in *Mythimna separata* after treatment with fraxinellone (Lu et al., 2010). Baker et al. (2012) mentioned that, larvae of *C. megacephala* fed on malathion treated diet have damaged midgut columnar cells and have atrophid microvilli. *Periplaneta amricana* showed enlargement in midgut epithelium and reduction in the lumen of midgut following treatment with N-nitroso- N-methylurea (Jain and Ahi, 2014). Yasmeen and Amir (2016) reported degeneration and distortion in the shape of midgut epithelial cells of *C. megacephala* following treatment with deltamethrin. The results obtained by Abdelsalam et al. (2016) showed that, the epithelial cells of the midgut of males of *Rhynchophorus ferrugineus* that were fed with spinosad treated diet reveal signs of apoptosis, including vacuolization.

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ARABIC SUMMERY

التأثيرات الفائقة الدقة للكلوربيريفوس والفنثوات علي المعي الوسطي ليرقات كريزوميا البيسيبس(ثنائية الأجنحة: كاليفوريدي)

كريمة خاطر

قسم علم الحيوان – كلية العلوم – جامعة الزقازيق – الزقازيق٤٤١٥٩ –الشرقية –مصر

يتناول البحث التأثيرات التركيبية الفائقة الدقة لكلوربيريفوس وفنثوات علي العمر اليرقي الثالث لذبابة الكريسوميا البيسيبس و ذلك بواسطة الميكرسكوب الالكتروني النافذ. واتضح ان الكلوربيريفوس أكثر فعالية من الفنثوات فقد قل عدد العذاري وخروج الطور البالغ بعد المعاملة بجميع التركيزات للمبيدين ووجدت غلبة لنسبة الذكورعلي الاناث (٥و٤ : ١) على التوالي بعد المعاملة بتركيز١٥جزء في المليون من الكلوربيريفوس.

وبدراسة الي التغيرات التركيبية الفائقة الدقة في خلايا المعي الوسطي بعد المعاملة بالفنثوات فقد أظهرت الخلايا كل من وجود أنوية متقلصة وغيرمنتظمة الشكل ونويات مجزئة وكروماتين غير متجمع وانتفاخ الشبكة الاندوبلازمية الخشنة واخيرا وجود الفجوات وضمور الزغبيات وقصرها. وبعد المعاملة بالكلوربيريفوس رصد ظهور عدد كبير من الفجوات في أطراف الخلايا بينما أظهرت الميتوكوندريا درجات مختلفة من التشوهات من تجلط الزوائد الداخلية علاوة على تقلص وقصرالزغيبات. واجمالا فان تحلل معظم مكونات الخلية هي الصفة المميزة بعد المعاملة بالكلوربيريفوس.