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RESEARCH REPORT

Cadmium exposure induced oxidative stress and histopathological disruption in the body wall of the freshwater leech *Limnatis nilotica* (Savigny, 1822)

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Abstract

Cadmium (Cd) is considered as one of the most highly toxic heavy metal that is released into the environment on a large scale, and its concentrations in water have significantly increased as a result of human activities. In particular, contamination of rivers and drinking water may easily occur, especially in locations close to industry or mines. In this study, we examined the impact of Cd exposure on the body wall of the freshwater leech *Limnatis nilotica* at concentrations of 100, 200, and 300 µg/l. The findings showed that the exposure caused histopathological changes as well as oxidative stress (increased malondialdehyde concentrations (MDA)) and changes in antioxidant activity (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Gpx)). In fact, numerous histological changes were observed, such as cuticle deterioration, a marked increase in the number of secretory cells, increased mucus production, vacuolization of the epithelium, altered epithelial cell borders, and mucus cell hyperplasia. The histological results are consistent with the biochemical findings because we found that MDA levels significantly increased in a dose-dependent manner. Additionally, the Cd exposure disrupted the levels of the antioxidant levels. Indeed, SOD, CAT, and Gpx activities were increased in the group treated with the lower dose (100 g/l), whereas their levels dramatically decreased at higher Cd doses (200 and 300 g/l). Therefore, the presence of this heavy metal in freshwater habitats may have severe ecological risks that may lead to leech aquatic habitat destruction and fragmentation.

Key Words: leeches; toxicity; cadmium; oxidative stress

Introduction

In aquatic ecosystems, organisms easily bioaccumulate heavy metal traces in their tissues either from the alimentary chain or from the surrounding aquatic medium (Zhang *et al.,* 2021). Among these metals, we can mention Cadmium (Cd) which is a non-biodegradable heavy metal that has harmful effects on aquatic organisms (Wei *et al.,* 2020). Its release in the environment occurs both naturally and anthropogenically through volcanic eruptions, waste incineration, and phosphate fertilizers (Ismael *et al.,* 2019; Kechiche *et al.,* 2021).

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Cd exposure has been associated with a variety of adverse effects including morphological dysfunctions and biochemical alterations (Zhang and Reynolds, 2019). It can weaken the reproductive function thereby causing germ cell apoptosis, necrosis, DNA damage as well as disruption of the endocrine system (Alharthi *et al.,* 2020). Both acute and chronic exposure to Cd was also be associated with the generation of metallothionein and reactive oxygens species (ROS) causing oxidative damage and loss of membrane integrity and function (Kubier *et al.,* 2019). In the oriental river prawn (*Macrobrachium nipponense*) that was exposed to different concentrations of Cd, a significant increase in mortality was noted as well as histological changes of hepatopancreas showing abnormal lumen shape, vacuolation, and necrosis (Tavabe *et al.,* 2019). Similarly, decreased respiration intensity, DNA

Fig. 1 Morphological changes of *L. nilotica* exposed to Cd. A: control; B: *L. nilotica* exposed to 100 µg/l of Cd, no morphological changes were noted. C & D: *L. nilotica* exposed to 200 µg/l and 300 µg/l of Cd respectively. Changes in body shape including contraction, coiling, and mucus secretion (arrows) were noted. (A-B): Scale bar = 1 cm; C: Scale bar = 0.8 cm; D: Scale bar = 0.7 cm

damage, abnormal behavior and high mortality rate were shown after Cd exposure in mollusks (Karim, 2022; Sharov *et al.,* 2022). Additionally, exposure to 0.125 µg/L of Cd, caused deleterious effects on the mud crab *Scylla paramamosain* resulting in immune system decrease, intestinal damage, alteration of the microbial community and disruption of the metabolic function (Cheng *et al.,* 2023).

Among invertebrates, leeches represent suitable organisms for aquatic biomonitoring (Khaled *et al.,* 2016). They are regarded as an appropriate environmental biomarker to evaluate the effects of pollutants in aquatic environments in an easy and rapid way. Indeeed, they exhibit an important scope of diversity, physiology, morphology, reproductive behaviors, and feeding (Khaled *et al.,* 2016; Khaled *et al.,* 2017; Bodó *et al.,* 2020). Given their sensitivity, simplicity of keeping under laboratory conditions as well as simplicity of measured biochemical and physiological parameters, leeches have been applied by pharmacologists and toxicologists as a pertinent tool for several investigations (Petrauskienne, 2005). As well, leeches can be used as a dependable indicator in cases of heavy metals contamination. In our previous researches, it has been reported that exposure to cadmium induced deleterious effects such as oxidative stress and histologic injuries on

the testes and ovaries of the freshwater leeches *Liminatis nilotica* (Khaled et al, 2022b; Khaled et al. 2023). Also, in his study, Kazlauskienne *et al*. (2010) reported that leeches behavioral responses such as avoidance, mobility, change in body shape, and feeding activities at different phylogenetic and ontogenetic level can be successfully used to evaluate the toxicity of oil hydrocarbons in ambient water. In the present study, we aimed to investigate the effect of Cd exposure on body wall structure in the freshwater leech *L. nilotica* by evaluating biochemical and histopathological responses. In fact, the body wall is mainly considered as the primary entry point of pollutants, and it could be prone to toxic injuries (Kutlu *et al.,* 2010; Kılıç, 2011; Annabi *et al.,* 2013; Baranzini *et al.,* 2020; Bodó *et al.,* 2020). In particular, we seek to evaluate oxidative stress markers in the body wall since they have been extensively used to evaluate the level of tissue injuries and organ contamination (Nna *et al.,* 2017; Wan *et al.,* 2018; Zhang *et al.,* 2021; Khaled *et al.,* 2022). They represent the first line of defense and play a fundamental role in cell protection against xenobiotics and ROS attacks (Çiftçi *et al.,* 2015; Park and Kwak, 2020; Belabed and Soltani, 2022; Khaled *et al.,* 2022a). To our knowledge, there are no previous studies about the toxic effects of cadmium on leech's body wall structures.

Materials and methods

Sample collection

Animals of *L. nilotica* were collected in May 2022 from Tamerza waterfall: a small village in the southwest of Tunisia. Leeches were mature, having a length ranging between 9 and 11 cm. Collected specimens were maintained in laboratory conditions in aerated and pure water for 15 days before experiments for the physiological adjustment to new environmental changes. Specimens of *L. nilotica* were fed weekly with calves' blood.

Experimental design

To perform the bioassay, healthy and sexually mature leeches were chosen and used in the experiment. Leeches were divided into four groups with 10 leeches per group. The first group served as a control, the second group was treated with 100 µg/l of Cd, the third group was treated with 200 µg/l Cd, and the fourth group was treated with 300 µg/l of Cd. Assays were run in triplicates. The Cd concentrations chosen in this study were based on previous studies that focused on leech Cd exposure (Khaled *et al*., 2022). All products used in the assay are bought from Sigma-Aldrich.

Histopathological assessment

For histopathological assessment, five specimens from each group were used and tissues from the leech body wall were fixed in neutral buffered formalin (NBF) 4% for 24 hours. After being dehydrated in increasing concentrations of alcohol each of 20 min, specimens were incorporated in paraffin wax for 24 hours. Sections 6 µm thick were stained with eosin and hematoxylin and examined under Leica Dm 500 light microscopy.

Evaluation of oxidative stress biomarkers

Five specimens of *L. nilotica* from each group were dissected and tissue from the leech body wall was removed and homogenized in 2 ml ice-cold Tris-buffered Saline. Then, after centrifugation at 5000 g for 15 min obtained supernatants were maintained at 4 °C for biochemical assay.

Determination of malondialdehyde (MDA) levels

The thiobarbituric acid (TBA) assay was used currently as a prominent index for lipid peroxidation which is based on the reactivity of MDA with TBA according to Buege and Aust (1978). 125 µl of supernatant was mixed with 15 µl of 20% trichloroacetic containing 1% butylated hydroxytoluene. Centrifugation at 1000 g for 10 min at 4 °C was carried out and 200 µl of the supernatant was mixed with hydrochloric acid (HCl) and 160 µl Tris-TBA for 10 min at 8 °C. The absorbance was measured at 530 nm. MDA level was expressed in nmol of MDA/mg of protein.

Determination of superoxide dismutase (SOD)

SOD activity, as previously described by Beyer and Fridovich (1987), was determined by measuring the inhibition of nitro blue tetrazolium (NBT) reduction. Briefly, 50 µg/l of the supernatant was mixed with 1ml of EDTA/methionine (0.3 mM), 892.2 µl of tampon phosphate Buffer (pH 7.8), 85.2 µl of NBT and finally 22.6 µl of riboflavin was added. The mixture was then exposed to light (lamp) for 20 mn. One unit of SOD was defined as the amount of enzyme required to inhibit NBT reduction by 50% at 25 °C.

Catalase (CAT)

CAT activity was assayed according to Aebi (1984): 20 µl of supernatant, 780 µl of phosphate Buffer (100 mM; pH 7.5), and 200 µl of H_2O_2 (500 mM) were mixed. Absorbance was measured at 20 nm every 15 sec for 1 mn. The measurement of CAT activity was based on the dismutation of H_2O_2 into water and oxygen at 240 nm. CAT activity was expressed as µmol H_2O_2 $min^{-1}mg^{-1}$ protein.

Determination of glutathione peroxidase (GPx) activity

Glutathione peroxidase (GPx) activity was measured by using the method described by Flohé *et al*. (Flohé *et al*., 1984) thereby estimating the oxidation of NADPH at 340 nm. Briefly, 100 µl of supernatant was mixed with 100 µl of EDTA Na_2, 200 µl of GSH (0.1 mM) of H_2O_2. After incubation at 27 °C for 15 mn, 0.5 mL 5% TCA was added. The mixture was then centrifugated at 1500 g for 5 min and the supernatant was recovered. 700 µl of DTNB and 200 µl of phosphate buffer were added to 100 µl of reaction supernatant.

Statistical analysis

Data were presented as mean ± SD and analyzed using GraphPad Prism 9. The basic statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. Value $P<0.05$ were considered statistically significant.

Results

Behavioral alteration

In all Cd-tested treatments, no mortality occurred. Compared to control, (Fig. 1A), no morphological changes were noticed in the group treated with 100 µg/l of Cd (Fig. 1B). However, a permanent contraction and shortening of the whole body, and an excessive amount of mucous secretion was observed in animals that were exposed to higher doses of Cd (200 µg/l and 300 µg/l) (Fig. 1C, 1D).

Histological study

In the untreated leeches (Fig. 2), the body wall appeared with the typical- pattern: the outer surface of the epidermis consists of a rigid cuticle and a layer of large columnar epithelial cells lie beneath and in direct contact with the cuticle. A narrow band of connective tissue lies between the basal lamina of epithelial cells and the musculature of the body wall. The epithelial cells are linked by well-developed junctional complexes. A few secretory cells are found between the epithelial cells.

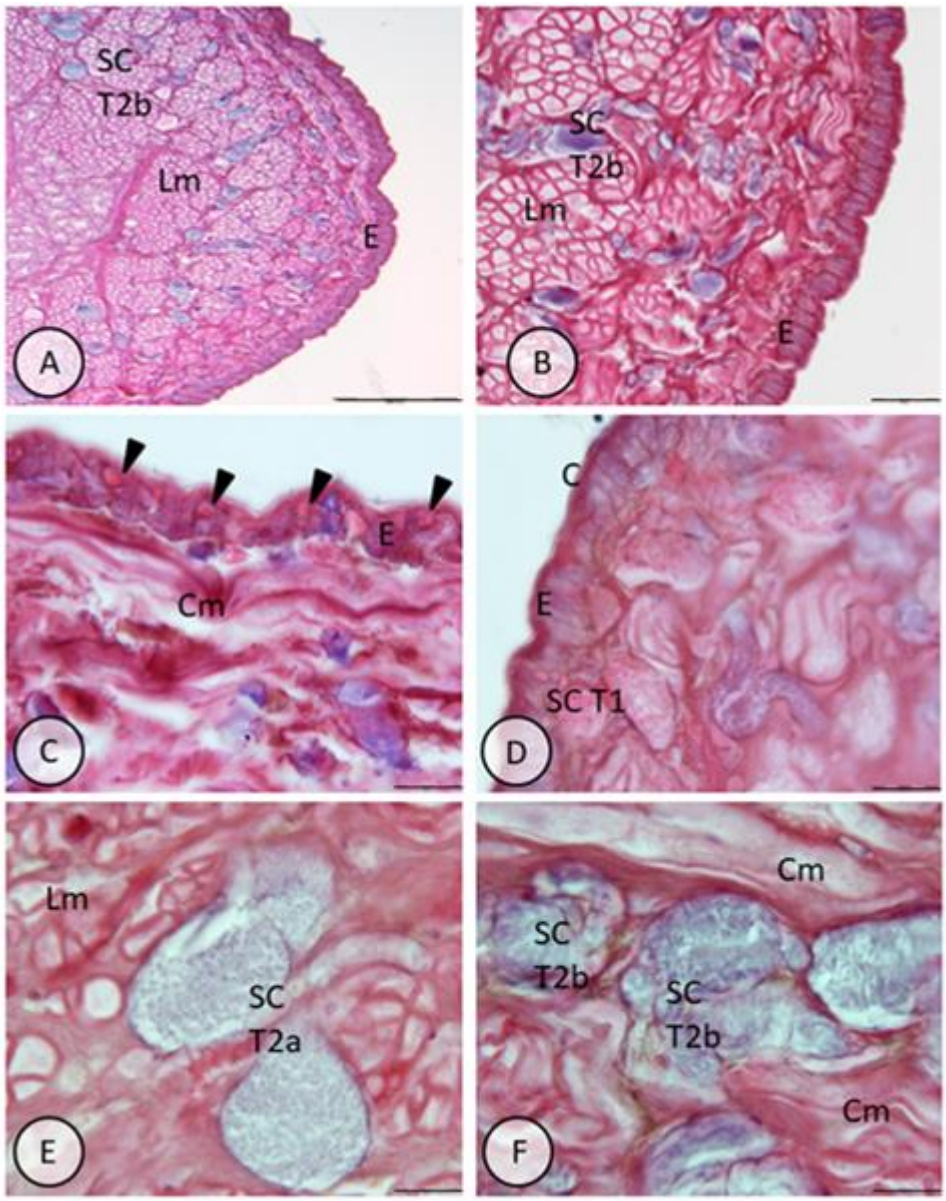

Fig. 2 Transversal section of the body wall of *L. nilotica* (H&E staining). A & B: General view of the body wall in control showing a normal structure. C & D: details of the epidermis (E) and surrounding area (close to the epidermis) including type 1 secretory cells (SCT1, arrowheads) and circular muscles (CM) (X100). E &F: Secretory cells: Type 2a (SCT2a), Type 2b (SCT2b), circular muscles (CM), and longitudinal muscles (LM) (H&E x 400). Epidermis epithelial cells (E), secretory cells (SC), longitudinal muscles (LM), circular muscles (CM), and secretory cells type 2 (SCT2) (H&E x 100). A: Scale bar = 300 µm; (B-F): Scale bar = 50 µm

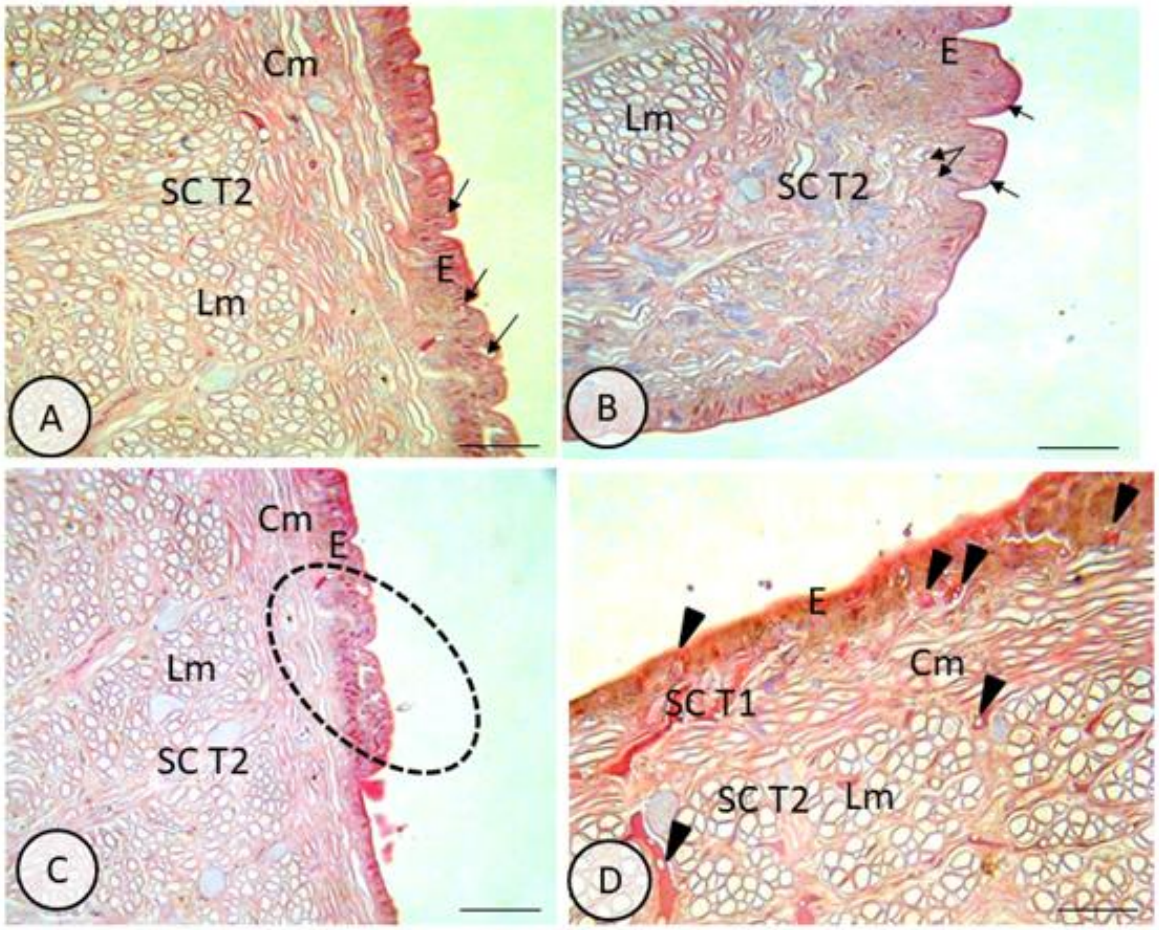

Fig. 3 Group of animals that were exposed to 100 µg/l of Cd. Stain: HE. A - D: Transversal section of the body wall of *L. nilotica*. A: the appearance of vacuolar degeneration in the epidermis (E) (arrows). B: proliferation of secretory cells type 2 (SCT2) without change in their morphology, change in circular cells (CM) morphology with increasing of vacuolization (arrows). C: enlargement and vacuolization of epidermis (ellipse). D: vacuolization and change of the morphology of secretory cells type 1 (SCT1) (arrowheads), the secretory cells type 2 (SCT2) seems not affected (H&E x 100). Scale bar = 50 µm

Cd-exposed leeches displayed several histopathological alterations as shown in Figs. 3, 4 and 5. These histopathological changes are mainly shown in all exposed leeches. In leeches exposed to 100 µg/l of Cd, there was a degeneration of the cuticle, the epidermis appeared with degeneration vacuole, a marked increase in the number of secretory cells type 2 and change of the morphology of secretory cells type 1 (Fig. 3). In leeches exposed to 200 and 300 µg/l of Cd, the body wall showed an increasing number of the secretory cells (type 1 and 2) and changing of their morphology. Moreover, we observed a cuticle and epidermis deformation and increased vacuolization with large degeneration vacuole (Fig. 4 and 5).

The effect of cadmium on MDA levels

As shown in Fig. 6 exposure to 100 µg/l, 200 µg/l and 300 µg/l of Cd afforded a perceptible increase in MDA levels ($p<0.0001$) in a dose-dependent manner as compared to control.

The effect of cadmium on SOD, CAT, and GPx activities

The activities of antioxidant enzymes were evaluated in leeches exposed to 100 µg/l, 200 µg/l, and 300 µg/l for 7 days (Fig. 7). Cd exposure avidly influenced antioxidant enzyme activities in leeches in a dose-dependent manner. The group treated with 100 µg/l of Cd marked a significant increase in SOD, CAT, and GPx activities ($p<0.0001$) compared with the untreated group. In groups treated with 200 and 300 µg/l of Cd, SOD, CAT, and GPx activities exhibited a marked decrease trend from the control group ($p<0.0001$).

Discussion

Contamination of aquatic ecosystems with heavy metals is gradually intensified in many aquatic organisms as a result of human activities given their environmental persistence and tendency to accumulate in living organisms (Mutlu *et al.,* 2016;

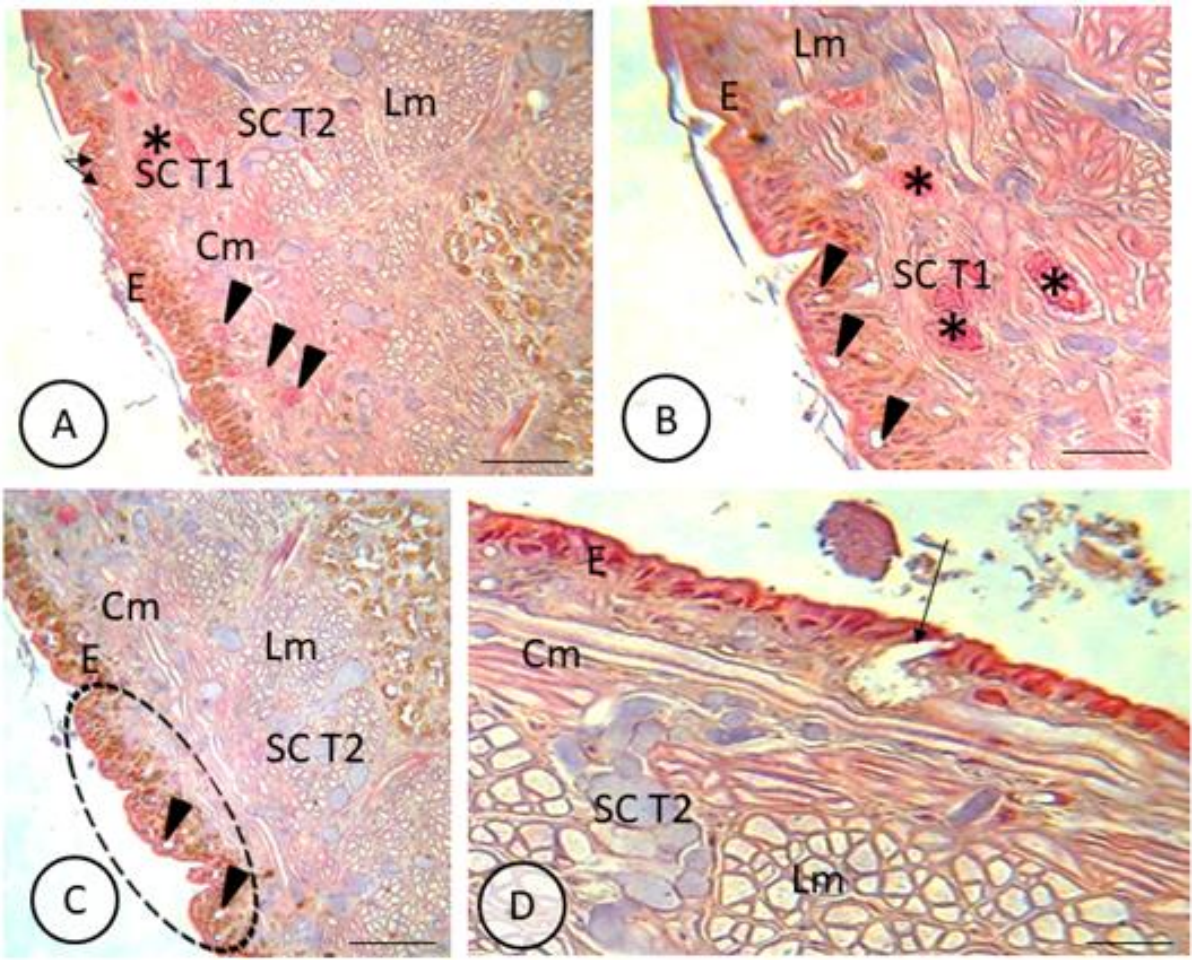

Fig. 4 Group of animals that were exposed to 200 µg/l of Cd (H&E staining). A - D: Transversal section of the body wall of *L. nilotica*: a general view of the body wall. A&B: The epidermis (E) is damaged showing an increasing number of vacuolar degeneration (arrowheads). Note also the damaged aspect of the secretory cells type 1 (SCT1) showing an irregular membrane (asterisk). The secretory cells type 2 (SCT2) seems not affected. C&D: the epidermis displayed increased vacuolization (arrowheads) with increased degeneration vacuole size (arrow), enlargement and torsion of epidermis (ellips). Note also the increasing number of the secretory cells type 2 (SCT2), longitudinal muscles (LM) and circular muscles (CM) are visible and seem not affected (H&E x 100). Scale bar = 50 µm

Ensibi and Yahia, 2017). Leeches, used as a model in the current study, are considered as one of the most important components of freshwater streams, lakes, rivers, and in several terrestrial and marine habitats not only as a part of the food chains but also as parasites of other hydrobiontes (Kaygorodova *et al.,* 2014; Saglam, 2018; Faleh *et al.,* 2019). There has been extensive research into their mechanisms of reproduction and development (Grimaldi *et al.,* 2013; Schorn *et al.,* 2015; Khaled *et al.,* 2022).

Morphological changes could be applied as pertinent biomarkers in toxicity monitoring of specific chemical pollutants as well as the assessment of the chronic and acute exposure of organisms to the impacted environment (Fontanetti *et al.,* 2011; Aguzie *et al.,* 2021). In this context, such morphological biomarkers could detect many levels of damage in several tissues and cell types and indicate qualitative evidence of a functional adaptation to external factors (Fontanetti *et al.,* 2011). In the current study, a comparison of the body wall histologic structure of unexposed leeches with those treated with Cd showed marked histological structure alteration of the epidermis layer and secretory cells. We found that animals of the groups 3 and 4 developed cell and tissue damage, loss of the structural integrity of their body wall. Interestingly, mucus cell proliferation in the epidermis of leeches that were exposed to 200 and 300 µg/l of Cd was very accentuated. These results correlated well with many previous studies which revealed the sensitivity of epithelial cells to environmental pollutants such as pesticides, fungicides, and heavy metals (Selvanathan *et al.,* 2013; Malik *et al.,* 2020; Pat *et al.,* 2022). Indeed,

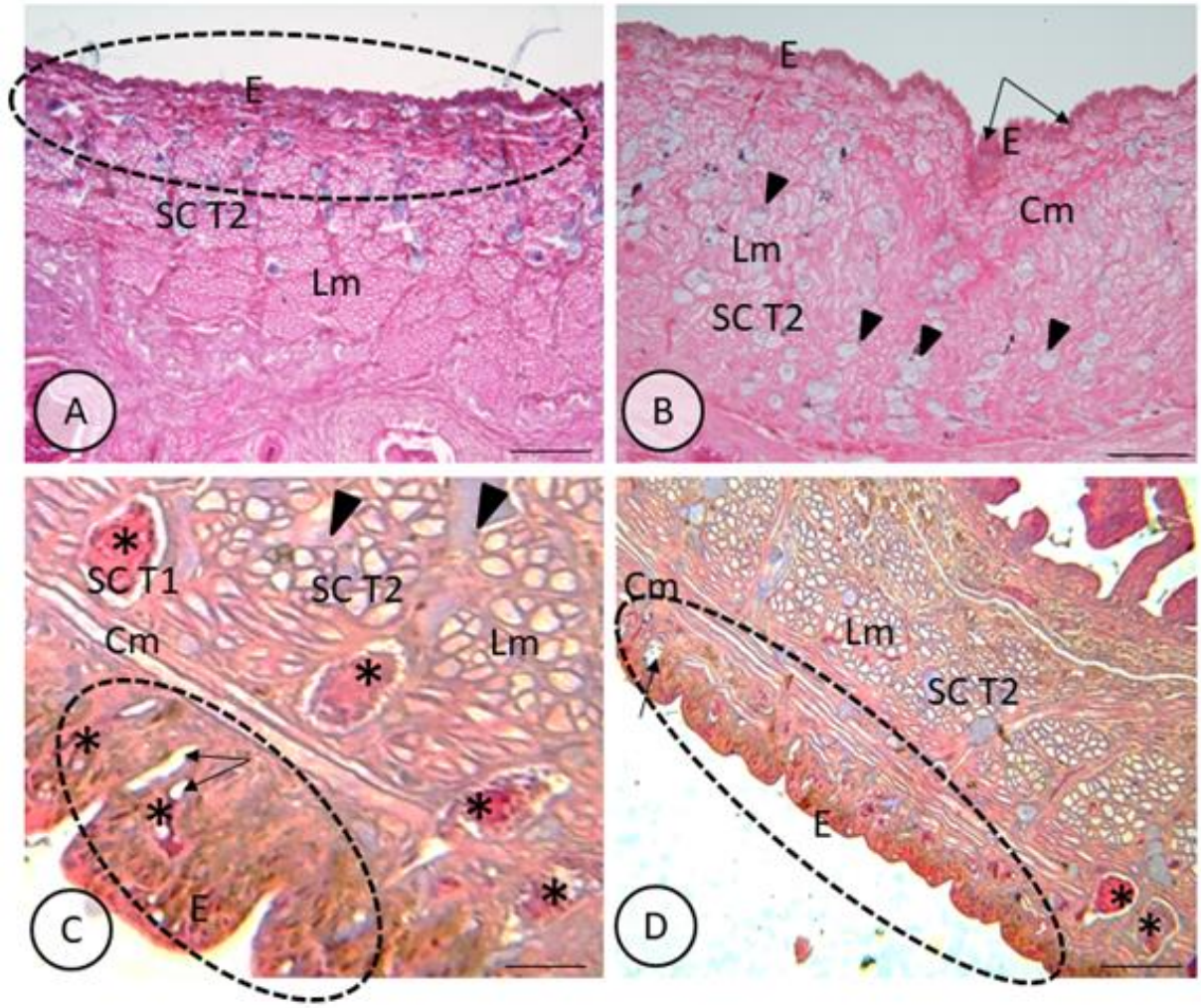

Fig. 5 Group of animals that were exposed to 300 µg/l of Cd (H&E staining). A - D: Transversal section of the body wall of *L. nilotica*: a general view of the body wall. A: note the alteration of the epidermis epithelial cells (E) (ellipse), Secretory cells type 2 (SCT2) and longitudinal muscles (LM) are seen. B: note the change of the epidermis morphology (arrows), the morphology of secretory cells type 2 (SCT2) are also affected (arrowheads). Epidermis epithelial cells (E), longitudinal muscles (LM), and circular muscles (CM) are visible and seem not affected (H&E x 100). C&D: Increased vacuolization of the epithelial cells (ellips) showing the appearance of large vacuole (arrows) and alteration of secretory cell type 1 (SCT1) are visible (asterisk). The arrowheads point to the damaged secretory cells type 2 (SCT2) (H&E x 400). Scale bar = 50 µm

epithelial cell alteration was a common finding in many other studies that focused on the adverse effect of noxious metal exposure and organic pollution (Reddy and Rao, 2008; Das *et al.,* 2019). Swelling, necrosis, separation of cells from the basal lamina, vacuolization in the cytoplasm of epithelial cells, and mucus cell hyperplasia were the most common reported alterations, in agreement with our results (Soegianto *et al.,* 2013; Das *et al.,* 2019; Ismael *et al.,* 2019; Wang *et al.,* 2021). In fact, epithelial tissue represents an important barrier against pathogens (Gül and Çakıcı, 2022). It has been shown that chronic exposure to pesticides in early life could harm crucial barrier integrity, especially skin epithelium, intestine, and airways, leading to the loss of epithelial barrier integrity, inflammation, vacuolization and development of immune-mediated inflammatory disease (Lima *et al.,* 2022). Similarly, the exposure of the medicinal leech *Hirudo verbena* to 0.2 mg/l of copper induced degenerated epithelial cells, disorganized muscle fiber and decreased cuticula fold (Kutlu *et al.,* 2010). On the other hand, the exposure of the earthworm *Lumbricus terrestris* to 50 ppm of mercury led to adverse effects on the body wall including cuticle rupture, laceration, cell shrinkage and loss of their characteristic features (Fawzy Salman *et al.,* 2022). The excessive epidermal mucus secretion may be an early reaction to contact with organic and metal pollution. A similar reaction was reported in many other annelids during exposure to cadmium, uranium, and copper (Lagauzère *et al.,* 2009; Esra

and Arman, 2016; Stabili, 2019). Mucus secretion was a protective reflex and detoxification process that could limit exchanges between the leeches and the Cd. In Annelids, mucus secretions prevent dehydration, absorb metabolites, and protect against parasites due to their antimicrobial activity. In addition, mucus absorption prevents heavy metals from entering the skin (Gül and Çakıcı, 2022). It can also represent a defense mechanism against pollutants and ROS attacks since it could act as an antioxidant and radical scavenger due to the carbohydrate content of its mucoproteins (Moraes *et al.,* 2006; Lagauzère *et al.,* 2009; Stabili, 2019).

In recent years, many studies reported that oxidative stress is a crucial process of metal-induced toxicity and Cd exposure provokes oxidative stress generation in most aquatic organisms that were studied (Das *et al.,* 2019; Zhang and Reynolds, 2019; Zhang *et al.,* 2019). Oxidative stress caused by toxins such as xenobiotics and trace metals often results in the production of reactive oxygen species (ROS) and free radicals that get higher with increasing metal and toxin concentration (Das *et al.,* 2019; Shahriari *et al.,* 2023). Our results showed a considerable increase in MDA level in the body wall of Cd-exposed leeches suggesting that the increase of

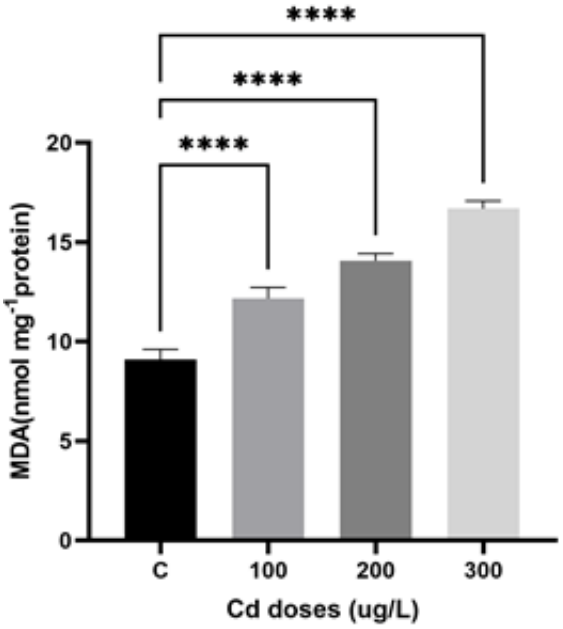

Fig. 6 Effect of Cadmium on MDA levels in the body wall of the freshwater leech *L. nilotica*. All data are described as mean ± standard deviation: **** p< 0.0001

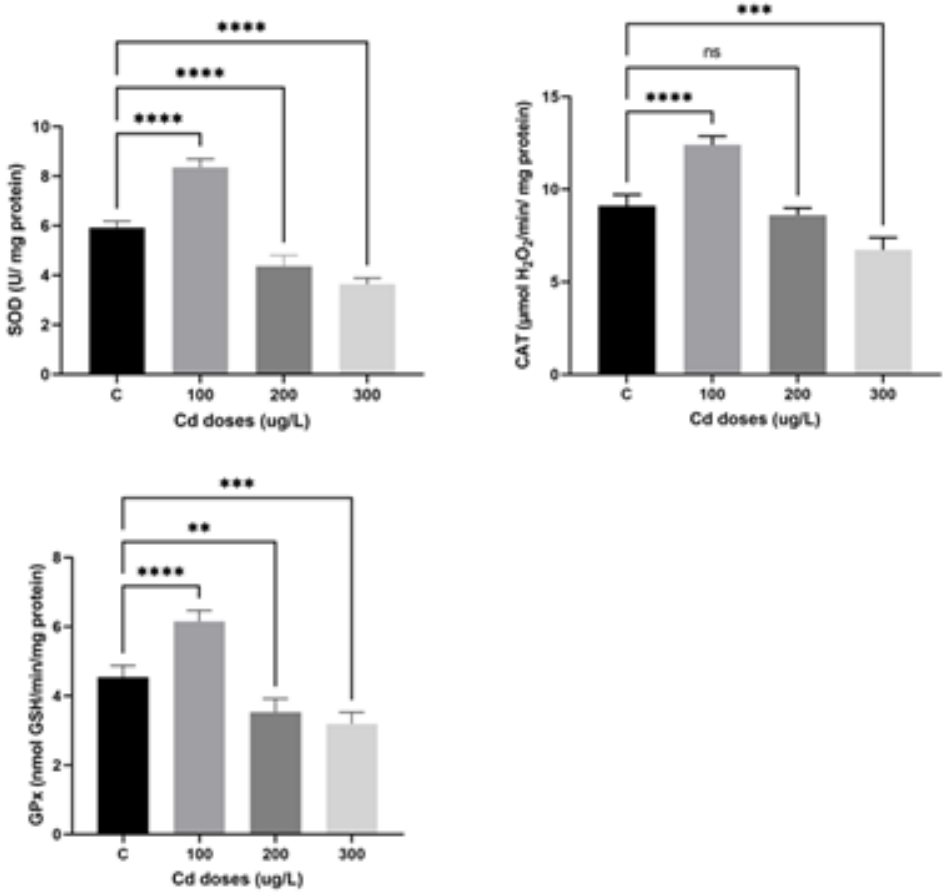

Fig. 7 Effect of Cadmium on (a) SOD, (b) CAT, and (c) GPx activities on the body wall of the freshwater leech *L. nilotica*. All data are expressed as mean ± standard deviation: **** p< 0.0001

lipid peroxidation implied an excess generation of ROS in the body wall of *L. nilotica* under Cd attack which resulted in the membrane structure damage. These findings are in agreement with many other studies that observed an increase in MDA levels, apertinent marker for the harm of oxidative cell damage (Ensibi and Yahia, 2017; Zhou *et al.,* 2017; Das *et al.,* 2019; Kechiche *et al.,* 2021). Many studies revealed that heavy metals including Cd are pro-oxidants and led to the impairment of antioxidant defense systems (Ensibi and Yahia, 2017). In the current study, our results showed that Cd exposure at lower concentrations (100 µg/l), leads to an increase in the activities of antioxidant enzymes SOD, CAT, and GPx in the body wall of *L. nilotica.* This indicates that SOD, CAT, and GPx activities were subsequently stimulated due to H_2O_2 generation. This could be explained by the defense role of the antioxidant system against the harmful attack of Cd resulting in the activation of antioxidant enzymes as a first line of defense. This increase was more likely to be a defensive strategy to counteract oxidative stress and ROS generation. However, with increasing Cd concentration (200 and 300 µg/l) SOD, CAT, and Gx activities were substantially reduced. The inhibition of antioxidant enzyme activities could be due to the substantial increase of ROS concentration as evidenced by the increase of MDA level, and ultimately these enzymes lost their defensive properties due to the increasing toxicity in the leech body wall. Eventually, an exceeding range of Cd concentrations could lead to the impairment of antioxidant enzyme functions. These findings were consistent with many recent studies which interfered with the same results regarding biochemical indexes under Cd exposure. In a recent study conducted by Zhang *et al*., 2020, the exposure of the common carp *Cyprinus carpio* to $4*10^{-5}$ mol/l of Cd caused oxidative stress, lipid peroxidation, apoptosis, and necrosis. Similarly, in the mud crab *S. paramamosin,* exposure to Cd caused *a* significant decrease of SOD and CAT activities and total antioxidation capacity (T-AOC) as well as a significant increase of MDA levels (Chen *et al*., 2021). Similar toxic effects induced by exposure to different concentrations of Cd in the freshwater snail *Bellamya aeruginosa* were linked to the generation of ROS and increased lipid peroxidation (Liu *et al*., 2019).

Conclusion

The present study aimed to investigate the impact of Cd on the body wall of the freshwater leech *L. nilotica.* The toxic effect was evaluated on the basis of histological results and biochemical examinations. We found that Cd exposure led to several disruption in antioxidant stress enzymes, MDA levels, and histopathological alterations in the body wall, mainly alteration of the epidermis layer and secretory cells. Thus, histopathology, lipid peroxidation, and biochemical responses could be pertinent biomarkers to monitor cadmium pollution in aquatic environments.

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