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Histological study on the effect of sodium bentonite nanoparticles on the earthworm, *Allolobophora caliginosa* (Lumbricidae: Annelida)

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ABSTRACT

Bentonites are used on a large scale, by farmers and the feed industry, as a feed supplements to adsorb mycotoxins. The presence of mycotoxins in feed affects its quality, animal safety, and human health. Food additives must be used carefully as they may contaminate food and became harmful to the environment and food consumers. The present work studied the effect of two different concentrations of sodium bentonite on the body wall, gut, and coelomic cells of earthworms. The results revealed a change in the behavior of earthworms and the occurrence of a great alteration in the tested samples but no mortality was recorded. Coelomic cells aggregated, vacuoles and pseudopodia were formed.

INTRODUCTION

The use of chemicals to control insects, snails, slugs and other pests increased crops productivity but at the same time it has a harmful effect on the environment and animals which feed on crops contaminated with pesticides residues **(Raikwar and Nag, 2003)**.

Nanoparticles (NPs) diameter does not exceed 100 nm. So they have special chemical and physical properties that differ from materials found in the environment **(Ostiguy *et al*., 2006)**. Due to their special physico-chemical properties nanoparticles (NPs) have negative effects on different animal tissues **(Schrand *et al*., 2010)**.

There are several types of NPs which can be distinguished. Natural NPs can be detected in soils and volcanic dust. They can be formed by biological and geological processes. Many organisms can adapt with toxic NPs and survive in environments rich in natural NPs **(Handy *et al*., 2008)**.

Nanotechnology is widely used in medicine, agriculture, chemistry, material sciences, physics, and electronics **(Matranga and Corsi, 2012; Sharma *et al*., 2014; Zhang and Zhang, 2014)**.

Nickel nanoparticles (Ni-NPs) are used in many applications due to their magnetic and catalytic properties **(Park *et al*., 2005)**. But they have a toxic effect on human lung epithelial cells **(Ahamed, 2011)**.

Earthworms improve the soil structure by shredding, mixing organic matter and stimulating microbial growth in the soil **(Massey *et al*., 2013)**.

Earthworms are in contact with the solid and aquatic phases of the soil so they exposed to NPs that deposited to the soil and can accumulate environmental pollutants within their body (**Lahive *et al*., 2014; Panzarino *et al*., 2016)**. **Spurgeon and Hopkin (1996)** reported that earthworms can be used as bioindicators to monitor the pollution of soil by metals.

Earthworm coelomocytes (leukocytes) are responsible for innate immunity such as encapsulation and phagocytosis against pathogens and parasites. **Cooper (1996)** reported the presence of three main leukocytes population: granular amoebocytes, hyalineocytes, and chloragocytes. Granular amoebocytes and hyalineocytes of earthworms are capable of encapsulation and phagocytosis. **Cancio *et al*. (1995)** suggested that the chloragosomes that identified in the cytoplasm of chloragocyte are lysosomal in origin.

Bentonite is the clay rock material which mainly composed of montmorillonite clay **(Eisenhour and Brown, 2009)**. There are two types of bentonites: sodium and calcium bentonites **(Wright, 1968)**.

According to the European Food Safety Authority **(EFSA, 2010)** bentonite is a soil natural component, and is widespread in the environment, used as feed additive and not expected to have a harmful effect on the environment.

In the present study, earthworm used as bio-indicator to test the harmful effect of sodium bentonite nanoparticles on the terrestrial environment.

MATERIALS AND METHODS

Sodium bentonite nanoprticles were obtained from Sigma-Aldrich Co.

Characterization of sodium bentonite nanoparticles (Na bentonite NPs):

X-ray diffraction (XRD): Sodium bentonite NPs was identified according to **Abdou *et al.* (2013)** using Phillips X-ray diffractometer (Model PW 1720, Holland) equipped with a graphite monochromator and automatic divergence slit. Measurements were swapped from 2θ= 30° to 2θ= 80° with a copper ray tube operated at40 kV and 40 mA with CuKα radiation with wavelength of 1.5406 A.

TEM: The morphology and size of Na bentonite NPs was investigated and micrographs of the sample were taken by the Ttransmission Electron Microscopy according to **Abdou *et al.* (2013)** (TEM; JEOL [model: JEM-100 CXII]) in Assiut University, Electron Microscope Unit.

Earthworm collection and culture:

Healthy earthworms of the species *Allolobophora caliginosa* (Family Lumbricidae, Phylum Annelida) were collected from Assiut University farm, Assiut, Egypt. Earthworms were maintained at the laboratory conditions for thirty days for acclimatization.

Experimental design and bentonite NPs exposure:

The acclimatized earthworms were randomly divided into 3 groups, control and two treated ones. Each group contains 12 specimens in plastic boxes covered with perforated plastic lid for ventilation. Dry powder of bentonite NPs were thoroughly mixed into the soil to achieve the desired final concentrations of 0, 0.05, 0.4 g/kg dry soil. For each test concentration, the mixed soil was sprayed using 10 ml distilled water each two days. Earthworms (200–240 mg fresh weight) were added to 300 gm of the contaminated soil of different concentrations along with a control group (uncontaminated soil), contained in plastic boxes and samples were examined daily.

Earthworms that did not respond to a gentle prodding with forceps were considered to be dead. Earthworms exposed to bentonite NPs for 15 days.

At the end of the experiment smears of the coelomic cells were prepared on clean slides, fixed in absolute methanol for 10 seconds, and after drying at room temperature; the slides were stained with Hematoxylin & Eosin (H&E) followed by dehydration in ascending grades of alcohol (30%, 50%, 70%, 90% and absolute). Finally the slides were cleared in xylene and permanently mounted by DPX **(Pascoe and Gatehouse, 1986)**. Examined slides were selected based on staining quality.

The anterior part of each specimen were fixed in neutral formalin and prepared for histological study. Longitudinal sections (7µm) longitudinal sections were prepared and were stained with Haematoxylin and Eosin (HE) **(Gray, 1952)**. Body wall and gut were examined under light microscope.

RESULTS

1.Characterization of sodium bentonite NPs:

1.1.X-ray diffraction (XRD): XRD pattern of Na bentonite NPs confirmed the crystalline nature of the nanoparticles (Fig. 1). Four Bragg reflections at 7.06°, 20.038°, 29.62° and 62.183° corresponding to the (001) and (020) sets of lattice planes, confirming the presence of bentonite in the sample.

1.2.TEM: The shape and size of Na bentonite NPs were determined by transmission electron microscopy. The common particles size range from 28.9 nm to 34.3 nm (Fig.2) and this confirms that Na bentonite is in the form of nanoparticles (particle size less than 100 nm).

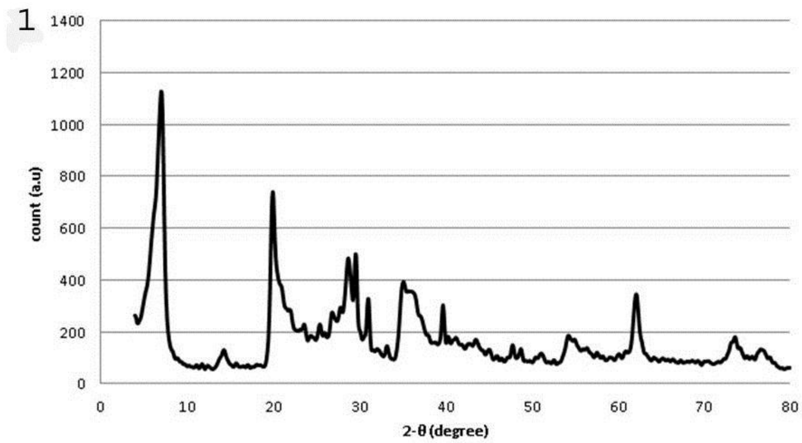

Fig.1: X-ray diffraction patterns of Na bentonite nanoparticles

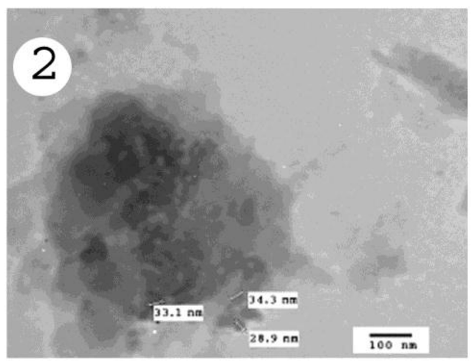

Fig. 2: TEM micrograph of Na bentonite nanoparticles showesthe morphology and size of Na bentonite NPs.

2.Examination of body wall:

The body wall of untreated earthworms (control) showed normal structure. Abnormal behavior was noticed after treatment with both 0.05 and 0.4 concentrations; the earthworms shrunk and its body writhing.

The outer layer of the body wall is the epidermis which consists of one layer of columnar epithelial cells with many unicellular glands. It is covered with a thin layer of cuticle. Below the epidermis there are two muscular layers (Fig. 3).

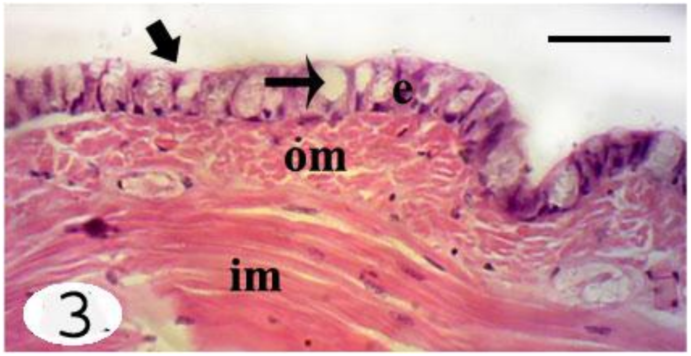

Fig. 3: Normal structure of body wall; epithelial cells (e), outer muscle layer (om), inner muscle layer (im), unicellular gland (thin arrow), cuticle (thick arrow). Scale bar = 50 µm. (H&E stains).

After treatment with concentration of 0.05 g/kg dry soil, the morphological and histological examinations showed that, the body wall became folded (irregularity of the peripheral rim of the worm body), the epidermis covered with thick layer of mucous, many unicellular glands in the epidermis lost their secretion, and the nuclei of the columnar cells became more elongated, in addition to rupture of the outer and inner muscle layers. As well as detecting the fibrosis and foci of nuclei of degenerated muscle fibers in the outer muscle layer (Fig. 4).

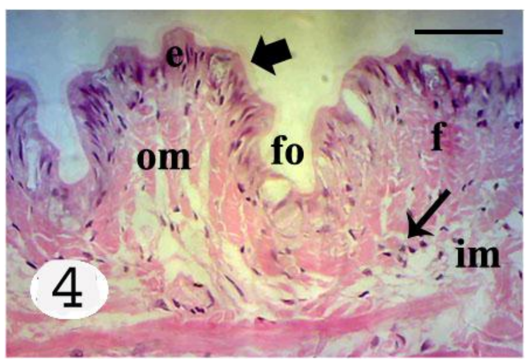

Fig. 4: Body wall of earth worm treated with concentration 0.05 g/kg soil shows: thick mucus layer (thick arrow), epidermal cells with elongated nuclei (e), enfolding of the outer rim of the body wall (fo), degeneration of the outer muscle layer (om), appearance of fibrosis (f) and aggregation of degenerated muscle cells nuclei (thin arrow), inner muscle layer (im). Scale bar = 50 µm. (H&E stains).

After exposure to concentration of 0.40g/kg dry soil, enfolding of the outer rim of the body wall increased, the epidermal layer degenerated, some columnar cells lost their contents, many glandular cells lost their content due to elaboration of the mucous substance and the mucous layer detached from the epidermis, angiogenesis was detected below the outer muscle layer (formation of new blood capillaries). The epidermal layer increased in thickens (became more than one layer of columnar epithelial cells) due to the formation of new epithelial cells. The muscle layers lost their normal architecture and fibrosis increased. The outer muscle layer became compact and degenerated (Fig. 5). Cuticle thickness increased in some earthworm and the muscle layer completely degenerated and was represented by appearance of empty spaces (Fig. 6).

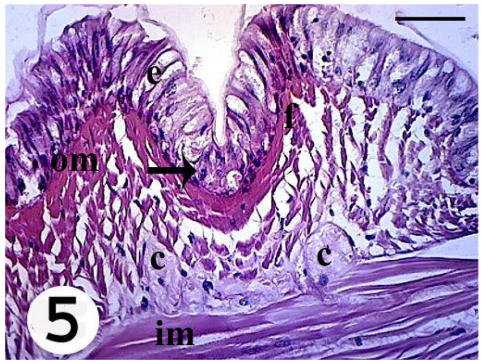

Fig. 5: Body wall of earth worm treated with concentration 0.4 g/kg soil shows: epithelial cells lost their contents (e), newly formed epidermal cells (arrow), fibroses (f) of the outer muscle layer (om), formation of blood capillaries (c), degeneration of the inner muscle layer (im). Scale bar = 50 µm. (H&E stains).

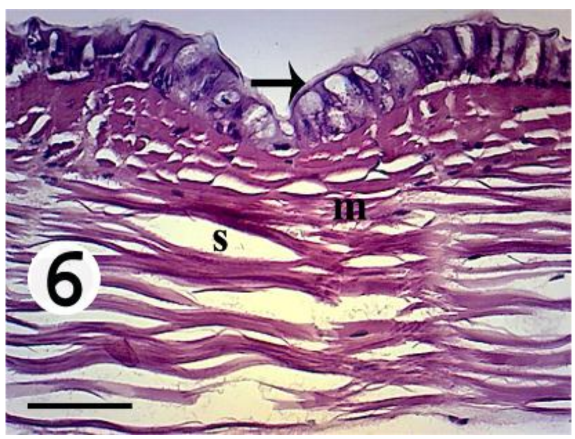

Fig. 6: Wall of earth worm treated with concentration 0.4 g/kg soil shows: thick cuticle (arrow), degeneration of the muscle layers (m), appearance of empty spaces (s). Scale bar = 50 µm. (H&E stains).

3.Examinations of gut:

Gut of untreated earthworm consists of three layers: inner mucosa, middle sub mucosa and outer muscularis. Mucosa consists of single layer of columnar epithelial cells, while the sub mucosa composes of connective tissue layer but muscularis consists of circular muscle layer (Fig. 7).

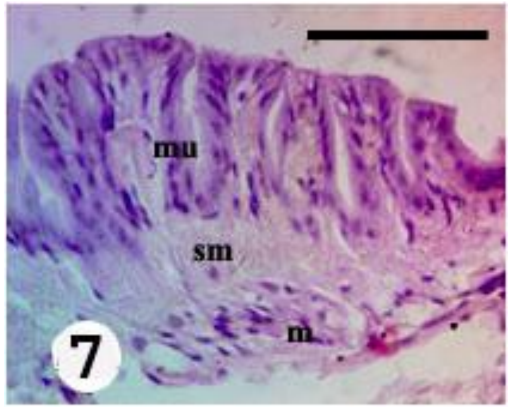

Fig. 7: Photomicrograph of gut of untreated earthworm shows: mucosa (mu), submucosa (sm), and muscularis (m). Scale bar = 50 µm. (H&E stains).

The treatment earthworms with 0.05 g/kg of Na bentonite NPS leads to disorganization in gut layers. The columnar cellsmucosa lost their shape and borders. The connective tissue of the sub mucosa disintegrated. The muscle layer lost their normal structure and separated from submucosa.In addition, empty spaces and red materials were detected in both sub mucosa and muscularis (Fig. 8).

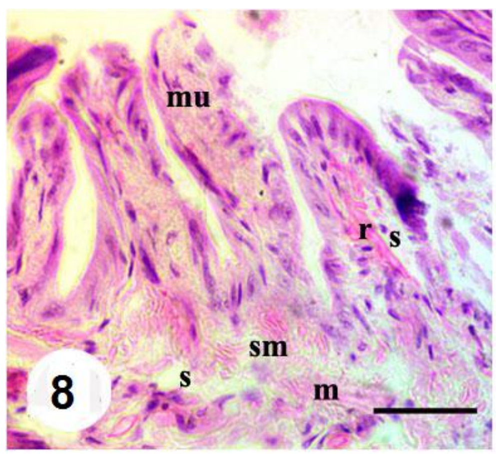

Fig. 8: Photomicrograph of gut of earthworm treated with concentration 0.05 g/kg soil shows: ruptured submucosa (sm) and muscularis (m), appearance of red material (r), and space (s). Scale bar = 50 µm. (H&E stains).

Gut of earthworm treated with concentration 0.4g/kg: The present results showed that, the columnar cells of mucosa replicated and lead to the formation of multiple cell layers. Thick layer of mucous was secreted to line the mucosa layer. The submucosa and muscularis completely lost their normal structure and could not be distinguished from each other. The empty spaces became wider (Fig. 9). In one earthworm the mucosa layer destroyed, while submucosa and muscularis were completely ruptured (Fig. 10).

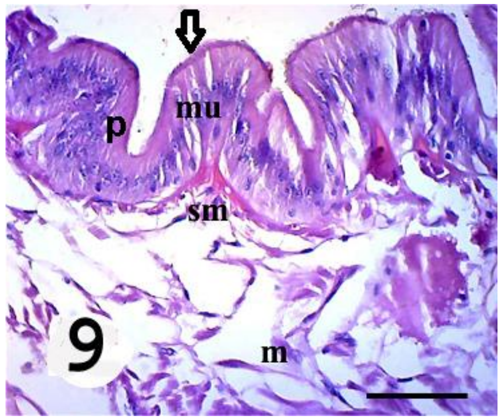

Fig. 9: Photomicrograph of gut treated with conentration 0.4 g/kg soil shows: multiplication of the columnar cells (p) of the mucosa (mu), complete destruction of submucosa (sm) and muscularis (m).Thick layer of mucus (arrow). Scale bar = 50 µm. (H&E stains).

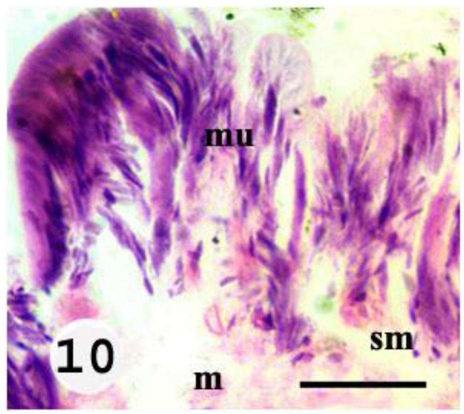

Fig. 10: Complete destruction of gut layers after treatment with concentration 0.4 g/kg soil; Mucosa layer (mu), submucosa (sm) and muscularis (m). Scale bar = 50 µm. (H&E stains).

4.Examination of coelomic cells:

Coelomic cells of the untreated earthworms can be distinguished into two main types according to the staining affinity of their cytoplasm; acidophilic cells (Fig.11) and basophilic cells (Fig.12). These cells comprised agranulated or granulated with peripheral or central nuclei and there are several pseudopodia or vacuoles (Fig. 13). The peripheral rim of the cytoplasm has a deep color than the rest of the cytoplasm (Figs. 11 b, d, f, p and 13 b, f-h). Most of the vacuolated basic cells were surrounded by acidophilic secretion (Figs. 12 e, f, h, i). Cells may contain basophilic (Fig. 13 a), or acidophilic granules (Figs. 13 b, f). Refractive droplets were recorded (Figs. 13 a, e, g).

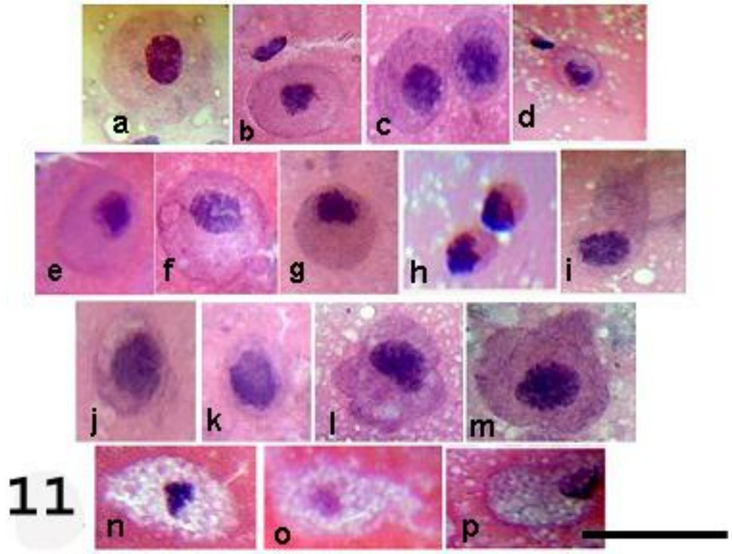

Fig.11: Photomicrograph of acidophilic coelomic cells of untreated earth worm shows: coelomic cells with central (a,b,k) and peripheral nuclei (c-j), pseudopodia (I, l-o) and vacuolated cells (n-p). Scale bar = 20 µm. (H&E stains).

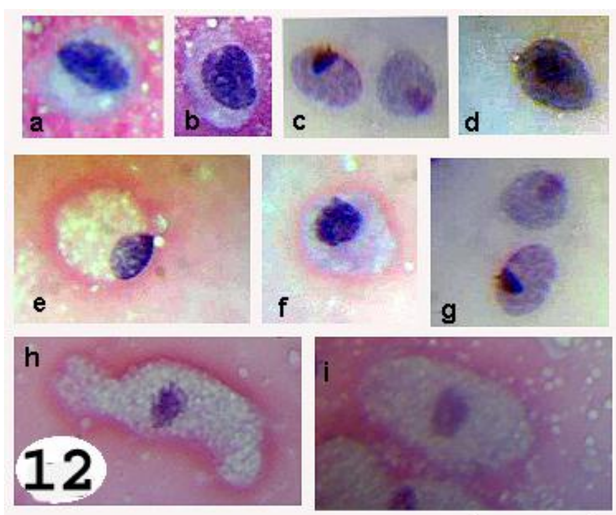

Fig. 12: Photomicrograph of basophilic coelomic cells of untreated earth worms shows: cells with central (a,i) and peripheral (b-g) nuclei, vacuolated cells with acidophilic secretion around them (e,f,h,i), cells with pseudopodia (h). Scale bar = 20 µm. (H&E stains).

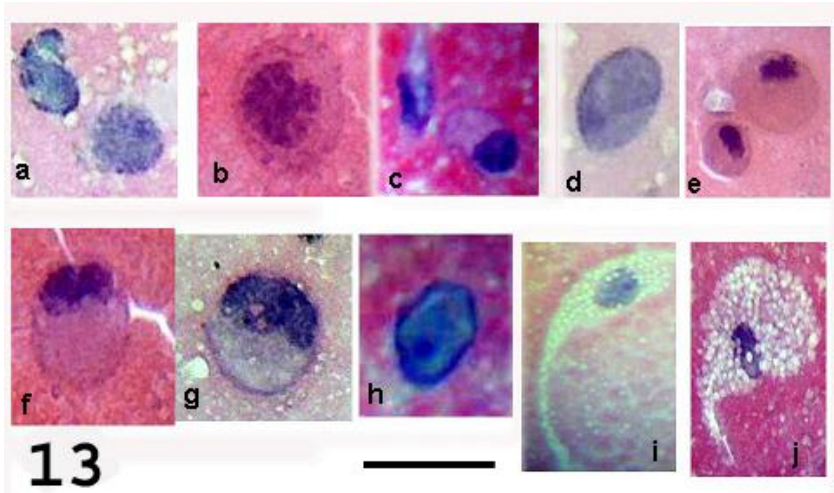

Fig. 13: Photomicrograph of coelomic cells of untreated earthworms shows: granulated basophilic cell (a), granulated acidophilic cell (b), agranulated basophilic cells with peripheral nuclei (c,d), agranulated acidophilic cell (e), cells with peripheral rim of deeply stained cytoplasm (f-h), cells with pseudopodia and vacuoles (i,j). Scale bar = 20 µm. (H&E stains).

Coelomic cells of earthworms treated with 0.05g/kg concentration: The present results showed that, many agranulated acidophilic cells increased in size (Fig. 14 a, b). Some of them surrounded by hyaline secretion and have many vacuoles (Fig. 14 c-f). On the other hand, granulated cells increased in number (Fig. 15 a-f). The nucleolus may be completely coated with granules (Fig. 15 a), which may be concentrated near the nucleus (Fig.15 b), or scattered in the cytoplasm (Fig. 15 c-f).

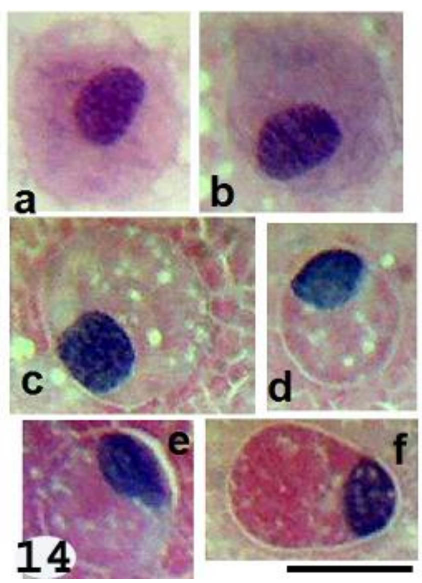

Fig. 14: Coelomic cells of earth worm treated with concentration 0.05 g/kg soil shows: large agranulated acidophilic cells (a,b), cells surrounded with hyaline secretions and contains vacuoles (c-f). Scale bar = 20 µm. (H&E stains).

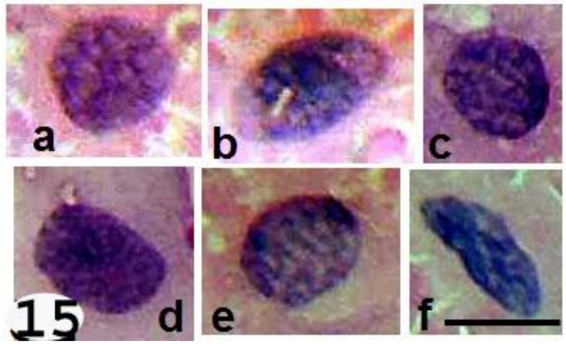

Fig. 15: Coelomic cells of earth worm treated with concentration 0.05 g/kg soil shows: nucleus coated with granules (a), granules aggregated around the nucleus (b), cells with distributed granules (c-f). Scale bar = 20 µm. (H&E stains).

Coelomic cells of earth worms treated with 0.4g/kg concentration: The obtained results indicated that, cells treated with this concentration tend to be aggregated in clusters (Fig. 16 a. b), associated with division of many nuclei (Fig. 16 c, d), and observed many cell fragments (Fig. 16 e-i); while vacuolated cells decreased in number (Fig. 16 j, k).

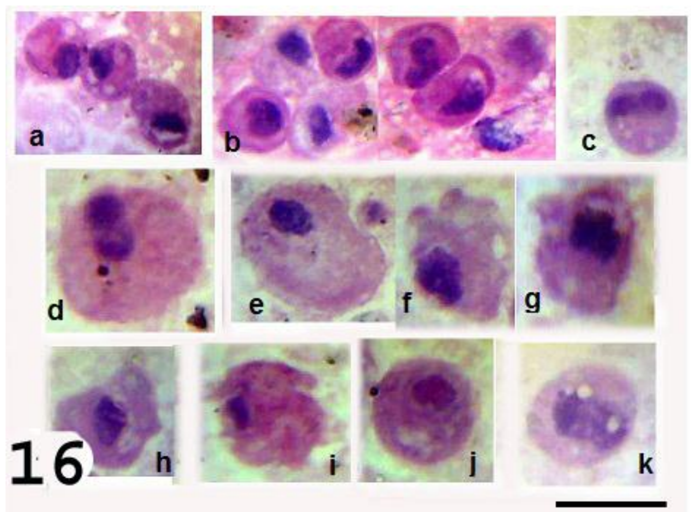

Fig. 16: Coelomic cells of earth worm treated with concentration 0.4 g/kg soil shows: aggregated coelomic cells (a.b). cells with divided nuclei (c,d), cell fragments (e-i), vacuolated cells (j,k). Scale bar = 20 µm. (H&E stains).

DISCUSSION

Bentonite is a term refers to clay consists of any minerals that contain saponite and montmorilloite regardless of their physical properties and origin **(Grimand and**

Giiven, 1978). Bentonites are widely used in industry due to their unique chemical and physical properties **(Harben and Bates, 1990)**.

According to **Kloprogee *et al.* (2002)** there are different types of bentonite. For example: sodium bentonite and Calcium bentonite. If sodium is the dominant element it is called sodium bentonite and vice versa.

Sodium bentonite is characterized by its high ability to absorb water and form viscous suspensions **(Eisenhour and Brown, 2009)** due to its negative charge, pore volume, high surface area, swelling ability **(Eliotte *et al*., 2020)**.

The size of nanoparticles has some special properties that interfere with chemical, physical, and biological activities of organisms **(Handy *et al*., 2008)**. Due to the small size of nanoparticles they have the ability to penetrate the cell membrane and enter the cell and reach the different organelles in the cell and modify the cell metabolism which may lead the cell death **(Hondroulis *et al.*, 2014)**.

Earthworms are common and abundant in the agriculture soil so they are affected by different chemicals that contaminate the agricultural soil **(Calisi *et al*., 2011)**. Earth worms have an important role in soil fertility so they are valuable organism to assess the toxic effect of chemicals that contaminate the soil **(Spurgeon *et al*., 2004)**.

In the present study the two tested concentration did not induce any mortality as similar results which were obtained by **Shoults-Wilson *et al*. (2011)** they revealed that silver nanoparticles affect the development and growth of earthworms and no mortality was recorded.

Earthworms have the ability to absorb the dissolved chemicals in the soil through their body wall due to the presence of the interstitial water and also ingested them by mouth and finally passes to the gut **(Yadav, 2015)**. This explain the behavior of tested earthworms after exposure to sodium bentonite nanoparticles; their bodies shrunk to avoid the exposure to the tested material and the columnar cells of the gut proliferate to form multiple cell layers covered with thick layer of mucous. Both sub mucosa and muscularis lost their architecture.

Abbas *et al.*, (2018) studied the effect of ZnO NP on earthworms. On their examination of the body wall they reported that; exposure to ZnO NPs leads to fibrosis, vacuolation and proliferation of mucous. This result explains the presence of empty spaces which detected in the muscle layer of the body wall of tested animals. At the same time these spaces may be due to hydropicedema (fluid retention).As in the present study they also detected loss of architecture of the body wall of exposed earthworm. They agree with the present study in that the gut of treated animals showed degenerated villi and that the abnormality of the body wall and the gut increases by increasing the dose of nanoparticles.

In the present study hyperplasia in the mucosa of the gut was recorded. It leads to the formation of multiple layers in the mucosa which may be to avoid the toxic effect of nanoparticls. Hyperplasia in the epidermis of earthworms was detected by **van der Ploeg**

***et al.* (2014)** and **Lahive *et al.* (2014)** after exposure to AgNPs and CeO2 NPs respectively. Fibrosis of the gut muscles was detected as reported by **van der Ploeg *et al.* (2014)**. **Lahive *et al.* (2014)** explained the presence of hyperplasia of the epidermis as mucocytes proliferation. Also erosion of the connective tissue matrix was reported by **Lahive *et al.* (2014)** as in the present study where sub mucosa of the gut of tested earthworms showed erosion and empty spaces in the connective tissue layer.

Coelomic fluid of earthworms contains different types of cells (coelomocytes). They classified according to the cytochemical and morphological criteria. In the present study two main types of coelomocytes were identified according to the staining affinity of their cytoplasm: acidophilic and basophilic cells with or without granules, pseudopodia and vacuoles.

Engelmann *et al*. (2002) classified coelomic cells of earth worm into three main types; eleocytes (chloragogen cells), granular and hyaline amoebocytes. Chloragogen cells have nutritive functions while granular or hyaline amoebocytes have a role in the defense functions for example phagocytosis. **Fugere *et al*. (1996)** reported the inhibition in phagocytic activity of coelomocytes of earthworm after exposures to zinc, mercury and cadmium.

In the present study coelomocytes of treated earth worms tend to be aggregated in clusters. Many cells undergone nucleic division, cell fragments, pseudopodia and formation of large vacuoles were detected. Large sized acidophilic coelomcytes with or without granules were also detected. Many cells were surrounded by hyaline secretion. The changes occurred in the coelomic cells are immune response from the treated earthworms to deal with the effect of Na bentonite NPs.

CONCLUSION

Sodium bentonite nanoparticles which used as an example of nano-caly have hazard effect on the body wall, gut and coelomocytes of earthworms during this study. So, it will affect their functions which consequently affect the terrestrial environment. In future the use of sodium bentonite must be in concentrations and amounts that have no harmful effect on the environment.

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