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# Histological abnormalities in the alimentary canal and Malpighian tubules of *Culex* sp. mosquitoes larvae treated with zinc oxide nanoparticles

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**ABSTRACT**

Culicid mosquitoes pose a threat to human and animals due to their capability of disease transmission. In this study, the impact of zinc oxide nanoparticles (ZnONPs) was addressed in the larval stages of *Culex* mosquito 3 days post treatment. Furthermore, the treated larvae were allowed to recover from the particle-effect for additional 3 and 6 days to address whether the particle impact is sustainable or temporary. To this end, a sub lethal concentration (LC30) of ZnONPs was used to address their impact on the tissues of the alimentary canal and Malpighian tubules of *Culex* larvae. The treated larvae suffered negative signs on foregut, midgut , hindgut and Malpighian tubule epithelia which appeared in the form of columnar cell vaculation and damage of the microvilli. In the meantime, the larvae which allowed recovering for 3 and 6 days didn't show signs of improvement in the alimentary canal and Malpighian tubules suggesting sustainable impact of the particles on the larval tissues. Future work will be directed toward ultra-structural studies in the gut cells. This study suggests that ZnONPs can be used as promising tools in controlling mosquito larvae.

## INTRODUCTION

Mosquitoes are one of the best-known insect vectors responsible for a wide range of infectious diseases [1]. Many species belonging to genera *Anopheles*, *Aedes* and *Culex*, are the mechanical and biological vectors for the pathogens of various diseases that affect both animals and humans [2-6]. Culicids have 44 genera and 3500 species with medical importance [7]. They play an important role in the transmission of parasites and pathogens of high public health concerns including malaria, dengue, filariasis, yellow fever, Japanese encephalitis, West Nile and Zika virus [8-9]. Control of these diseases

depends on the understanding of the transmission mechanisms of the disease, which are generally complex because of the indirect mode of transmission [10].

Protection against mosquito borne diseases can be obtained by controlling mosquito populations rather than targeting the parasite or the causative agent itself. Controlling the population of mosquito vectors is one of the best strategies for combating such vector-borne diseases. The control of immature stages now appears to be the most powerful means of reducing target populations of Culicidae. Mosquitoes management mainly was depending on the application of several chemical pesticides such as; organochlorines, organophosphates, carbamates, Dichloro Diphenyl Trichloroethane's (DDT) and others, especially to control their increased resistance and residue contamination of human foods and toxicity and pollution of the environment [11-15]. Some of the mentioned pesticides are still using while other are banned by WHO. The increased use of chemical control methods has led to the selection of physiological, behavioral, and biochemical resistance mechanisms leading to a crucial need to alternative new insecticides for controlling mosquitoes. The suspected alternatives should be more environmentally safe , biodegradable and minimizing resistance development.

Nanotechnology concerns with the development of the synthesis of nanoparticles (NPs) of different sizes, shapes and controlled disparity with size range of less than 100nm [16]. Most of known nanoparticles share unique characteristics as they are non-hazardous, biodegradable, having greater surface to volume ratio, cost effective and biocompatible [17- 18]. Metal oxide nanoparticles are most commonly used in catalysis, sensors, environmental remediation and personal care products [19-20]. Metal oxide nanoparticles have different significant application, such as anti-microbial, cell line studies and dye degradation properties [21].

The objective of this study was to addressing the effect of zinc oxide nanoparticles (ZnO NPs) on the histological appearance of the alimentary canal and Malpighian tubules tissues in *Culex* larvae. Additionally, It was needed to understand whether the particle effect on the gut histology is reversible or not which was analyzed by allowing *Culex* larvae to recover from particle-effect and readdressing the histological characteristics.

## MATERIALS AND METHODS

- **Insect rearing**

The eggs of *Culex* mosquito were obtained from a laboratory culture reared in the laboratory of Entomology, Assiut University and allowed to hatch under controlled laboratory conditions of temperature, humidity and photoperiod (28 ± 2◦C, 70–85%,14:10 h (light/dark)), respectively. Male mosquitoes were fed on 10% of sucrose solution while females fed on a rat blood meal. The larvae were maintained in dechlorinated tap water and were fed with artificial diet containing 17% protein.

- **Chemicals and experimental design**

Extra pure ZnONPs (10-30 nm) in this research were purchased from Sky Spring Nanomaterial (Texas, USA). Complete characterization of the used nanoparticle is available at the website of manufacturer (http://www.ssnano.com). First instar *Culex* larvae were treated with ZnONPs for 3 days and transferred to fresh container containing fresh artificial diet and allowed to recover for additional 3 and 6 days. Samples were fixed in 70% ethanol to be used in histological examinations.

The toxicity values of ZnONPs were calculated based on an assay using different concentrations of ZnONPs (Ibrahim et al., unpublished data). Based on the data of Probit analysis the LC30 of ZnONPs against the first instar of *Culex* larvae was calculated as (0.24355 g/L). Aqueous suspension of ZnONPs was prepared by suspending 0.024355 gram in 100ml distilled water and used as the rearing volume of larvae.

- **Histological observations**

Histological changes in the gut epithelial cells of both control and treated larvae were observed. Larvae that were alive after 3 days of nanoparticle treatment at LC30 and recovered for 3 and 6 days were collected for examination. The larvae were rinsed with distilled water before fixation with 70% ethanol for 1h, followed by dehydration in graded ethanol series cleared with methyl benzoate. Larvae were embedded in paraffin, then sectioned at 5 µm with a microtome and stained with Hematoxylin and Eosin before the examination and photographing using light microscope.

## RESULTS

**1.1.** ZnONPs induce chronic histological implications in the digestive canal of *Culex* larvae:

**1.1.1.** Histological changes in the foregut

The normal digestive canal of mosquitoes larvae is divided into 3 regions, the fore gut (gastric caeca), midgut (anterior and posterior) and hindgut (Fig. 1).

The gastric caeca (foregut) which are lined by tall and large cuboidal cells containing heterogeneous cytoplasm with basophilic granules. The apex is slightly stained acidophilic with curved boundary and regular microvilli. The nuclei is vesicular , rounded and centrally located (Fig. 2a). In larvae treated for 3 days, the gastric caeca

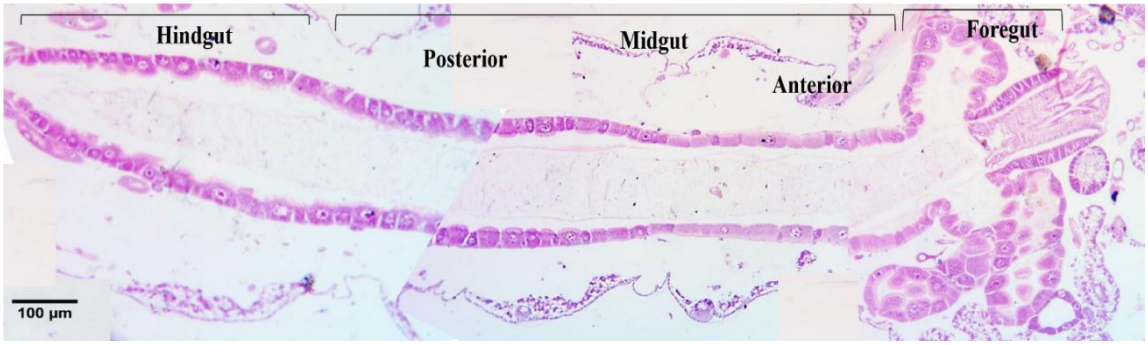


**Figure 1.** Longitudinal section of the normal alimentary canal of a *Culex* mosquito larva. The alimentary canal is divided into foregut, midgut (anterior and posterior) and the hindgut. This micrograph is prepared by merging 3 different images.

were suffering shrinkage with small cubic cells deeply stained and having granule-containing basophilic cytoplasm and well clear acidophilic brush borders (Fig. 2b). Our results showed that cells of the gastric caeca recovered for 3 days suffered shrinkage and appeared highly vacuolated specially at their basal region. Additionally, they showed aggregation of cytoplasmic materials at their apical border and flat nuclei (Fig. 2c). Gastric caeca in larvae recovered for 6 days showed degeneration in lining cells , with basal nuclei (Fig. 2d).

**1.1.2.** Histological changes in the midgut

The anterior mid gut is lined by flattened and regenerative cells. The flattened cells have homogenous acidophilic cytoplasm containing tiny granules with open phase nuclei located in the center of cells and well observed brush borders. Tall regenerative cells lie between the flat cells with deeply stained basophilic cytoplasm (Fig. 3a,e).

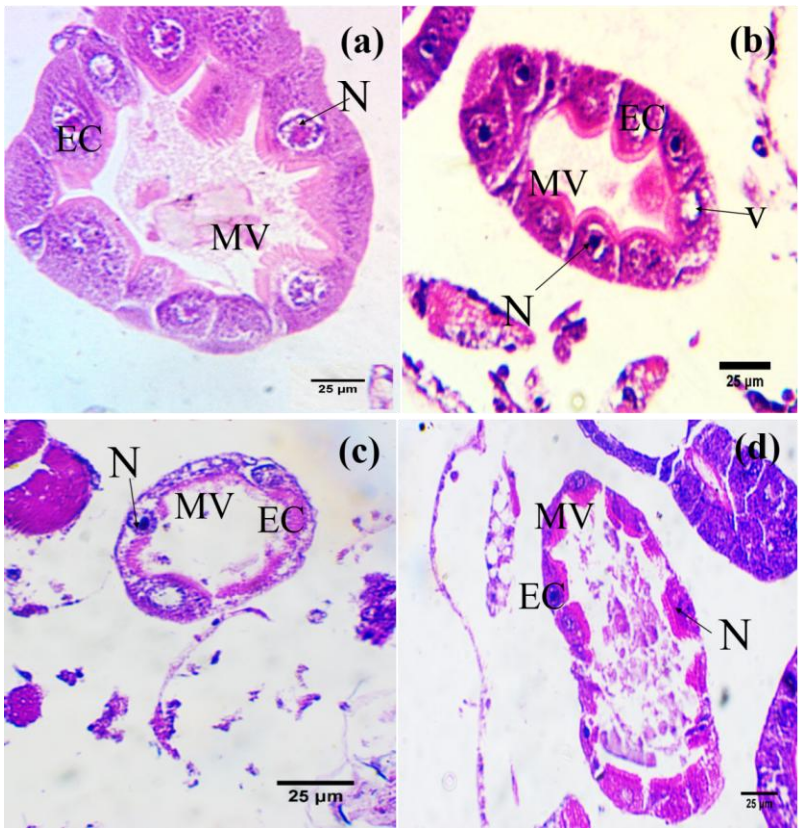


**Figure 2.** Transverse sections of gastric caeca of control *Culex* larvae (a), larvae treated with sublethal concentration (LC30) of ZnONPs for 3 days (b), larvae subjected to recovery from nanoparticle effect for 3 days (c) and larvae recovered 6 days (d). EC, epithelial cell; N, nucleus; MV, microvilli; V, vacuoles.

In treated larvae, the anterior midgut epithelial cells contained heterogeneous basophilic cytoplasm and numerous vacuoles and the nuclei appeared slightly elongated (Fig. 3b,f). Three days recovery period negatively affected the anterior midgut showing shrinkage of the lining epithelium with heterogeneous acidophilic cytoplasm and tiny vacuoles (Fig. 3c,g). After 6 days recovery period, it showed fine ruminant of the basement membrane , acidophilic cytoplasm with shrunk and deeply stained nuclei (pyknotic) (Fig. 3d,h).

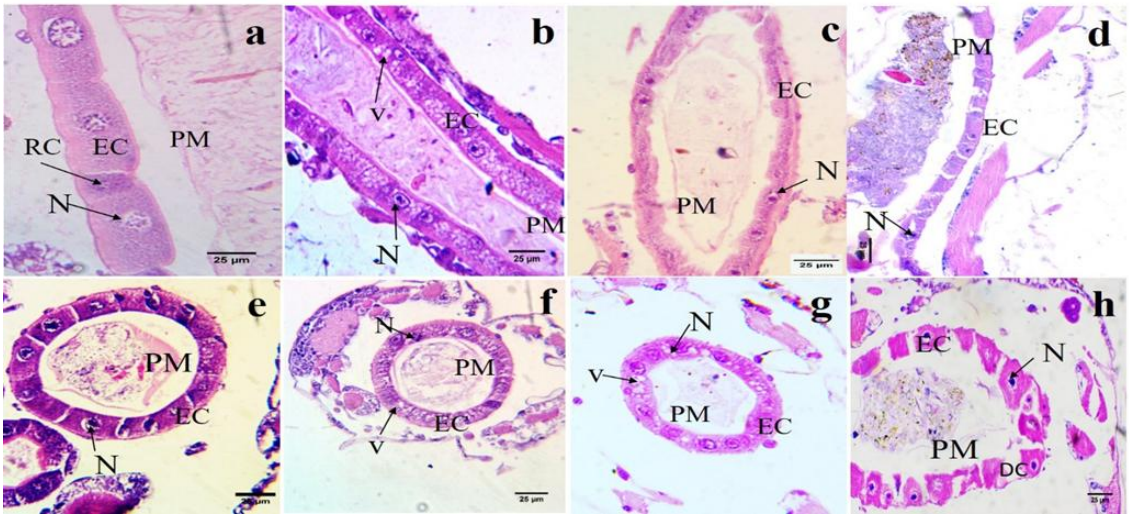


**Figure 3.** Longitudinal sections in anterior midgut of control *Culex* larvae (a), larvae treated with sublethal concentration (LC30) of ZnONPs for 3 days (b), treated larvae recovered for 3 days (c) and treated larvae recovered for 6 days (d). Transverse sections in anterior midgut of control *Culex* larvae (e), treated for 3 days (f), recovered for 3 days (g) and recovered for 6 days (h). EC, epithelial cell; RC, regenerative cell; MT, Malpighian tubules; PM, peritrophic membrane; N, nucleus; MV, microvilli; DC, degenerated cell; V, vacuoles.

The posterior midgut was characterized by two types of cells, tall cuboidal and regenerative cells, which rest on a basal membrane. Tall cells contains deeply stained heterogeneous cytoplasm with rounded vesicular nuclei located in the center of cells with thick acidophilic stained brush border. The regenerative cells lie in the cleft between tall cells (Fig.4a,e). In treated larvae (3 days), the posterior midgut showed atrophy in the epithelial lining with deeply stained cytoplasm and degeneration in the nuclei (Fig. 4b,f). The posterior midgut did not show any signs of improvement either after 3 or 6 days of recovery where it appeared suffering atrophy in the epithelial lining with deeply stained acidophilic cytoplasm containing large vacuole, loss of microvilli with shrunk nuclei (Fig. 4c, g,d,h).

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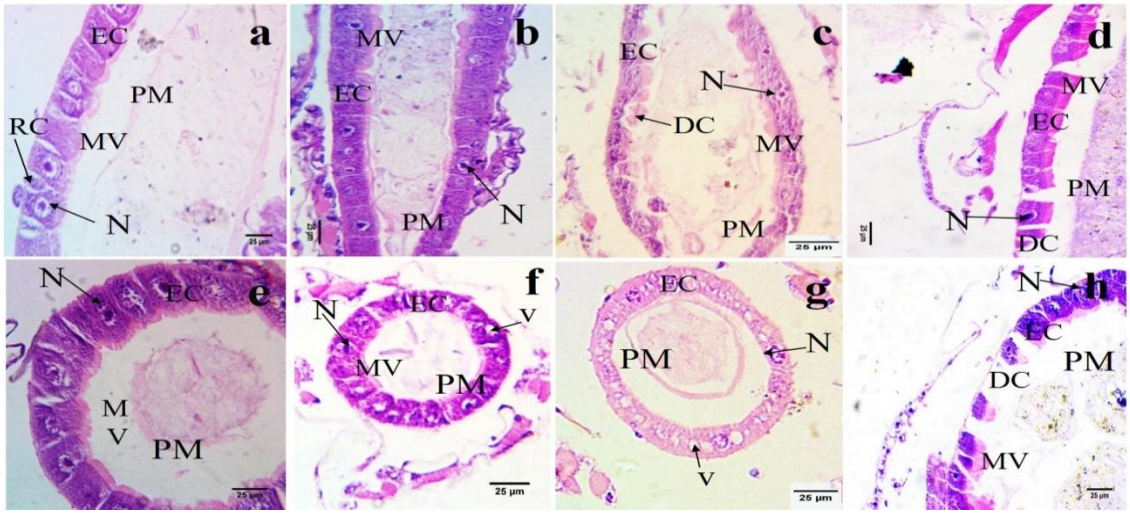


**Figure 4.** Longitudinal sections in the posterior midgut of control *Culex* larvae (a), larvae treated with LC30 of ZnONPs for 3 days (b), recovered for 3 days (c) and recovered 6 days (d). Transverse sections in posterior midgut of control larvae (e), treated for 3 days (f), recovered for 3 days (g) and recovered for 6 days (h). EC, epithelial cell; RC, regenerative cell; MT, Malpighian tubules; PM, peritrophic membrane; N, nucleus; MV, microvilli; DC, degenerated cell; V, vacuoles.

### **1.1.3.** Histological changes in the hindgut

The hind gut presents a single layer of columnar and regenerative epithelial cells which rest on a basement membrane. The columnar cells have acidophilic cytoplasm with fine granules and their curved border contains apocrine cells with rounded , vesicular nuclei. Pyramidal shape regenerative cells were located in the fissure between columnar cells (Fig. 5a,e). In treated larvae, the hindgut cells contained deeply stained cytoplasm with basophilic granules and clear brush border. Furthermore, many cells were degenerated and detached (Fig. 5b,f). Hindgut cells recovery period did not show different signs from larvae treated with ZnONPs for 3 days where hindgut cells contained deeply stained cytoplasm with basophilic granules and clear brush border. Furthermore, many cells were degenerated and detached (Fig. 5c,g,d, h).

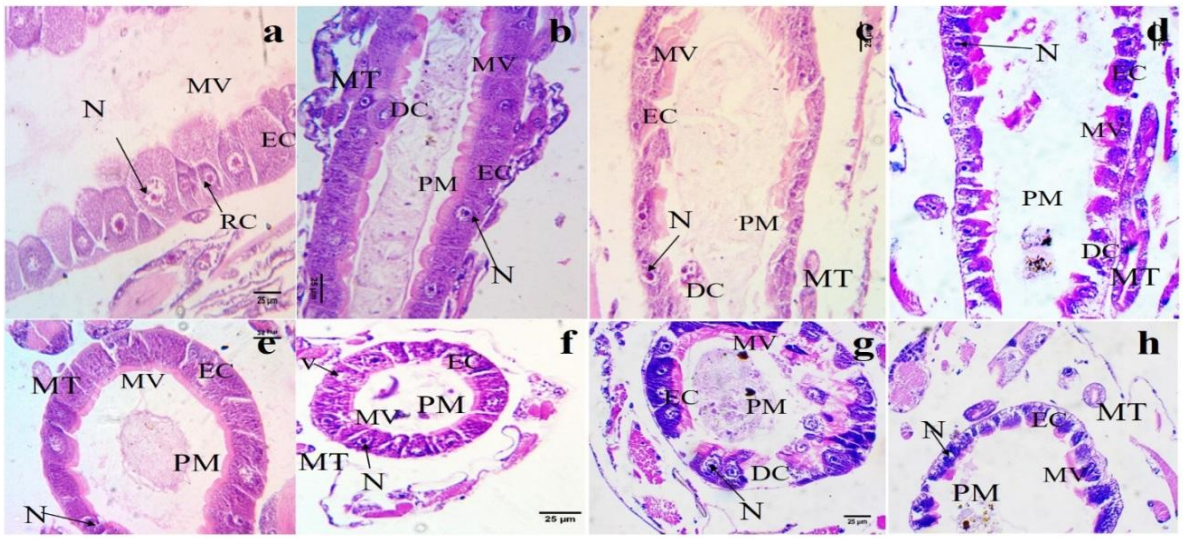


**Figure 5.** Longitudinal sections in hindgut of control *Culex* larvae (a), larvae treated with LC30 of ZnONPs for 3 days (b), larvae recovered from NPs for 3 days (c) and recovered for 6 days (d). Transverse sections in hindgut of control larvae (e), treated for 3 days (f), recovered for 3 days (g) and recovered for 6 days (h). EC, epithelial cell; RC, regenerative cell; MT, Malpighian tubules; PM, peritrophic membrane; N, nucleus; MV, microvilli; DC, degenerated cell; V, vacuoles.

### **1.2.** ZnONPs target Malpighian tubules of *Culex* larvae:

Malpighian tubules of the control larvae showed large epithelial cells resting on a basement membrane with fine basophilic granules and open phase nuclei (Fig.6a). Malpighian tubules treated with LC30 of ZnONPs showed narrowing of tubules and shrinkage of cells with vacuoles and vesicular nuclei were noticed (Fig. 6b). on the other hand, Malpighian tubules showed amelioration in cell shapes with large, rounded and deeply stained nuclei (Fig. 6c,d).

## DISCUSSION

In recent years, nanoscience has been grown in different fields including agricultural research and medicine. The unique characteristics of such materials at the nanoscale opened new line to study their effects on insects either as potential management agents or to analyze their possible negative effects on the ecosystem [22]. Current research is directed toward application of nanoparticles in management programs especially after

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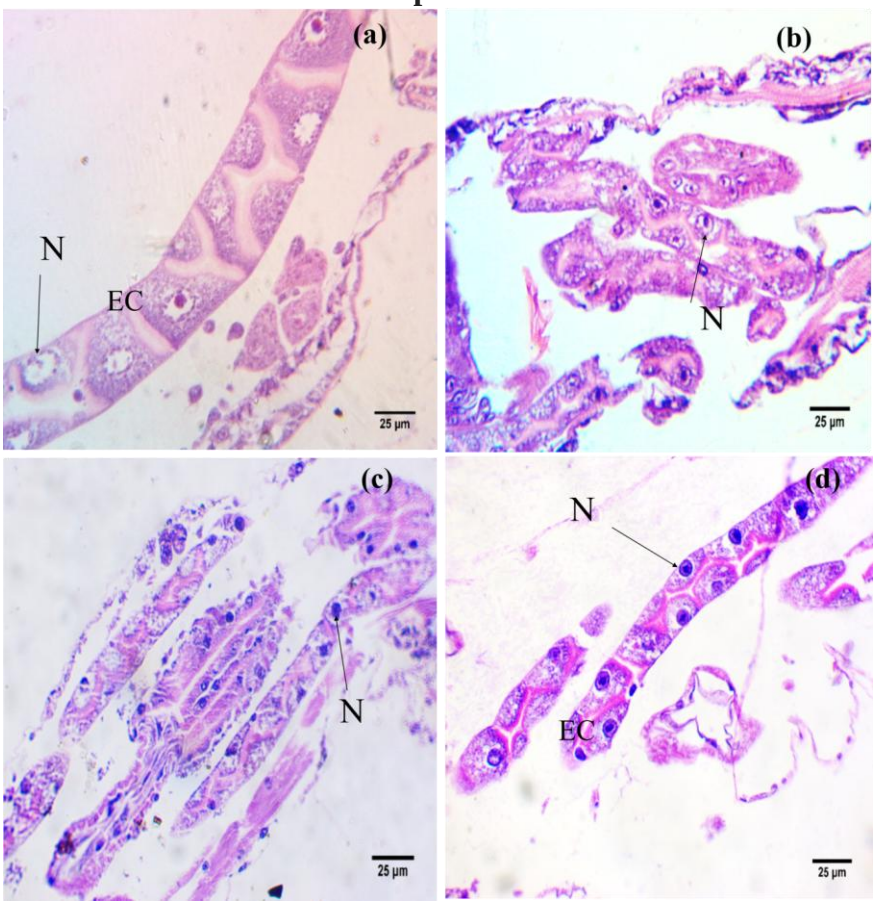


**Figure 6.** Longitudinal sections in the Malpighian tubules of: Control *Culex* larvae (a), larvae treated with LC30 of ZnONPs for 3 days (b), larvae recovered from ZnONPs effect for 3 days (c) and larvae recovered for 6 days (d). EC, epithelial cell; N, nucleus.

emergence of eco-friendly methods for preparation of nanostructures such as the green synthesis methods. To this end, larvae were treated with NPs and allowed to recover from its effect. The recovery period was initially tested to analyze the chronic effects of nanoparticles on the larval tissues of *Culex* mosquito.

The complete description of mosquito alimentary canal and Malpighian tubules is performed in many genera [23- 26]. Our results indicated *Culex* larvae share similar histological appearance with other genera of mosquito.

Treatment of *Culex* mosquito with ZnO nanoparticles induced several forms of histopathological signs along the alimentary canal and Malpighian tubules. These signs

appeared in the form of vaculation in the cytoplasm, shrinkage of the cells and appearance of granules in the cytoplasm. Generally, treatment with insecticidal compounds affect the tissue structure in insects. For instance, the essential oils of garlic and lemon induced several histological abnormalities in the midgut of the cotton leaf worm [27]. Heavy metals also cause histological abnormalities in some ground beetles [28]. In both cases (essential oils and heavy metals), the structural changes were in the form of vacuolization of the epithelial layer, clumping of the nuclear chromatin and degeneration of the peritrophic membrane. Nanoparticles were observed to reflect similar symptoms in insects where nickel oxide nanoparticles caused appearance of vaculation, dense vesicle and lytic areas in the midgut of the ground beetle *Blaps polychresta* [29]. Furthermore, there was a report highlighting the histopathological signs in mosquito larvae treated with nanoparticles [30]. The abnormalities appeared mostly in the midgut epithelia, muscles and adipose tissue.

Previous reports addressed in detail the effect of pesticides and nanoparticles on the anterior parts of the alimentary canal (gastric caeca and midgut). Therefore, it was needed to give some attention to the posterior portions of the canal including the Malpighian tubules and the hind gut. The Malpighian tubules and hindgut in insects are considered as the homologous structures to the renal excretory tissues of other animals; they keep hemolymph water and solute homeostasis and contribute to detoxifying metabolic wastes and xenobiotics in the insect hemolymph [31]. Malpighian tubules also were reported to suffer negative cytotoxic effects in insects treated with insecticides [32]. However, the current study is the first to highlight the histological abnormalities in Malpighian tubules in insects treated with nanoparticles.

This study revealed irreversible effect of ZnONPs on the tissues of *Culex* larvae. In other words, larvae were unable to avoid the negative impacts of ZnONPs. The ability of insects to recover from the insecticides is a negative characteristic in the insecticide as it means that the activity of the compound is guaranteed only by multiple application times. Ideal pesticides should have a unique characteristic of being applied into the environment at a single application. Our results on the insecticidal activity of ZnONPs showed

promising effects even at low doses (Ibrahim et al., unpublished data). This activity is supported by the current histological effects.

Mosquito control programs are suffering key challenges, such as the current and previous outbreaks of novel mosquito borne viruses and the development of pesticide resistance in several genera [33]. Several forms of nanoparticles were reported to be used against phytophagous insects and mosquitoes [34,35]. Interestingly, progress in the fabrication methods of nanostructures is running in very fast rate. Production of cost effective, ecofriendly and environmentally safe nanostructures is currently achieved by redox reactions of metal salts by plant-derived extracts [30]. These cheap methods allow mass production of such pesticides help in using these chemicals in pest management.

## CONCLUSION

The current study clearly shows that nanoparticles of ZnO are promising tools in the control of mosquito larvae. This fact is supported by the clear impact of ZnONPs on the histological abnormalities of the whole alimentary canal and Malpighian tubules in treated larvae when compared to control larvae. Interestingly, larvae allowed to recover from the negative effect of ZnONPs did not show any signs of improvement either in the parts of the alimentary canal or Malpighian tubules suggesting irreversible damage in the larval tissues.

## ACKNOWLEDGEMENT

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