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Histopathology of Nitrate Toxicity on Hepatopancreas of the Terrestrial Isopod, *Porcellio laevis*

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The intensive addition of nitrogenous fertilizers to soil leads to the accumulation of soil nitrate that in turn affects soil fauna. The hepatopancreas is the major metabolic organ in crustaceans. It has an important function in the assimilation of nutrients and structurally includes two types of cells (B and S). This study was designed to investigate the negative effects of nitrate on the hepatopancreas of the terrestrial isopod *Porcellio laevis*. The isopod was subjected to different concentrations; 30, 60 and 90 ppm of $NaNO_3$ in food diet to study the histopathological changes on the hepatopancreas. The study revealed that the hepatopancreatic epithelium of the exposed isopod displayed numerous pathological changes varied from destruction of the apical cell membrane with the appearance of deeply stained cytoplasm to complete disintegration of B cells especially in 90 ppm treated group. A trend toward histochemical changes of protein, neutral mucopolysaccharides, acidic and sulphated mucopolysaccharides in hepatopancreatic epithelium were observed. Quantifying these changes in the hepatopancreas were assessed, using image analysis, it indicated the presence of dose-related changes in the area of hepatopancreatic cells, nuclei and lumen. It could be concluded that the recovery process in *P. laevis* from nitrate toxicity is a slow one.

Keywords: Nitrate concentration, Hepatopancreas, Isopoda, Image analysis, Histology, Histochemistary, Recovery.

INTRODUCTION

Nitrogen is essential to plant growth, yet the excessive use of nitrogenous fertilizers in agriculture is generally a major cause of nitrate pollution. Human intervention in the nitrogen cycle due to intensive agricultural activities lead to nitrate accumulation in natural and agricultural ecosystems, resulting in leaching of nitrogen to groundwater and surface water due to applied higher amounts of natural and mineral fertilizers (1). Recently, agriculture fertilization represents a major source of soil and water nitrate pollution, which is difficult to be eliminated (2 and 3). Overuse of nitrogen fertilizers result in diminishing crop returns (4) and leads to diminished environmental quality and human wellbeing (5 and 6).

Nitrate ion (No_3) is a naturally occurring form of nitrogen in soil, highly

soluble in water and used as a food by plants for growth and production. NO_3 has a tendency to form coordination complexes with other metal ions in solution (7), which causes a real problem resulted in excessive environmental risks of soil pollution that affects soil fauna.

A broad range of tests has been used to evaluate the effects of agricultural fertilizers on soil organisms (8). Isopods are suitable indicators of toxicity (9; 10; 11 and 12).

Macroinvertebrates particularly terrestrial isopods in field, exposed to high concentrations of nitrate pollutants, from insecticides and fertilizers, can evoke protective cellular adaptations, or/and pathological changes in the hepatopancreas, and eventually lead to death (13). Terrestrial isopods are also regarded and considered as suitable organisms for environmental studies, and are one of the best investigated invertebrates in soil for ecotoxicological experiments (13;14; 15; 16; 17; 18; 19; 20 and 21).

Hepatopancreas is a major metabolic organ in crustaceans, that is prominent in the midgut, accomplishing intestinal, hepatic and pancreatic functions and represent 2-6% of the body weight of crustaceans (22). Hepatopancreas considered as the largest digestive gland in these animals. It has an important function in the assimilation of nutrients, as well as in providing the energy reserves necessary for growth and metabolism in crustaceans (23). The structure of hepatopancreatic cells of isopods is known to reflect influences of internal and external factors (24 and 25). Many pollutants have been demonstrated to cause hepatopancreatic toxicity, resulted in alterations of cellular ultrastructure, such as pesticides and heavy metals (22; 26 and 27).

Quantitative method represents a biomarker tool to evaluate the toxicity and histological changes in the structure of hepatopancreas, (28) determined and quantified the effect of cadmium exposure on the histological structure of the hepatopancreas of *Porcellio laevis* using image analysis software for cellular area determination. Changes in the quantity and quality of energy reserves in the hepatopancreas of the isopod *Armadillidium vulgare* can result from many ecological factors, or active avoidance of contaminated food (29).

The objective of this study was to:

1- Identify the histological structural changes in the hepatopancreatic cells of the terrestrial isopod *Porcellio laevis*, in both control and nitrate treated individuals, and to evaluate the recovered capacity of hepatopancreas.

2- Detect and quantify the histochemical alteration induced by nitrate in the hepatopancreatic cells, using five different stains (H&E; Bromophenol blue;

PAS; PAS /Alcian blue double stain and Alcian blue).
3- Detect and quantify histopathological changes expressed by the surface area of the hepatopancreas using image analysis software program.

MATERIALS AND METHODS

1-Isopod collection:

Pitfall traps and hands were used to collect adult males of isopods from the field. A number of adult isopods were collected from different sites during the period of late spring and summer seasons, the laboratory experiments were carried out on the males specimens of the isopod, *Porcellio laevis.*

2-Design of the laboratory experiment to study the effects of food containing different concentrations of nitrates on *Porcellio laevis*:

The preliminary analysis of soil nitrates indicated that nitrate concentration reached up to 60 ppm which exceeds the considered safe levels for the biota (5-10 ppm, WHO). On this basis the pattern of offered concentrations of food was chosen in the laboratory experiment.

In the laboratory, adult males of isopods were divided into three groups, each group contained 10 (adult isopods), that exposed to different food containing different concentrations of nitrate which included: 30 ppm or (T1), 60 ppm or (T2) and 90 ppm or (T3), plus untreated (control group). Before offering food to isopods, animals were starved for two days. The food pellets were prepared from dried citrus leaves with 40% rabbit food. Two types of food pellets were prepared, control food (without nitrate addition) and nitrate containing food (30, 60, 90 ppm). In the exposure experiment, nitrate contaminated food pellets (weighed two grams) of each concentration, were offered to the three treated groups (30, 60, 90 ppm). The exposure experiment lasted for ten days. At the end of the experiment, the treated group of 60 ppm was chosen for undergoing a recovery experiment.

At beginning of the recovery experiment, individuals were transported to other boxes supplied with freshly moistened food, to be fed like untreated group on uncontaminated diet. The recovered animals were sacrificed after 20 days.

3- Histological preparation:

Three specimens of isopods were randomly chosen from each exposure group, and embedded in Kahle's solution fixative for 24h. Impregnated with Paraffin wax at 58 C°. Slides of 6 µm thickness were prepared from each group (control, treated) and stained. Certain slides from each group were

chosen to be stained and examined to show distinct section of hepatopancreas in all.

In the histochemical investigation, the slides number fifteen to nineteen of each treatment and control group were stained using five methods includes five different stains. Each method was used to indicate the presence or absence and intensity of specific cellular substances in both treated and control group (Pearse, 1972).

3.1. Hematoxylin and eosin (H&E) method:

Hematoxylin has a deep blue-purple color and stains nuclei. Eosin is pink and stains proteins. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extra- cellular matrix have varying degrees of pink staining.

3.2. Bromophenol blue method:

It can be used as a color marker and as a biological stain, and it greatly used to stain total proteins and nucleic acids. Bromophenol blue is an intermediate and is used as an acid-based indicator in the pH range of 3-4.6 where the color changes from yellow to blue.

3.3. Periodic acid-Schiff (PAS) method:

It most commonly used to highlight molecules with a high percentage carbohydrate content, this staining method used to detect neutral mucopolysaccharides such as glycogen, and mucosubstances such as glycoproteins, glycolipids and mucins in tissues. This stain reacts to give a purple- magenta color, the tissue will be stained purple and the nuclei will be stained blue.

3.4. Alcian Blue/PAS combined method:

The combined result from the double staining method is to stain both acidic- neutral- and mixtures of acidic and neutral mucins. When using a double staining method of alcian blue and PAS, neutral mucopolysaccharides are stained "red" with Schiff's reagent, and acid mucopolysaccharides are stained blue with alcian blue.

3.5. Alcian blue (pH 1.0):

This dye was used to stain sulphated polysaccharides or acidic mucopolysaccharides such as glycosaminoglycans. The tissue parts that specifically stained by this dye become blue to bluish-green after staining and are called (Alcianophilic).

4. Image analysis of the hepatopancreatic sections:

Image analysis software package was used for measurements of surface area of the hepatopancreatic cells, nuclei and lumen. This analysis included the measurement of surface area in pixels, the surface area of the control and treated groups (T_1, T_2 and T_3) were measured. The area of the lumen was subtracted from the area of the total section to calculate the area covered by cells. Measurement of about overall 60 cells surface areas mm (pixels) were carried out. In the second step, the surface areas of the nuclei and lumen were measured in the same way as well. The same program also converted the color values of the different histochemical stains that were used in the histochemical technique, to their corresponding optical density (Intensity/area).

5. Statistical analysis.

Statistical analysis of the data was performed using SPSS16 software package. Data were expressed in the form of mean±SD. One way ANOVA was used to analyze the parameters of the control and exposed groups. Followed by the multiple range test (Duncan's test) which was used to detect differences between means.

RESULTS:

1. Histological observations on the hepatopancreas of *Porcellio laevis.*

1.1.Histological structure of the normal hepatopancreas of *P. laevis*

The normal hepatopancreas usually consists of four blind-ending tubules, which open into the stomach. The tubules lined with epithelial monolayer comprising large dome-shaped B cells and small wedge-shaped or cylindrical S cells, both with finger-like microvillus brush border. Generally, the hepatopancreas is surrounded by a muscle layer of neuromuscular network and resting on a thin basal lamina. B cells protrude into the tubule lumen for most of their height, and contain apical vacuoles. In most B cells, the nuclei were found in basal position, where sometimes they located almost centrally. In some sections of the epithelium, B cell is flattened with a pair of nuclei.
In (Fig. 1): B cells appears eosinophilic and the cytoplasm is slightly darker than that of the S cells. S cells are characterized by a lightly stained cytoplasm with only one large rounded nucleus occurred per cell, and usually found in the basal position. S cells alternate with B cells in somehow regular manner, and are much shorter than B cells. B and S cells show basal lamina and brush borders.

1.2. Effect of nitrate contaminated food with 30 ppm nitrate on the hepatopancreas of *P. laevis*.

Generally, the toxicity with NO_3 induced changes both in the general morphology of the tubules and in the structure organization of epithelial cells in hepatopancreas.

Hepatopancreatic cells of animals in this case displayed numerous alterations. The majority of B cells and S cells didn't hold over the same shape and size of untreated form, with appearance of slightly condensed cytoplasm (Table 1), and elongation of some B cells toward lumen followed by holocrine secretion of these cells into the lumen to be incorporated in faeces. This treated group recorded the highest measured value for the ratios of (Cell/Lumen) and (Cell/Whole section) (Table 2). A slight breaking in the basal lamina of the hepatopancreatic section associated with appearance of intercellular spaces were observed as a result of the holocrine secretions of some B cells with a slight increase in the apical vacuoles of the rest B cells (Fig. 2 and Table 1). Only a small number of B and S cells had damaged cell apex. The measured ratio of (Lumen/Whole section) recorded here and that measured in the control group appeared nearly the same (Table 2). The muscle layer that surrounds the hepatopancreas showed no clear damages.

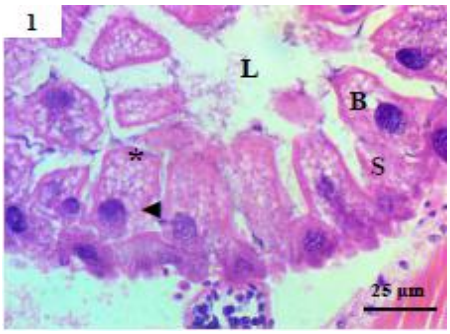


Fig. (1): Photomicrograph of transverse section showing hepatopancreas of untreated isopods stained with H&E stain and cell apex (*); nucleus (arrowhead); dome shaped B cells (B) lumen (L); S cell (S).

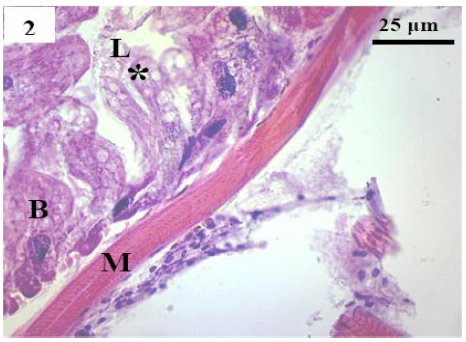


Fig. (2): Photomicrograph of transverse section showing hepatopancreas of treated isopods with 30 ppm NO_3 stained with H&E stain and vacuolation of B cell (*), B cells (B); lumen (L); muscle layer (M).

1.3. Effect of nitrate contaminated food with 60 ppm nitrate on the hepatopancreas of *P. laevis*.

The cytoplasm of B and S cells appeared condensed compared with the cytoplasm of the hepatopancreatic cells in the previous mentioned groups (Table 1). B cells exhibited irregular shape and lost their contour with enlarged hypertrophied nuclei in some, and increasing in the apical vacuoles

(Fig. 3). This concentration of nitrate induced a hyperplasia and cell proliferation with a considerable increasing of cell number and nuclei. The basement membrane began to ripple and exhibit irregular folds. The ratio of (Cell/Lumen) and (Cell/Whole section) recorded a reduced value compared with 30 ppm treated group, but the opposite was observed with respect to (Lumen/Whole section) ratio, it was higher than that of 30 ppm (Table 2).

1.4. Effect of nitrate contaminated food with 90 ppm nitrate on the hepatopancreas of *P. laevis*.

Exposure of isopods to this concentration induced great alterations in the morphological and histological structure of cells. Dramatic changes including; cell apex disorganization, great tendency to cytoplasmic vacuolation in B cells (Table 1), in some cells the cytoplasm was completely filled with vacuoles. Large portions of the cytoplasm in the apical cell regions were particularly condensed with clearly damaged cell apex. The muscular layer appeared exhausted and damaged, while the basal lamina was completely disappeared from some regions (Fig. 4). Destroyed cells releasing their content into the lumen, so the ratio of (Lumen/Whole section) of this group was the highest (Table 2). In contrary, the (Cell/Lumen) and (Cell/Whole section) ratios were lower than that observed in the control and other treated groups (Table 2).

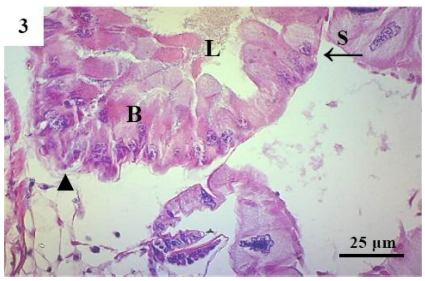


Fig. (3): Photomicrograph of transverse section showing hepatopancreas of treated isopods with 60 ppm, stained with H&E stain and hyperplasia of epithelial cell. Note rippled basement membrane (arrowhead), B cell (B); lumen (L); S cell (S).

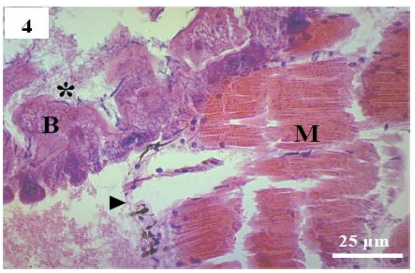


Fig. (4): Photomicrograph of transverse section showing hepatopancreas of treated isopods with 90 ppm NO_3, stained with H&E stain and excretion of cellular contents (*). Note ruptured part of the basement membrane (arrowhead), B cell (B); muscle layer (M).

1.5. Histological observation of *P. laevis* hepatopancreas after recovery period.

At the end of the exposure experiment, the treated group of 60 ppm was chosen for undergoing a recovery experiment. It was the most treated group suitable for achieving the recovery experiment, because the isopods hepatopancreas of this group exhibited more obvious histological changes

than those of 30 ppm treated group, while the isopods of 90 ppm treatment showed exhausted hepatopancreatic section, and most individuals died during exposure due to the lethal effect of this concentration.

After recovery period, 60 ppm treated group, showed the presence of both B and S cells in better appearance, regeneration of basement membrane was evident (Fig. 5), also double nucleated B cells were renewed, with clearly pink staining. The vacuoles in B cells were obviously decreased. The lumen stills filled with cellular content that were secreted from B cells during the period of treatment with nitrate.

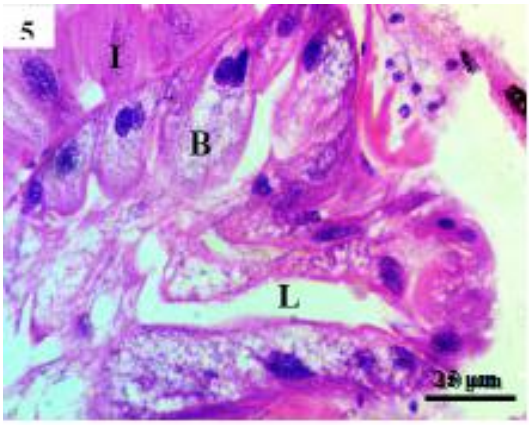


Fig. (5): Photomicrograph of transverse section showing hepatopancreas of 60 ppm treated isopods after recovery period stained with H&E stain and double nuclei B cells, B cell (B); intestine (I); lumen (L).

2. Histochemical study of the hepatopancreas of *Porcellio laevis* treated with different doses of nitrate.

2.1 Bromophenol blue staining.

The reaction of Bromophenol blue in untreated group showed moderate staining in the B and S hepatopancreatic cells (+ve) (Table 3). The hepatopancreatic cells of the 30 ppm nitrate group showed (++ve) reaction especially in the granules of B cells that surrounded vacuoles (Fig. 6). The holocrine secretions of the cells that fall in the lumen acquired a highly positive reaction. Isopods treated with 60 ppm nitrate showed a different pattern of staining with a positive weak reaction in the apical part (+ve) and a high affinity to staining in the basal lamina (Fig. 7). While a high positive reaction was recorded (+++ ve) in the 90 ppm of NO_3 (Table 3) with intense staining in the basement membrane that was started to fade away in some parts. Elevation of nitrate concentration in food results in developing darker color in the lumen with increasing detachment of cells (Fig. 8).

After recovery period, *P. laevis* hepatopancreas of 60 ppm showed positive staining for protein in the B cells which exhibited large faint stained vacuoles, while the surrounding of the apical vacuole was stained heavily, as well as the cell nuclei, cell membrane (Figs. 9).

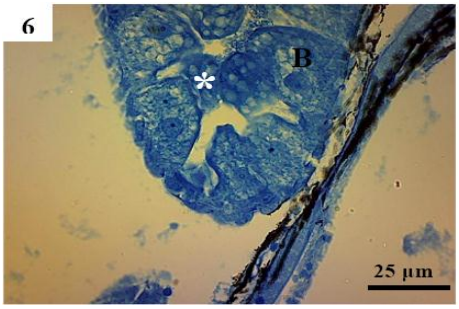


Fig. (6): Photomicrograph of transverse section showing hepatopancreas of treated isopods with 30 ppm NO_3 stained with Bromophenol blue and a holocrine secretion (*) into lumen from B cell (B).

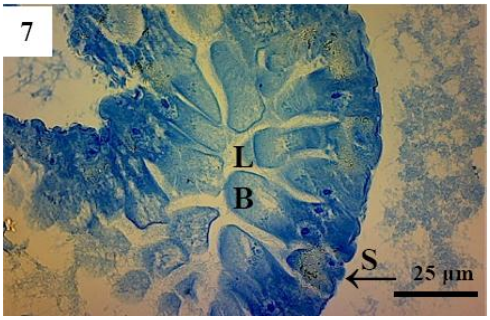


Fig. (7): Photomicrograph of transverse section showing hepatopancreas of treated isopods with 60 ppm NO_3 stained with Bromophenol blue and elongated B cells with faintly stained apices, B cell (B); lumen (L); S cell (S).

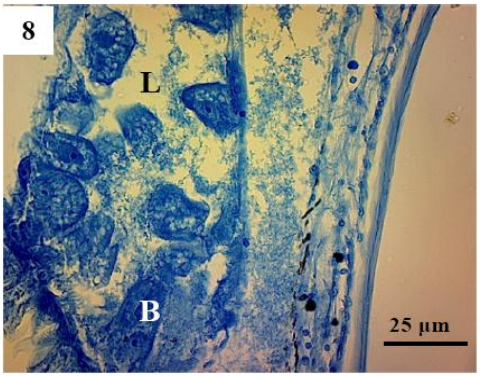


Fig. (8): Photomicrograph of transverse section of hepatopancreas of treated isopods with 90 ppm NO_3 stained with Bromophenol blue. Note heavily stained section, B cell (B); lumen (L).

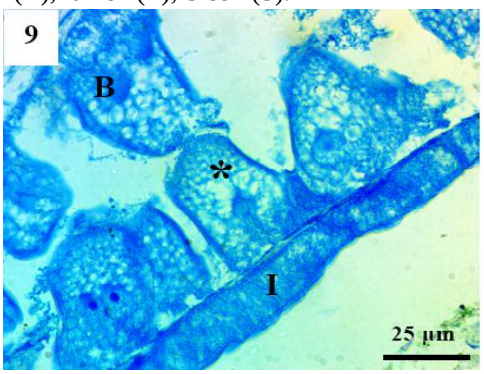


Fig. (9): Photomicrograph of transverse section showing hepatopancreas of 60ppm treated isopods after recovery period stained with Bromophenol blue and non-stained vacuole within cells (*), B cell (B); intestine (I).

2.2 Periodic acid-Schiff (PAS) technique.

It was noticed that in both control and treated groups the color reaction was pale, indicating the presence of a few polysaccharides granules in B and S cells (Figs. 10-12) (Table 3). Treated groups with different doses showed light staining or weak reaction at the basement membrane and also the same around cell nuclei. No traces of polysaccharides were detected in the luminal area of these treated groups.

After recovery period of 60 ppm treated group, the cytoplasm remained faintly stained, while the cell apex, cell membrane and the thin layer adjacent to the nuclear membrane acquire a weak positive reaction. This reaction to PAS technique showed that these cells contained small amounts of polysaccharides (Fig. 13).

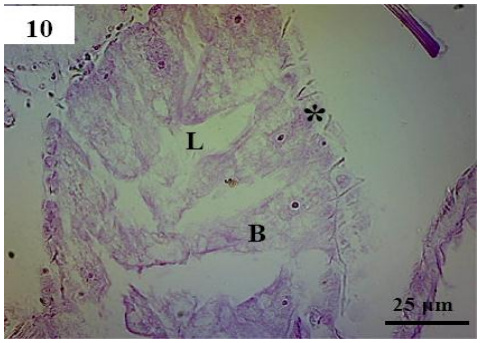


Fig. (10): Photomicrograph of transverse section of hepatopancreas of treated isopods with 30 ppm NO_3 stained with PAS. Note weak positive staining at the basement membrane (*), B cell (B); lumen (L).

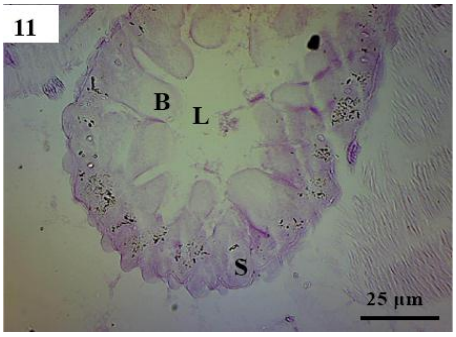


Fig. (11): Photomicrograph of transverse section of hepatopancreas of treated isopods with 60 ppm NO_3 stained with PAS. Note weak positive staining in the nuclei surrounded area, B cell (B); lumen (L); S cell (S).

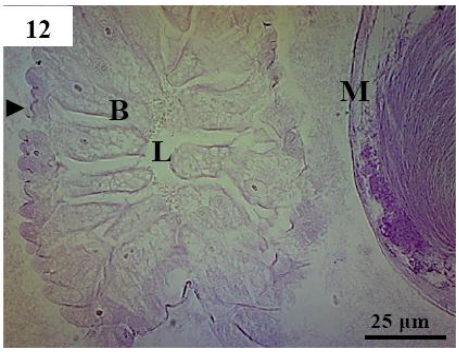


Fig. (12): Photomicrograph of transverse section showing hepatopancreas of treated isopods with 90 ppm NO_3 stained with PAS and positive staining at the basement membrane (arrowhead), B cell (B); lumen (L); muscle layer (M).

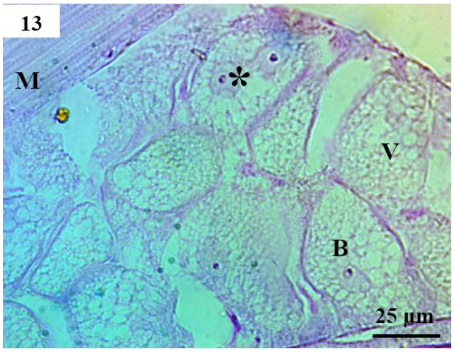


Fig. (13): Photomicrograph of transverse section showing hepatopancreas of 60 ppm treated isopods after recovery period stained with PAS and double nucleated B cells (*), B cell (B); muscle layer (M); vacuoles (V).

2.3 PAS/Alcian blue staining (double stain).

PAS/Alcian blue staining of neutral and acidic polysaccharides was detected in hepatopancreatic tissues of *P. laevis*. Acid mucopolysaccharides were concentrated mainly inside apical vacuoles of B cells in both treated and untreated or control groups. In 30 ppm nitrate concentration B cells acquired a high positive staining (+++ ve) at the basal cytoplasm and basal lamina, the color density exceeded that of the control group (Fig. 14, Table 3), the luminal region exhibited negative reaction to this stain. Acid

mucopolysaccharides were detected also in the nucleus of the hepatopancreatic B and S cells, and at the basal lamina. Neutral mucopolysaccharides which supposed to acquire magenta red color were masked. This in turn confirmed the observation of the presence of small amount of neutral mucopolysaccharides early detected by PAS technique.

The predominance of mucopolysaccharides inside the cells vacuoles decreased in 60 ppm nitrate group (Fig. 15). The color intensity in 90 ppm showed a highly positive (+++ve) reaction. The rippled basement membrane and the rupture cell apex showed strongly positive staining than that observed in the vacuoles and its surrounding (Fig. 16).

After recovery, the hepatopancreatic section of 60 ppm treated group exhibited staining color concentrated at the basement membrane, while faint staining was detected within B cells around the vacuoles. The nuclei of these cells exhibited dense staining, but the destructive cell apex showed moderately positive staining (Fig. 17).

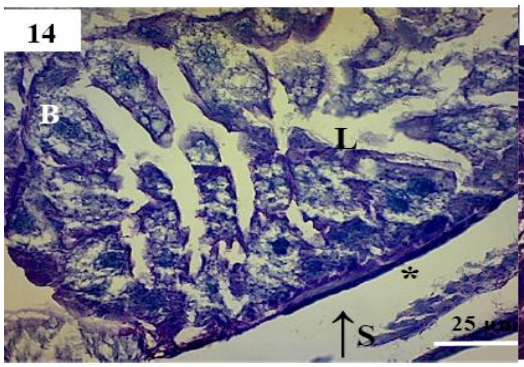


Fig. (14): Photomicrograph of transverse section of hepatopancreas of treated isopods with 30 ppm NO_3 treated group stained with double stain PAS/Alcian blue. Note positive staining at the basal lamina (*), B cell (B); lumen (L); S cell (S).

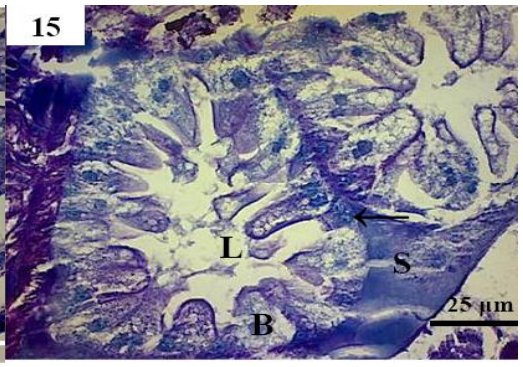
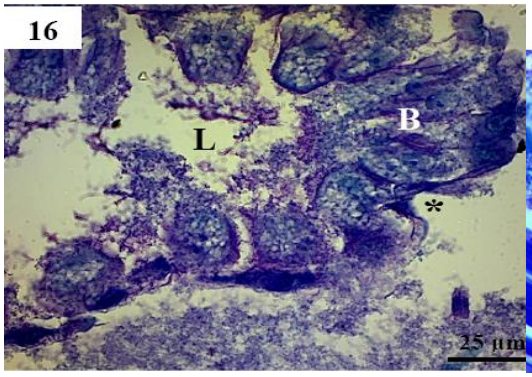


Fig. (15): Photomicrograph of transverse section of hepatopancreas of treated isopods with 60 ppm NO_3 stained with double stain PAS/Alcian blue, B cell (B); lumen (L); S cell (S).



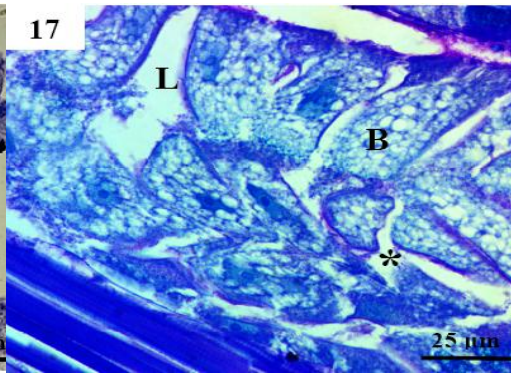


Fig. (16): Photomicrograph of transverse section of hepatopancreas of treated isopods with 90 ppm NO_3 stained with double stain PAS/Alcian blue. Note strong positive staining at the ruptured basement membrane (*), B cell (B); lumen (L).

Fig. (17): Photomicrograph of transverse section showing hepatopancreas of 60 ppm treated isopods after recovery period stained with double stain PAS/Alcian blue and damaged B cells (*), B cell (B); lumen (L).

2.4 Alcian blue staining (1.0 pH).

In 30 ppm treated group, the staining color was remarkable only around vacuoles of B cells, as well as at the basement membrane (Fig. 18). The treated group of 60 ppm showed a weak reaction of stain compared with the other treated and untreated groups (Fig. 19 and Table 3). The pyknotic nuclei within B cells were heavily stained, as well as the exhausted apices of these cells.

In the treated group with 90 ppm, the few remaining B cells had a strong positive reaction (+++ ve) to the stain. The exhausted basement membrane and cell apex of these cells showed also high positive color affinity (Fig. 20 and Table 3).

After recovery period, B cells of 60 ppm treatment showed high staining affinity, most of the vacuoles in B cells acquired also positive reaction; while the lumen contained cellular contents with moderately stain (Fig. 21).

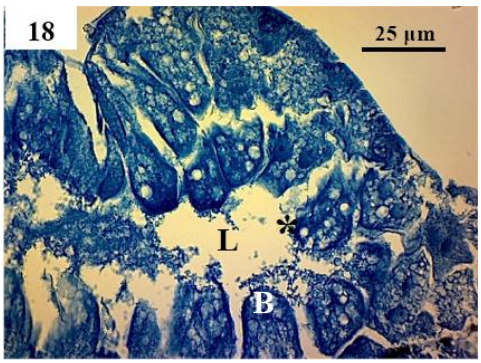


Fig. (18): Photomicrograph of transverse section of hepatopancreas of treated isopods with 30 ppm NO_3 stained with Alcian blue. Note staining only around vacuoles (*), B cell (B); lumen (L).

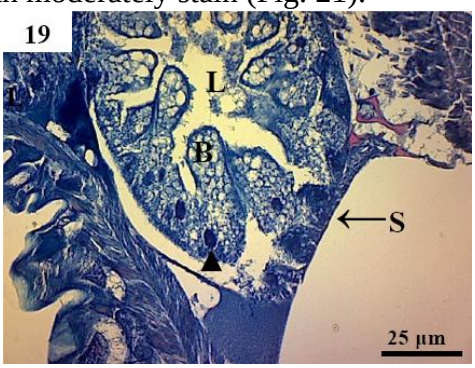


Fig. (19): Photomicrograph of transverse section of hepatopancreas of treated isopods with 60 ppm NO_3 stained with Alcian blue. Note heavily staining in the nuclei within B cells (arrowhead), B cell (B); lumen (L); S cell (S).

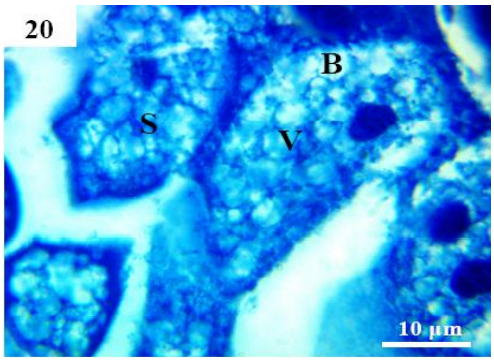


Fig. (20): Photomicrograph of transverse section. Showing part of hepatopancreatic B&S cells of treated isopods with 90 ppm NO_3, stained with Alcian blue, B cell (B); S cell (S); vacuoles (V).

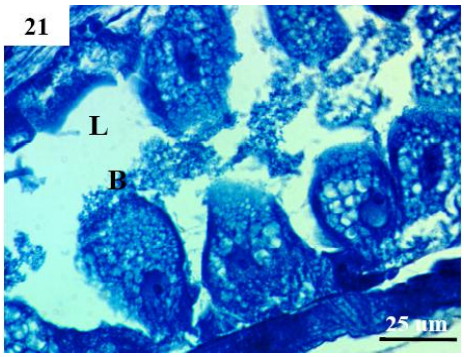


Fig. (21): Photomicrograph of transverse section of hepatopancreas of 60 ppm treated isopods after recovery period, stained with Alcian blue. Note positive staining in B cells and at the basement membrane, B cell (B); lumen (L).

Table (1): Comparison among histological observations of the hepatopancreatic B and S cells of *P. Laevis* affected by different nitrate concentration added to food.

Histology of B & S cells organiod	Control	(T_1) 30 (mg/kg)	(T_2) 60 (mg/kg)	(T_3) 90 (mg/kg)
Cell apex	-	-	+	++
damage	-	+	++	++
Ripple of basal	-	+	++	+++
Lamina	-	+	++	+++
Vacuolation appearance	-	-	-	+++
Condensed cytoplasm				
Cell disruption				

Histological damages in the hepatopancreas:
(-) No histopathology in this organiod.
(+) weak histopathological changes.
(++) moderate histopathological changes.
(+++) severe histopathological changes.

3. Image analysis of hepatopancreas of *Porcellio laevis*.

Three parameters were measured by image analysis software program in both treated and untreated groups. These parameters are cell surface area, nucleus area and lumen area. The ratios between these parameters area were calculated and the statistical analysis of the ratios (Cell/Whole section), (Lumen/Whole section) and (Nuclei/Cells) exhibited significant difference of

($p<0.01$) between the treated groups (Table 2). Non-significant difference between treatments was recorded in case of (Cell/Lumen) ratio.

The color intensity of the different histochemical techniques, were measured using the image analysis program. The intensity of a particular stain or staining procedure depends on the original strength of the stain, and how much a cellular substance of interest is present in the material. The results revealed that there were a significant difference among the different treatments at the color intensity of the same stain type except in the case of H&E, where non-significant differences were observed among treated groups (Table 3).

Table (2): Image analysis of the hepatopancreatic cells and lumen of *P. laevis* of control and different treated groups (mean ± SD).

Ratio	Control (M ± SD)	(T_1) 30 (mg/kg) (M ± SD)	(T_2) 60 (mg/kg) (M ± SD)	(T_3) 90 (mg/kg) (M ± SD)	P. value (Sig.)
Cells/Lumen	4.30 ± 1.6	6.37 ± 5.2	3.45 ± 1.7	3.39 ± 2.2	**0.120**^{NS}
Cells/Whole section	0.79^{a} ± 0.06	0.80^{a} ± 0.09	0.74^{ab} ± 0.09	0.70^{b} ± 0.12	**0.016***
Lumen/Whole section	0.20^{b} ± 0.06	0.19^{b} ± 0.08	0.26^{ab} ± 0.09	0.30^{a} ± 0.12	**0.017***
Nuclei/Cells	0.13^{a} ± 0.04	0.11^{ab} ± 0.05	0.08^{b} ± 0.03	0.13^{a} ± 0.06	**0.048***

Values in the same row which share the same superscript symbol are not significantly different.
Not significant. NS
Significant ($p<0.01$). ** Significant ($p<0.05$). *

Table (3): Image analysis of color intensity in the hepatopancreatic cells of the control and treated groups (Intensity/area).

Parameter		Control (M ± SD)	(T1) 30 (mg/kg) (M ± SD)	(T2) 60 (mg/kg) (M ± SD)	(T3) 90 (mg/kg) (M ± SD)	P. value (Sig.)
Stain	H&E	40607 b ± 785	41719b ± 3992	43131b ± 911	45226a ± 1028	0.119^{NS}
	Bromophenol	37093c ± 741	42062b ± 1750	29298d ± 1604	47716a ± 646	<0.001**
	PAS	30497a ± 1158	29640a ± 1267	26813b ± 972	29812a ± 926	0.014*
	PAS/Alcian blue	35466c ± 2355	40177b ± 1293	34352c ± 4186	46431a ± 826	0.001**
	Alcian blue	48573a ± 514	48715a ± 1028	33067b ± 1570	49601a ± 550	<0.001**

Values in the same row which share the same small superscript symbol are not significantly different.
Not significant. NS
Significant ($p<0.05$). * Significant ($p<0.01$). **

DISCUSSION

The effect of agricultural nitrogenous fertilizers on soil isopods can be measured as changes in the histological structure. In isopods, hepatopancreas is the first barrier against the poisoning of the organism (22). Therefore, changes or damage caused by pollutants should be detected first in this organ (14). At the present histological work, epithelial tissue of *P. laevis* contains only two types of cells (B and S cells). The main function of B cells are the storage and secretion of different enzymes beside absorption of different nutrients. B cell cycle can be divided into two stages extrusive and restitutive stage (31). S cells are specialized for the storage of metals and have long residence time in the hepatopancreas (32).

The experimental results showed notable degrees of damage between different treated groups. All NO_3 concentrations used in this experiment caused cellular damage. Most of the obvious histopathological damages was in the B cells of epithelium layer of hepatopancreas, this is may be due to the effect of nitrate in the lumen, that affect the apical membrane of nutrient absorbing B cells, resulting in the reduction of number of these cells, and leads to shrinkage of nuclei (18) reported that luminal cadmium contaminated food, when present in the lumen of the hepatopancreas affected the apical membrane of B cells, resulting in reduction of these cells that lead to marked reduction in body weight due to impaired nutrient absorption (33) emphasized this result, when study the effect of mercury pollution on isopods. The reduction in food uptake due to contamination was first mentioned by (34) who mentioned that *P. laevis* represents avoidance behavior to reduce food uptake, when used cadmium contaminated food.

1. Histological and histochemical changes in the structure of hepatopancreas after nitrate exposure:

In the present study, nitrate concentrations of 60&90 ppm induced much progressive changes appeared in vacuolation and subsequently disruption of cells. When the toxic potential of the gut content increases, lead to blabbing of hepatopancreas cell tips, then disruption of their outer membranes, which seemed affected the nutrient absorption. This cellular reaction could be considered as structural biomarkers of nitrate exposure. (35) described similar effects in the hepatopancreas of *Ligia oceanica* as indicators of necrotic cellular processes caused by a long starvation period.

The hepatopancreatic cells exhibited variation in the protein distribution after nitrate exposure. The moderate amount of protein was found in the control or untreated group, and reduced significantly in 60 ppm treated group, which may related to the reutilizing of the stored hepatopancreatic proteins during this stressful conditions. The toxic effect of nitrate at 90 ppm *P. laevis* caused a re-synthesize proteins. This re-synthesize proteins retained the highest significant estimated value which was the next step to reduce protein. This reaction may be related to synthesize of unknown protein as an antioxidant. Metallothionein (MT) is an antioxidant formed of proteins with low molecular weight containing cysteine, which is synthesized as a result of pollution and toxification to protect cells from exposure to oxidants (36.).

The present demonstration of PAS showed pale or weak positive color intensity that was restricted in the apical part of the epithelial cells, as well as in the basement membrane. (37) Illustrated that the basal cytoplasm and the cell apex of B cells, appeared moderately positive to PAS technique, in the hepatopancreas of the pink shrimp *Farfantepenaeus brasiliensis*. The present low intensity value of PAS color implies the absence or reduction of neutral polysaccharides, especially in the 60 ppm and 90 ppm treated groups, while in untreated group the intensity of PAS was higher which means that under the stressful toxic conditions, animals of these treated groups are forced to utilize most of the stored polysaccharides. These results confirmed by (38) who mentioned that reduction of polysaccharides in hepatopancreas of terrestrial isopods was a direct effect of nutritional stress. (37) Detected intense PAS staining inside the large apical vacuole of B cells in hepatopancreas of *Farfantepenaeus brasiliensis*, and found that the staining for polysaccharides decreases probably due to release of vacuolar material to the tubular lumen. In this study the absence of neutral polysaccharides in the hepatopancreatic cells after exposure indicates the utilization of energetic reserves due to the nutritional stress and inadequate toxic nutrition.

PAS/Alcian blue staining was strongly positive around the vacuoles at 90 ppm of nitrate concentration, this may be due to the secretion of more sulphated mucopolysaccharides from B cell, and extrusion amount of it to the luminal cavity in order to be utilized by the animal during stress conditions. Conversely, hepatopancreatic B cells are comparatively rich in sulphated mucopolysaccharides, but poor in neutral mucopolysaccharides at the same time. This result may relate to the antioxidant action of the sulphated polysaccharides excreted by the hepatopancreatic cells, especially under stressful conditions. (39) suggested that sulphated polysaccharides fractions extracted from the seaweeds samples represent natural effective indicators to antioxidant activity. Also (40) illustrated that sulphated polysaccharides and

β-glucans are able to activate cellular functions directly, that stimulate anti-oxidant defense mechanisms essential to functioning and survival of organisms. These immuno-stimulants are applicable to fish and shrimp (larval, juvenile and adult stages).

The hepatopancreatic cells become blue to bluish-green when stained with alcian blue. The abundance of sulphated polysaccharides in the hepatopancreas sections indicates a good physiological state of the animals of untreated and 30 ppm treated group. The measured color intensity was the lowest at 60 ppm treated group, this is due to the reduced amount of polysaccharides as a response to the stress which may reflect energy reserve mobilization, and reduced nutrients absorption by the hepatopancreatic epithelium due to cell apex damage. (41) studied the metal-containing granules in hepatopancreas of the isopod *P. scaber*, and reported that with increasing soil metal pollution animal uses the stored nutrients, so it showed a trend towards massive reduction in hepatopancreatic energy reserves. The response of *P. laevis* to the high concentration of 90 ppm of nitrate in food led to increased amount of sulphated mucopolysaccharides around vacuoles of B cells, which mean that B cells synthesized more sulphated acidic polysaccharides. That implies differences in energy metabolism after nitrate exposure. The stain intensity should be decreased in the 90 ppm NO_3, but according to image analysis of color, a significant increase was detected. The un-expected increase in the sulphated mucopolysaccharides may be partially explained by sulphur elevation associated with increasing in metallothionein, which had been documented to be metal-binding antioxidants responsible for detoxification (36). Also the cysteine fraction (sulfur-containing amino acid) of metallothionein represents nearly 30% of its constituent (42). In contrary (43) suggested the absence of a MT-like compound in the isopod *Porcellio scaber*, concluded that inducible metal binding compounds are not involved in metal tolerance in this animal.

2. Image analysis and surface areas measurements of hepatopancreas:

The cellular and histological alterations in terrestrial invertebrates reflect the health status of a cell, so the presence of toxicants should be measured (44). (45) Worked on hepatopancreatic ceca of *Gammarus pulex* achieved the comparable level of sensitivity from control and test treatment using semi-quantitive techniques. The present image analysis indicated that the value of cell surface area increased after nitrate exposure of 30 ppm, due to the elongation of B cells and appearance of excessive vacuolation within them, which was reflected on the ratio of (Cell/Whole section). The epithelium of the hepatopancreas regulates the transfer of toxic elements between the

digestive fluid and the blood, enabling P. *laevis* to detoxify a greater amount of toxicants (32). The (Lumen/Whole section) ratio of this group was lower than other treated groups; this was logic due to hyper-elongation of B cells since they occupied more of the lumen cavity space, in addition to accumulation of cellular contents that had been excreted from B cells.

Under toxic conditions of 60 ppm nitrate, the epithelium layer of the hepatopancreas or (Cell/Whole section) ratio, was thinner than that of the untreated and first treated group, this attributed to the damages in the cells apices and releasing the cell content into the lumen by exocytosis. This result confirmed by (46) who noticed a holocrine secretion of the digestive cell of the slug's digestive gland, that detached from the basal lamina connective tissues and fall in the acini's lumen to be incorporated into faeces. The ratio of (Lumen/Whole section) in 60 ppm of this study was larger than those of untreated and 30 ppm treated group, this is due to the changes in the cell shape and size as more space was available in the epithelium layer of this treatment.

The ratio (Cell/Whole section) of the third treated group 90 ppm recorded the lowest surface area compared to other groups. This concentration of nitrate seemed to activate and evoke pathological changes that lead to dehydration, cell shrinkage and eventually cell death. (47) worked on the snail *Helix aspersa* exposed to copper contamination indicated that the area of the digestive gland epithelia was significantly smaller after exposure. However the present image analysis of the lumen area of this group recorded the highest value. This attributed to the epithelial layer disorders, that cause cells collapse leaving intercellular spaces increasing the lumen area, beside the appearance of early aging newly formed cells as a clear sign of isopod modification with nitrate. A similar observation was recorded by (46) who worked on slugs digestive cells after exposure to high dose of cadmium.

According to image analysis, the nuclei surface area as well as the ratio of (Nuclei/Cells) of the 60 ppm treated group showed the lowest surface area, this was followed by dilatation, swelling and damage of nuclear envelop, then karyolysis was observed in the nuclei of the 90 ppm treated group, at this concentration the nuclei surface area and (Nuclei/Cells) ratio recorded higher values, that synchronized with the appearance of dark pigmented (pyknotic) nuclei. The pyknotic nuclei may be a response to binding toxin to chromatin (46). Karyolysis phenomenon is a final symptom which was the last form of cell death (48).

The image analysis recorded the highest intensity of the color values in 90 ppm, in different histochemical techniques except for PAS. This imply

that B cells in 90 ppm exhibited more intense color. The discussion of the dark and light variants of the same cell type in a tissue preparation, is a common factor explaining excessive cellular dehydration, which could occur in vivo, engendered by physiological or pathological states. According to (48) the dark cell phenomenon represented dead or dying cells at least in some instances. The condensed dark cytoplasm phenomena observed in 90 ppm was also mentioned by (18) in hepatopancreatic cells of *P. scaber* exposed to cadmium.

3. Histological changes in the structure of hepatopancreas after recovery period:

The hepatopancreatic cells of the 60 ppm treated group, after recovery period, tend to restore its normal conditions after detoxification, when the animal began to reduce the pollutant exposure in order to allow the recovery of the cellular damage.

In the present experiment, 20 days was insufficient for complete recovery, which means that the healing process from nitrate occurs at a slow rate which may be a result to low elimination capacity of nitrate. According to (49) who worked on the terrestrial isopods *Porcellionides pruinosus* exposed to Ag nanoparticles and $AgNO_3$ via soil and food, body concentration of Ag remained almost constant during the elimination phase, and a significant part of the Ag seemed to be stored in an inert fraction. (50) Showed that after recovery, *P. laevis* still exhibited amount of cadmium in the hepatopancreas, and related this to the accumulation of heavy metals in the hepatopancreatic S cells, so it couldn't release it throughout the recovery period. Similar result exhibited by (32) that isopods are not able to excrete cadmium. The same mechanism proposed to be carried out in the case of nitrate exposure. Also (51) showed that isopods maintain constant copper concentration during the year. In contrast, (52) found that cadmium metal loaded individuals of *P. scaber* fed on uncontaminated leaf during a period of recovery lost cadmium from their hepatopancreas at low, but constant rates; this means that *P. scaber* is able to eliminate certain fraction of the metal slowly.

Conclusion

Egypt must formulate relevant agricultural policies and recommendation to encourage farmers to reduce their fertilization rates by improving the balance of supplied nutrients in the fields. Furthermore, it is necessary also to reduce and control the quantity of nitrogen applied to the soil with fertilizers, since the tested concentrations of NO_3 had histopathological changes on the hepatopancreas of *P. leavis*. Most progressive changes induced by the 60 and

90 ppm of NO_3, and evident obviously in reduction the hepatopancreatic cells number, vacuolation and blabbing the cell tips, rupture the basement membrane and the apices of these cells. So it is recommended to focus the efforts on the exchange of thought's and opinions concerning the importance of the soil fauna.

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دراسة نسيجية مرضية لسمية النترات على البنكرياس الكبدي لمتشابهة الأرجل الأرضية (بوروسيليو ليفيز)

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استخدام الأسمدة الأزوتية (النيتروجينية) بكثافة يؤدى إلى تراكم النيترات في التربة والذي بدوره يؤثر على الكائنات الحية. البنكرياس الكبدي هو مكون أيضى رئيسي في القشريات حيث أنه يلعب وظيفة مهمة في التمثيل الغذائي وهو يتكون تركيبيا من نوعين من الخلايا وهما (خلايا B و S). صممت هذه الدراسة لتقييم التأثيرات السلبية للنترات على البنكرياس الكبدي لمتشابهة الأرجل الأرضية (بوروسيليو ليفيز). حيث تم تعريض متشابهة الأرجل لتركيزات مختلفة من نترات الصوديوم في الغذاء المقدم (٣٠، ٦٠، ٩٠ جزء من المليون) لدراسة التغيرات المرضية لنسيج البنكرياس الكبدي. أوضحت الدراسة أن الخلايا الطلائية للبنكرياس الكبدي لمتشابهة الأرجل المعرضة للمعاملة حدث لها العديد من التغيرات المرضية تفاوتت من تدمير غشاء الخلية القمى بالإضافة إلى تكثف السيتوبلازم بالخلية مما يؤدى إلى التدمير الكامل لخلايا B خاصة في المجموعة المعاملة بتركيز ٩٠ جزء من المليون. وأوضحت النتائج أيضا أن هناك اتجاه نحو حدوث تغيرات نسيجية للبروتين والسكريات العديدة المخاطية بنوعيها الحامضية والكبريتية. وقد تم تقييم هذه التغيرات باستخدام تقنية برنامج التحليل الكمي للصور الهيستولوجيه والتي أوضحت وجود تغيرات مرتبطة بالجرعات المختلفة على المساحة السطحيه لكل من خلايا وأنوية وتجاويف قنوات البنكرياس الكبدي. ويمكن تلخيص النتائج المتحصل عليها في أن متشابهة الأرجل الأرضية (بوروسيليو ليفيز) هي كائنات تستجيب ببطء للتخلص من سمية النيترات.