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Impact of Ag NPs and $AgNO_3$ on the histopathological studies of land slug (*Lehmannia nyctelia*) inhabiting Assiut city, Egypt

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ABSTRACT

It is known that silver nanoparticles (Ag NPs) and silver nitrate (AgNO3) have harmful effects on the surrounding animals, which may cause several damages to these animals, whether harmful or beneficial. The aim of the present study is to detect damage caused by Ag NPs and AgNO3 to land slugs *Lehmannia nyctelia*. In this study, land slugs have been exposed to different concentrations (0.04, 0.08, 0.4, 0.8 g/L) of Ag NPs and AgNO3 for 15 days. Histopathological examinations were performed in each of the following (digestive gland, kidney and gonads). The digestive gland appeared several alterations after exposed to different concentrations (0.04, 0.08, 0.4, 0.8 g/L). A clear change in the shape of the cells was noticed where, cells became more flattened and many cells were damaged. The cavity of each tubule increased in size and filled with some remains of damaged cells and the cells became empty from inside. The histological study of the kidney revealed a deterioration in some cells, widening of the spaces between tubules and an increased in connective tissue size than the normal size. The gonads showed clusters of cells in some places, spermatozoa lost the normal shape of the tail. In conclusion the results indicated that, the Ag NPs and AgNO3 can cause damage in histological structure of internal organs of the slug and may cause harmful effects on the terrestrial organisms.

INTRODUCTION

In the past decades, nanotechnology has the use of nanoparticles (ENP) in a wide variety of fields. Metal nanoparticles (NPs) it was a source of interest for many environmental studies concerned with the study of toxicology since the early 2000s.

Accumulated Nanomaterials in the soil through various activities such as sewage, atmospheric sedimentation, agrochemicals, and soil remediation[1]. Soil has been indicated pollutants [2].

The global productivity of Ag NPs ranges between 500 ton/year and 1000 ton/year [3, 4]. Ag NPs are used as an antimicrobial component in a lot of commercial products such

as paints, cosmetics, clothing, electrical appliances, food packaging, and biomedical materials [5-7]. This widespread use of Ag NPs has raised concerns about their environmental impact [8].

Due to the increased use of Ag NPs, it may be released into the environment, and this leads to the accumulation of Ag NPs in the soil, and it is known that it is harmful to the invertebrates that live in the soil. [9, 10]. Ag NPs after entering the environment, they undergo to physical, chemical, and biological transformations that change their physicochemical properties, which in turn affect their toxicity [11-13].

The toxicity mechanism of Ag NPs is the induction oxidative stress which damage components of cells [14, 15]. Negative effects of Ag NPs were shown in earthworms [15-18].

Gastropods are known to be efficient mineral scavengers and pollution-sensitive models; therefore, they are widely used as indicators of environmental pollution in soil [19, 20].

Land snails and slugs have a high capacity to accumulate heavy metals and are considered important species for monitoring the bioavailability of metal constituents in soil compared to other invertebrates [21].

The digestive gland is the main site for the storage of minerals in *Lehmannia nyctelia* [22, 23], and it plays a vital role in the detoxification process [24]. The epithelium of the digestive glands of land slugs consists of three differentiated cell types: digestive (the most common), excretory, and calcium cells [25]. Additionally, the digestive gland of terrestrial slugs living in metal polluted areas or who were exposed to metals during laboratory experiments had a high relative number of calcium cells and calcium cell hypertrophy [26, 27].

The aim of the present investigation was the studying histopathological changes of (digestive gland, kidney and gonads) of the land slugs after exposure to Ag NPs and AgNO3.

MATERIALS AND METHODS

2.1. Chemicals

Silver nitrate (Ag NO_3; purity 99.8% and Mw 169.873) in the form of a colorless crystalline was obtained from Sigma-Aldrich (USA).

Silver Nanoparticles (Ag-NPs) were powder with spherical-shaped particles (purity 99.9%, Mw 107.87 and Average Particle Size <100 nm size) obtained from Sigma-Aldrich (USA). Charactrisation of silver nanoparticles (Unpublished data)

2.2. Sample collection

Specimens of *Lehmannia nyctelia* (about 180 samples) (with a weight of 1 ± 0.4 g and a length of 1.5 ± 0.5 cm) were harvested during autumn at the Assiut University Farm, Assiut Governorate, Egypt, and transported to the Ecology Laboratory at Assiut University. The slugs were kept under normal laboratory conditions, kept in plastic containers with soil from their natur.

2.3. Experimental design

The slugs were randomly divided into nine groups (one control group and eight groups treated). Each group consisted of 20 samples and kept in plastic containers containing a mass of soil (30 g). Each container was covered with a perforated cloth for ventilation.

The control group was fed fresh lettuce impressed in 10 mL of distilled water. On the other hand, the treated groups were fed fresh lettuce impressed in various concentrations of Ag NPs and AgNO3 mixed with distilled water.

The first group was the control group, and the second, third, fourth, and fifth groups were exposed to 0.04, 0.08, 0.4, and 0.8g/L Ag NPs, respectively, for 15 days. The sixth, seventh, eighth, and ninth groups were exposed to 0.04, 0.08, 0.4, and 0.8g/L AgNO3, respectively, for 15 days. We used the alive slugs for histological studies at the end of the experiment, and we removed and counted the dead ones.

2.4. Histopathological change

At the end of the experiment, whole slug bodies were collected and immediately fixed in 70% alcohol. Fixed slugs were processed in the usual way for embedding in paraffin. Embedded tissues were sectioned 5-7 µm thick and then stained with hematoxylin and eosin (H and E). Sections were examined and examined using a BX50F4 OLYMPUS microscope (Olympus Optical Co., LTP, Japan).

RESULTS

3. Histopathological change (hematoxylin and eosin stain)

3.1. Kidney

3.1.1. Control groups

Kidney of control groups showed extensive folds. Each tubule lined by simple columnar epithelium which contain basal nuclei and faintly stained cytoplasm. There are two types of cells, the first type has apical vacuole and the second type without vacuole Fig.1 (A) and Fig.2 (A)

3.1.2. Treatments exposure to Ag NPs

1. Concentration of (0.04 g/ L Ag NPs)

Section of kidney showed increased connective tissues and shortening of columnar cells in some places. Vacuoles increased in size and the nuclei irregular in distribution Fig.1 (B).

2- Concentration of (0.08 g/ L Ag NPs)

Appearance of pyknotic nucleus, the vacuoles of the cells became larger in size inside the cells. Fig.1 (C).

3. Concentration of (0.4 g/ L Ag NPs)

Section of Slug kidney showed that cells boundaries became not clear. The nucleus lysis and all cells became vacuoles. Decreased the space between the kidney tubules, epithelial cells lost their normal shapes Fig.1 (D).

4- Concentration of (0.8 g/ L Ag NPs)

Showed some destroyed in the apical of some cells, and the cells became fused together Fig.1 (E).

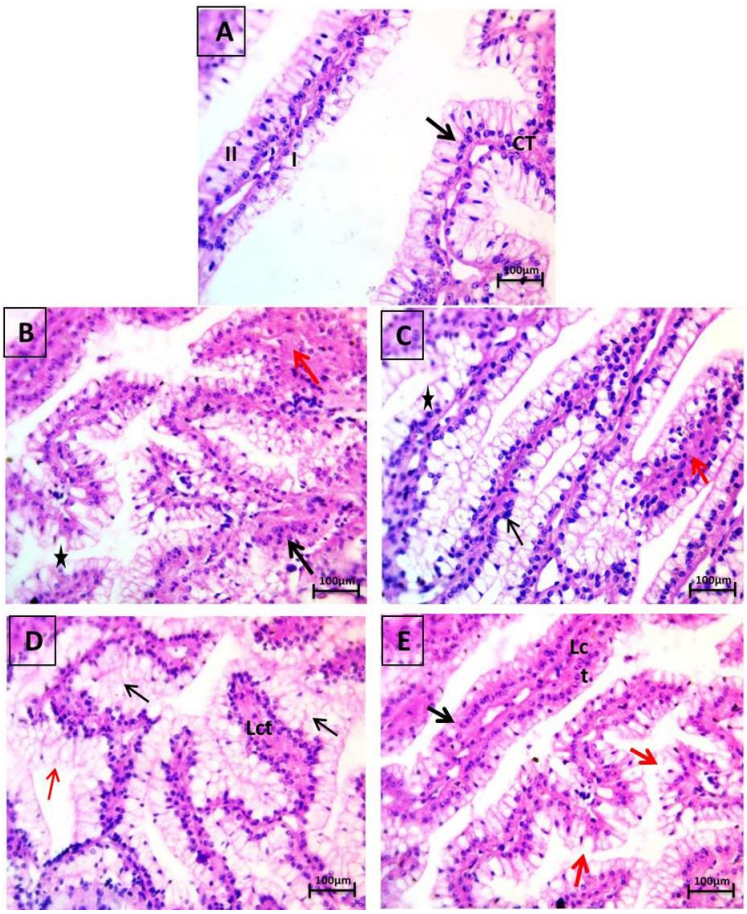

Fig. (1) Photomicrograph of kidney of Slug *Lehmannia nyctelia* after exposure to Ag NPs: (A); Control, two types of cells: (I) vacuole, (II) non vacuole, (arrows) basal nucleus and (CT) connective tissue. (B); 0.04 g/L, (arrow) irregular nuclei and (red arrow) thickened connective tissue, (stare) lysis some apical rim. (C); 0.08 g/L, (stares) large vacuole, (arrow) pyknotic nuclei, (red arrow) thickening connective tissue. (D); 0.4 g/L, (red arrow) lysis apical rim (arrows) lysis cell boundary, (Lct) large connective tissue. (E); 0.8 g/L, (red arrows) lysis apical rim, (arrow) lysis cell boundary and large connective tissue (Lct) (H &E stain, scale bar 100 µm).

3.1.3. Treatments exposure to silver nitrate

1. Concentration of (0.04 g/ L of $AgNO_3$)

Complete degenerated cells with complete fusion of epithelial cells and increased of intertubular space between cells. Increased the size of connective and many pyknosed nuclei were appeared with irregular distribution Fig.2 (B).

2. Concentration of (0.08 g/ L of $AgNO_3$)

Large vacuoles were appeared inside the cells. In some regions were observed as aggregated of pyknotic nuclei and in some places observed multiplication of cells Fig.2 (C).

3. Concentration of (0.4 g/ L of $AgNO_3$)

Showed shorting of some cells and more increased of intertubular space, degenerated of apical border of cells with lysis to nuclei. Complete fusion of epithelial was observed Fig.2 (D).

4. Concentration of (0.8 g/ L of $AgNO_3$)

Slug kidney showed fibrosis in some places between the tubules and complete damage of cells and nucleus Fig.2 (E).

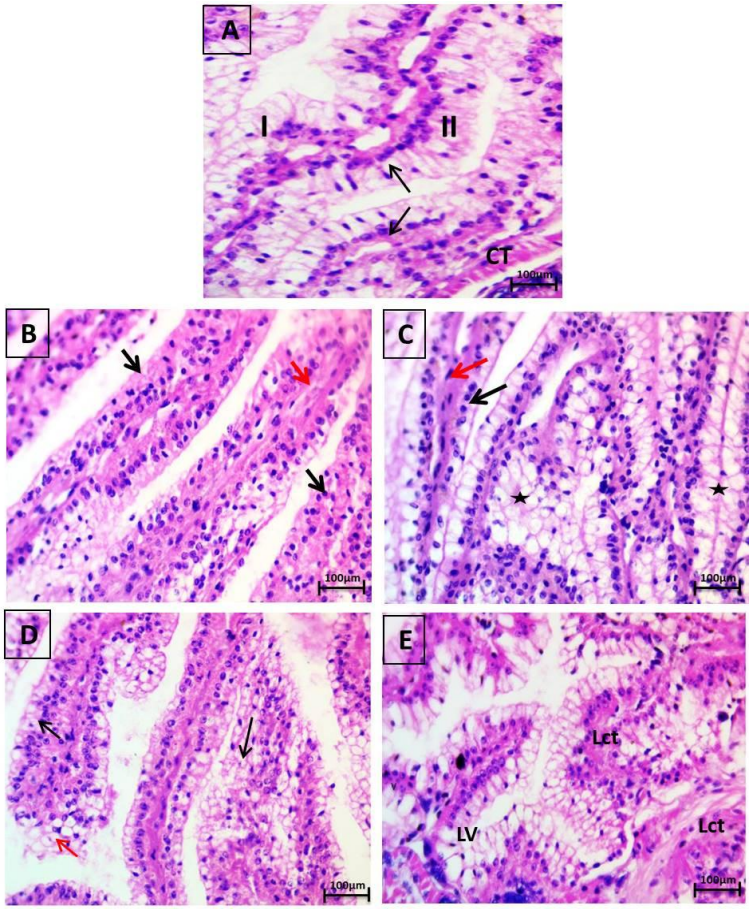

Fig. (2) Photomicrograph of kidney of Slug *Lehmannia nyctelia* after exposure to Silver Nitrate: (A); Control, two types of cells: (I) vacuole, (II) non vacuole, (arrows) basal nucleus and (CT) connective tissue. (B); 0.04 g/L, (arrows) irregular nucleus, (red arrow) large connective tissue. (C); 0.08 g/L, (arrow) pyknotic nucleus, (red arrow) connective tissue and (stars) large vacuoles. (D); 0.4 g/L, (arrows) destroyed cell, and (red arrow) apical rim. (E); 0.8 g/L, (LV) large vacuoles and (LCT) large connective tissue (H &E stain, scale bar 100 µm).

3.2. Gonads Section of Slug (*Lehmannia nyctelia*)

3.2.1. Control groups

In control gonads observed stages of both spermatogenesis and oogenesis, mature oocyte and sperms (head and tails) Fig.3 (A) and Fig.4 (A)

3.2.2. Treatments exposure to Silver Nanoparticles

1. Concentration of (0.04 g/ L of Ag NPs)

Showed thickening of simple squamous epithelium surrounding gonads tubules and unclear head of sperm appeared Fig.3 (B).

2. Concentration of (0.08 g/ L of Ag NPs)

Observed shorting tail and lysis of some head sperms and nuclei, more thickened epithelium surrounding the gonade tubule. Degenerated of stages of gonades Fig.3 (C).

3. Concentration of (0.4 g/ L of Ag NPs.

Some filtrations were appeared. Irregular and complete degeneration of stages with deeply stained nucleus. Increased the size of ova and some taken different size. Sperm head pointed like with deeply stained Fig.3 (D).

4. Concentration of (0.8 g/ L of Ag NPs)

Showed of degeneration of epithelia cells lining gonadal tubules and loss of germinative epithelium. accumulation of gonad stages with pyknosed nucleus. Unclear and degenerated stages were noticed. Lysis of sperm head and tails Fig.3 (E).

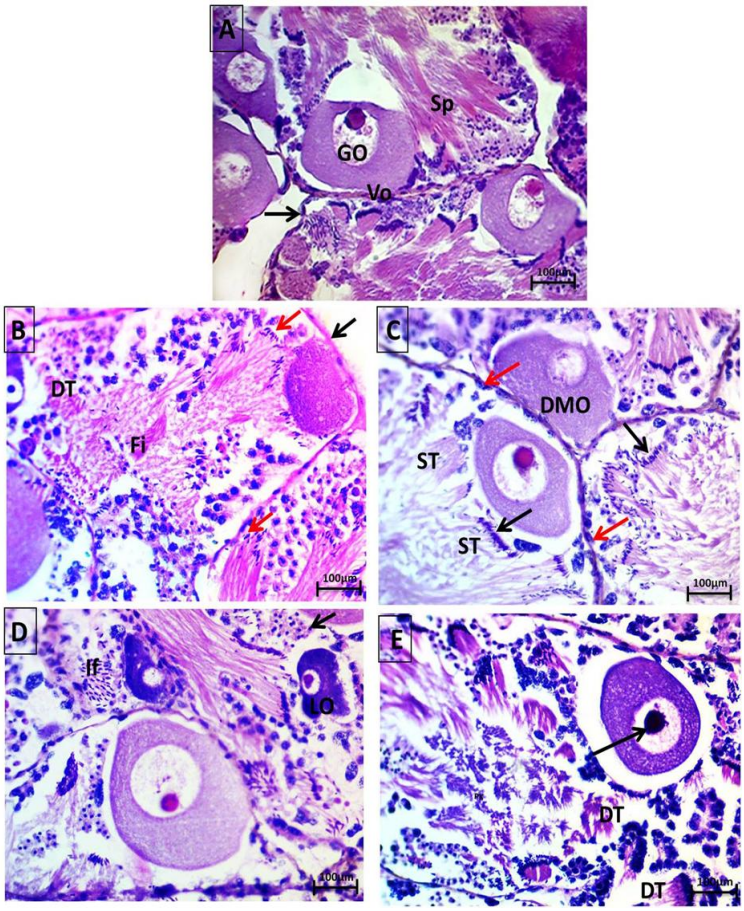

Fig. (3) Photomicrograph of gonads of Slug *Lehmannia nyctelia* after exposure to Ag NPs: (A); Control, (Sp) sperm, (Go) growing oocyte, (Vo) vitelline oocyte, (arrow) simple squamous epithelium. (B); 0.04 g/L, (arrow) thickness of simple squamous epithelium, (red arrows) unclear head, (Fi) fibrosis, (DT) degenerated tails. (C); 0.08 g/L, (DMO) Degenerated mature ova, (ST) short tail, (arrows) lysis sperm head, (red arrows) thickness of simple squamous epithelium. (D); 0.4 g/L, infiltration (If), (arrow) degeneration stage of gonad, (LO) lysis of oocyte. (E); 0.8 g/L, (arrow) pyknosed nucleus, (DT) degenerated tails (H &E stain, scale bar 100 µm).

3.2.3. Treatments exposure to silver nitrate

1. Concentration of (0.04 g/ L of $AgNO_3$)

Showed unclear stage of gonads, damage of simple squamous epithelium and confuse of nuclei. groups of sperm with pointed head and groups of aggregated head were noticed with shorted tails Fig.4 (B).

2. Concentration of (0.08 g/ L of $AgNO_3$)

Stages of developing cells with deeply stained nuclei and complete lysis Fig.4 (C).

3. Concentration of (0.4 g/ L of $AgNO_3$)

Aggregated of stages of gonads, noticed variation of ova size. Short tails of sperm and some pykonsed nucleus. Fig.4 (D).

4. Concentration of (0.8 g/ L of $AgNO_3$)

Increased different size of mature oocyte with pyknosed nucleus. Degenerated of stages of gonads Fig.4 (E).

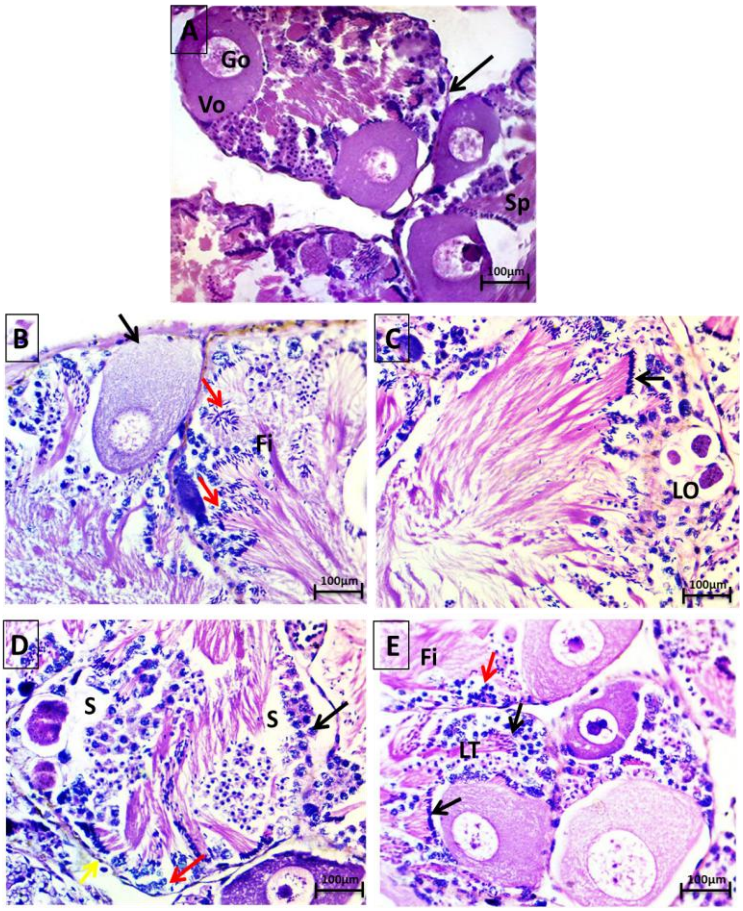

Fig. (4) Photomicrograph of gonads of Slug *Lehmannia nyctelia* after exposure to Silver Nitrate: (A); Control, (Sp) sperm, (Go) growing oocyte, (Vo) vitelline oocyte, (arrow) simple squamous epithelium. (B); 0.04 g/L, (arrow) damage of simple squamous epithelium, (Fi) fibrosis, (red arrows) degenerated sperm head. (C); 0.08 g/L (arrow) degenerated sperm head, (LO) lysis of oogonia. (D); 0.4 g/L, (arrow) pykonsed nucleus, (red arrow) degenerated gonad stage, (S) space, (yellow arrow) degenerated of epithelial. (E); 0.8 g/L, (LT) lysis tail of sperm, (arrows) lysis of head, (Fi) fibrosis, (red arrow) pyknosed nucleus (H &E stain, scale bar 100 µm).

3.3. Digestive gland

3.3.1. Control groups

Digestive gland of slugs surrounded by epithelia cell and connective tissue between the tubules, It has lumen and four types of cells, (I) digestive cells with columnar or elongated cells with rounded apical, (II) Excretory cells takes different shapes rounded or

void with central vacuole which containing yellow excretory granules, (III) Pyramidal shape calcium cell and (IV) thin cell Fig.5 (A) and Fig.6 (A)

3.3.2. Treatments exposure to Silver Nanoparticles

1. Concentration of (0.04 g/ L of Ag NPs)

Digestive gland of slugs showed deformation of tubules. Showed large lumen, most cells became cuboidal in shape. Increased of excretory and calcium cells and destroyed of connective tissue Fig.5 (B).

2. Concentration of (0.08 g/ L of Ag NPs)

Showed deformation and difficult to distingue between different cell types. Appeared spaces and fragment cells in the lumen Fig.5 (C).

3. Concentration of (0.4 g/ L of Ag NPs)

Degeneration of tubule basement membrane, appeared large spaces, many aggregated fragment cells Fig.5 (D).

4. Concentration of (0.8 g/ L of Ag NPs)

Sections of the digestive gland of slugs showed fibrosis and large space. Complete degeneration to the digestive tubule. Fig.5 (E).

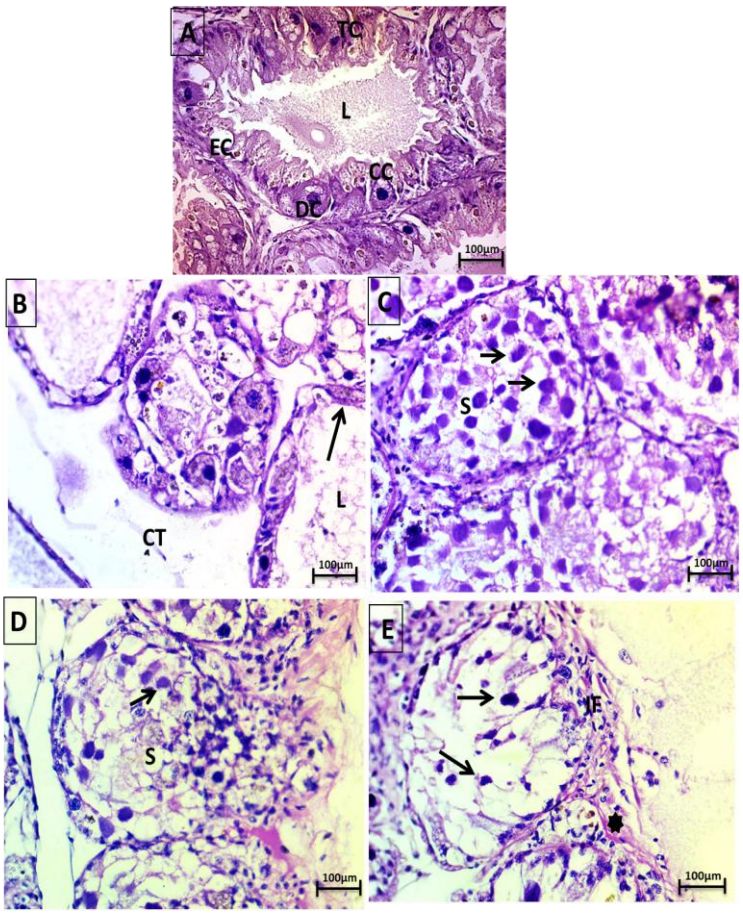

Fig. (5) Photomicrograph of digestive gland of Slug *Lehmannia nyctelia* after exposure to Ag NPs: (A); Control, (DC) digestive cell, (CC) calcium cell, (EC) excretory cell, (TC) thin cell and (L) lumen. (B); 0.04 g/L, (L) lumen, (arrow) cuboidal cell and (CT) connective tissue. (C); 0.08 g/L, (arrows) fragment cell, (S) space. (D); 0.4 g/L, (S) space, and (arrow) fragment cell. (E); 0.8 g/L, (arrows) fragment cell, (IF) infiltrations, (star) fibrosis (H &E stain, scale bar 100 µm).

3.3.3. Treatments exposure to silver nitrate

1. Concentration of (0.04 g/ L of AgNO3)

The digestive gland of slugs showed thickened of basement membrane of tubule and the cells became lysis and empty. Fig.6 (B).

2. Concentration of (0.08 g/ L of AgNO3)

Digestive gland showed infiltrations and fibrosis. Empty cells Fig.6 (C).

3. Concentration of (0.4 g/ L of AgNO3)

Showed aggregates of some digestive cells contain pyknosed nuclei and deeply stained nucleus. Whole cells lost their contained. Fig.6 (D).

4. Concentration of (0.8g/ L of AgNO3)

Whole cells deformed and lysis, appeared large space. fragment cells in the lumen. Fig.6 (E).

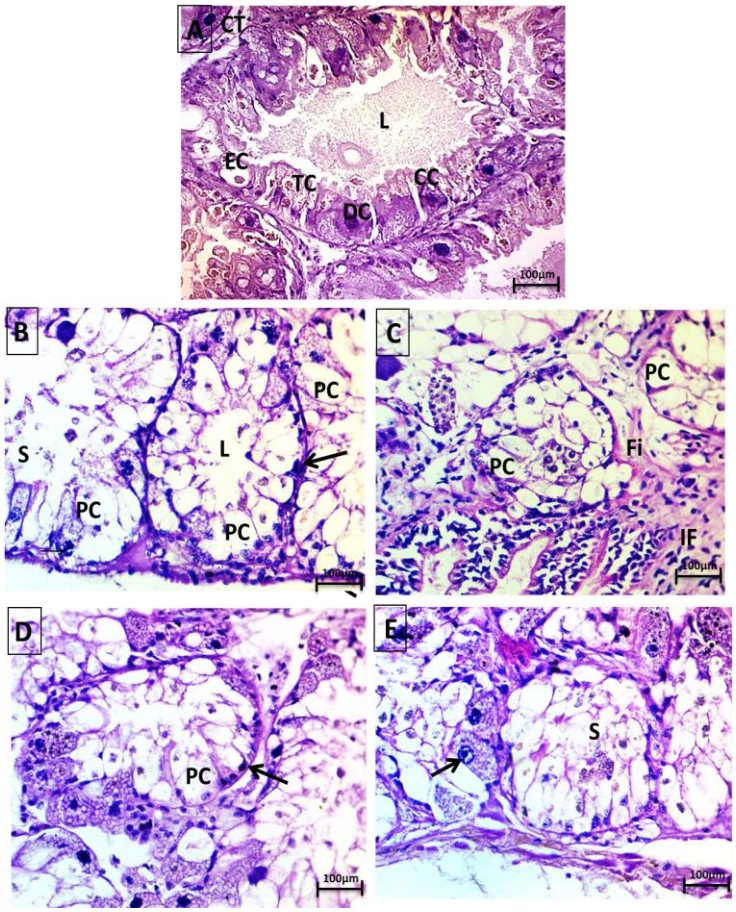

Fig. (6) Photomicrograph of digestive gland of Slug *Lehmannia nyctelia* after exposure to Silver Nitrate: (A); Control, (DC) digestive cell, (CC) calcium cell, (EC) excretory cell, (TC) thin cell, (CT) connective tissue and (L) lumen. (B); 0.04 g/L, (PC) empty cell, (L) lumen, (arrows) thickness of basement membrane and (S) space. (C); 0.08 g/L, (IF) infiltrations, (fi) fibrosis, (PC) empty cell. (D); 0.4 g/L, (arrow) pyknosed nucleus, (PC) empty cell. (E); 0.8 g/L, (arrow) pyknosed nucleus and (S) space (H &E stain, scale bar 100 µm).

DISCUSSION

Little is known about the impacts of AgNO3 and Ag NPs on the *Lehmannia nyctelia* in the soil, While, Land slugs are considered as serious pests in filed crops in many parts of the world [28].

Histological examination of the (digestive gland, gonads and kidney) of *Lehmannia nyctelia* treated with Ag NPs and AgNO3 showed fundamental changes with different concentration. Nanoparticles exposure can cause significant cytological alterations in digestive gland, which plays an important role in the detoxification of pollutants [29]. The histochemical and histological changes are expected to be useful biomarkers of AgNO3 and Ag NPs exposure [30]. Our histological study establishes the tissue damage in the digestive gland, gonads and kidney of *Lehmannia nyctelia* after exposure to the different concentration of toxicity of the metal particles (Ag NPs and AgNo3).

The digestive gland, similar in function to the human pancreas and liver, is the main organ involved in the absorption and storage of metals in molluscs. This gland is responsible for the production of digestive enzymes, nutrient absorption, endocytosis of certain food ingredients, and food storage and excretion [31]. The digestive gland is one of the most important organs in the gastropod , which works to expel and remove toxins [32]

Further histological examination in digestive gland in our study showed cellular degeneration, with a more expanded lumen, cell necrosis, with atrophy of the connective tissue of the digestive gland and degeneration of tubules lining cells with presence of calcium cells, Furthermore, spaces and presence of pyknosed nuclei. Therefore, the intercellular exchange. Accordingly, the diffusion of heavy metals increases in cells causing cellular necrosis [33]. According to Osterauer 2010 [34], the changes in digestive gland of snails (*Marisa cornuarietis*) were showed large hemolymph spaces between the tubules, enlarged tubule lumen, flattened epithelia, irregular shape of cells, increased amount of vacuoles in digestive cells, necrosis of digestive and basophilic cells at 50 and 100 g/l of PtCl. The same results were observed in *Planorbarius corneus* exposure to 0.4 mg/l of endosulfan [35]. These observations agree with previous findings.

Several digestive gland cells showed alterations in cell shape. With higher doses, the alterations became more severe, resulting in digestive gland tissue being destroyed completely [36].

The digestive cells are intracellular food digestion [37]. The calcium accumulate toxicants like excess copper [38].

The deterioration of digestive cells caused by ingestion of the nanoparticles [32, 39, 40]. These results are in accordance with other studies [41, 42] which have revealed structural alterations of digestive cells of the digestive gland of slug exposed to Cd, Pb and Zn. [43] they observed the acute toxicity of the metal oxides on the cells of the hepatopancreas on the species of *H. pomatia*.

As reported previously, the kidney is defined as a sac of some gastropods [44], they may play a part in the regulating of the Para cellular pathway followed by some molecules and ions.

Kidney of *Lehmannia nyctelia* treated with increasing doses of AgNO3 and Ag NPs, the histopathological examinations revealed the changes like lost normal shapes of cell, fusion of epithelial cells and their connective tissues increased in size and destructed

apical border. These results are in agreement with Russell, 1981 [40] observed in Cd treated *Helix aspersa*. Histopathological analysis revealed significantly of snail's kidney *Bellamya bengalensis* exposed to copper sulfate (3CuSO .5H O) and destructed epithelial lining [45].

Compared with the control, the kidney tubules of the group that received $AgNO_3$ and AgNPs had a number of histopathological changes, swelled cells, lost their normal structure, and tubules disintegrated. As a result of the presence of toxicants, the tubules swell as a result of cell proliferation.

In the present work, the histological alterations of ovotestis of the *Lehmannia nyctelia* due to exposed to $AgNO_3$ and Ag NPs was disruptions of acini and sperm head. Some alterations were observed such as, degeneration of germinal layer, degenerated of mature ova, thickening of lining gonadal tubules, Aggregation of spermatogenesis and oogenesis in groups. Germnative stages of gonad. Wangsomnuk, 1996 [46] noticed the same results in *Indoplanorbis exustus*. Zhou, 1993 [47] stated that, following niclosamide exposure, *Biomphalaria glabrata's* spermatozoa numbers and oocytes were reduced.

According to Mohammad, 2021[48], ZnONPs have been observed to disrupt the acini and alter some features of spermatocytic degeneration, oocyte disruption, and the appearance of fibrosis within acini induced by *Lehmannia nyctelia*.

That damage of the gonadal tissues implies that chronic exposure of slugs to AgNO3 and Ag NPs not only the structure of the gonadal tissues, but also the normal function of the gonads. AgNO3 and Ag NPs can consume in gonads and effect on energy for reproduction [49].

AgNO3 and AgNPs release in ecosystems may result in ecological consequences based on the results of this investigation. These biomarkers have also opened a wide perspective in terrestrial toxicology, as slugs *L. nectyle* is being exposed to environmental pollutants. Our results provide critical information to regulatory agencies and industry to determine the need for monitoring and regulation regarding AgNO3 and Ag NPs.

CONCLUSION

The present study has focused on the effects of AgNO3 and Ag NPs in the *L. nectyle* using Histopathological change to tackle their acute toxicity. This study confirmed that exposure to AgNO3 and Ag NPs could result in the damage of different organs of slugs *L. nectyle* and subsequently have negative impacts on the vitality and life of slugs *L. nectyle*.

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