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Histopathological effects of zinc (Zn) on mantle, digestive gland and foot in freshwater mussel, *Anodonta cygnea* (Linea, 1876)

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Abstract: Heavy metals are the most important pollutants in aquatic ecosystems that may cause adverse effects on its biota. In this study, histopathological effects of zinc (Zn) and their incidence time on swan mussel, *Anodonta cygnea*, were studied. Exposure to Zn was done during 18 days and histopathological investigations were conducted in mantle, digestive gland and foot in days 0, 4, 9, and 18. Histopathological changes observed were: damages of epithelium cells with increasing mucous cells (in mantle), atrophy of digestive tubules and haemocyte aggregation (in digestive gland), and hypoplasia, increasing mucous cells and myocyte swelling (in foot). Moreover, granuloma and tissue rupture were found in all organs. Primary histopathological changes were observed in fourth day of examination in all of studied organs. Results showed that sensitivity of digestive gland is lesser than mantle and foot in exposure to Zn. Also the results indicated the histopathological alterations in the organs of swan mussel can be considered as reliable biomarkers in biomonitoring of heavy metal pollution in aquatic ecosystems.

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Introduction

Heavy metal is a general name for a group of metallic elements that have toxic effects in concentrations higher than tolerable physiological levels of animals (Forstner and Wittman, 1983; Rainbow, 2002; Banfalvi, 2011). They have two categories: essential (with structural and biological functions) and non-essential (without any biological role) groups (Simkiss, 1981; Williams, 1981; Banfalvi, 2011). These pollutants may have adverse impacts on biota of aquatic habitats. Zinc (Zn) is one of the essential heavy metals contaminants of freshwater ecosystems. The major anthropogenic sources of this element are processing of ores, electroplating, domestic and industrial effluents, combustion of fossil fuels and soil erosion (Liobet et al., 1988; Buhl and Hamilton, 1990). The reported natural concentrations of Zn were in the range of 0.1-50 μg l^{-1} in freshwaters and 0.002-0.1 μg l^{-1} in marine ecosystems (WHO, 2001).

In last decades, aquatic organisms are used as bioindicators for monitoring chemical pollution of freshwater and marine environments. Bivalves are a group of molluscs that are appropriate for study of biological impacts of environmental pollutants in aquatic ecosystems (Livingstone et al., 2000) and are widely used as bioindicators in biomonitoring (Sanders et al., 1993). With high potential of accumulation of heavy metals in tissues and organs of bivalves, it is possible to detect and assess their occurrence using cellular and physiological responses (Van Duren et al., 2006). Long term exposure to heavy metals can increase susceptibility to disease and development of histopathological malformations (Zorita et al., 2006). Broad varieties of histopathological alterations in fish and bivalves

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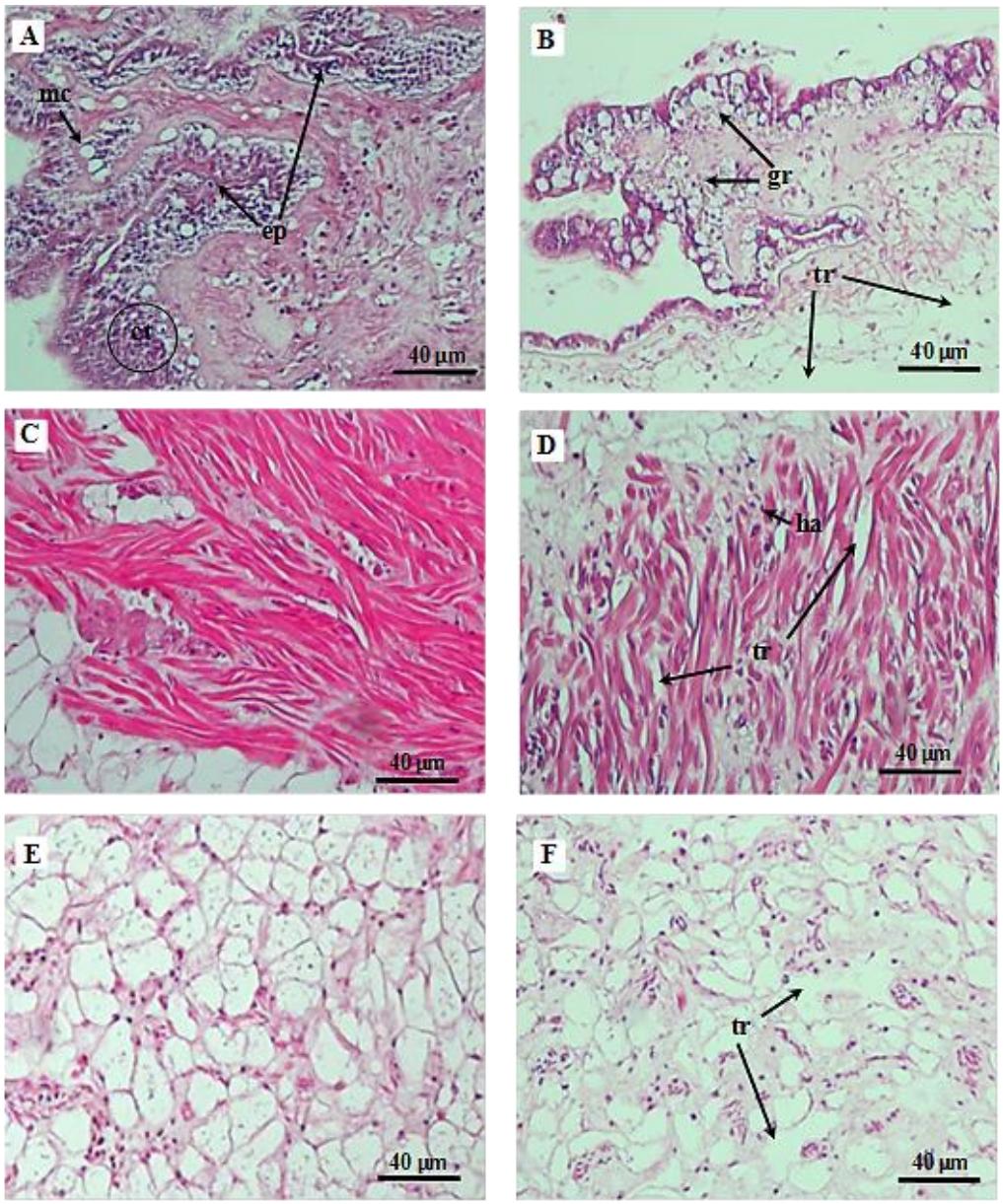

Figure 1. Light micrographs of transverse sections (H&E) of control mantle and metal-exposed specimens. (A, C and E) control and (B, D and F) exposed to Zn. mc: mucous cell; ep: epithelium; ct: connective tissue; gr: granuloma; tr: tissue rupture; ha: haemocyte.

have been developed and offered as biomarkers for monitoring of pollutants (Lowe, 1988; Cajaraville et al., 1992; Yevich and Yevich, 1994; Syasina et al., 1997; Au, 2004).

This study aimed to investigate the histopathological alterations in different organs of swan mussel, *Anodonta cygnea*, exposed to Zn. Swan mussel which belongs to family Unionidae, has a wide dispersal area including North America, Europe, northern Africa and Asia (Pourang et al., 2010). In Iran, this species is found in northern wetlands and rivers and in some coastal regions and due to its wide distribution area, can be considered as an appropriate bioindicator of heavy metal pollution in aquatic ecosystems.

Material and methods

Specimen collection and experimental condition: In October 2011, eighty specimens of *A. cygnea* were collected from the Tajan River estuary (36°48′46′′N, 53°6′57′′E) (Mazandaran Province, Iran). These specimens were transferred to 60L fiberglass tanks for adaption to laboratory conditions. These tanks have been equipped to a circulatory system and

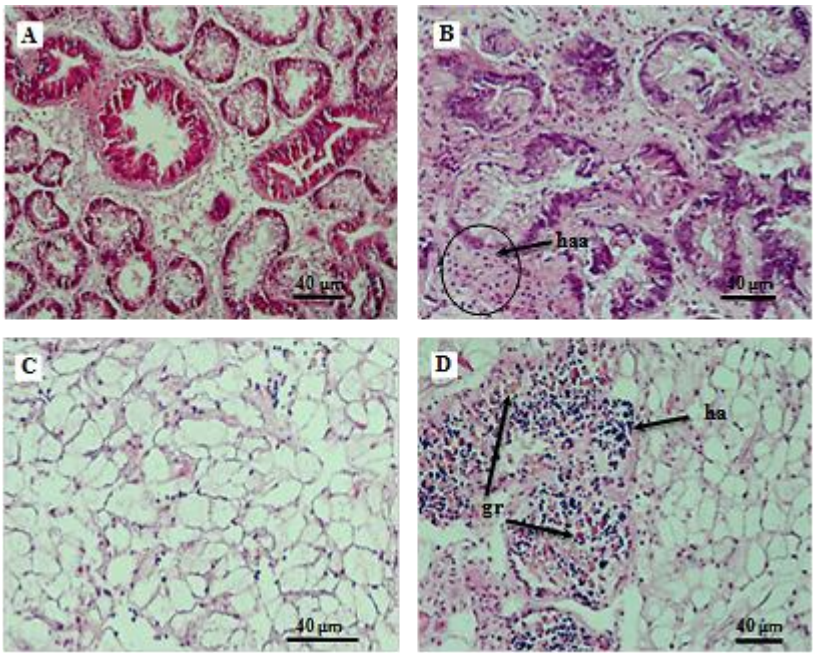

Figure 2. Light micrographs of transverse sections (H&E) of digestive gland of control and metal-exposed specimens. (A and C) control and (B and D) exposed to Zn. haa: haemocyte aggregation; gr: granuloma; ha: haemocyte.

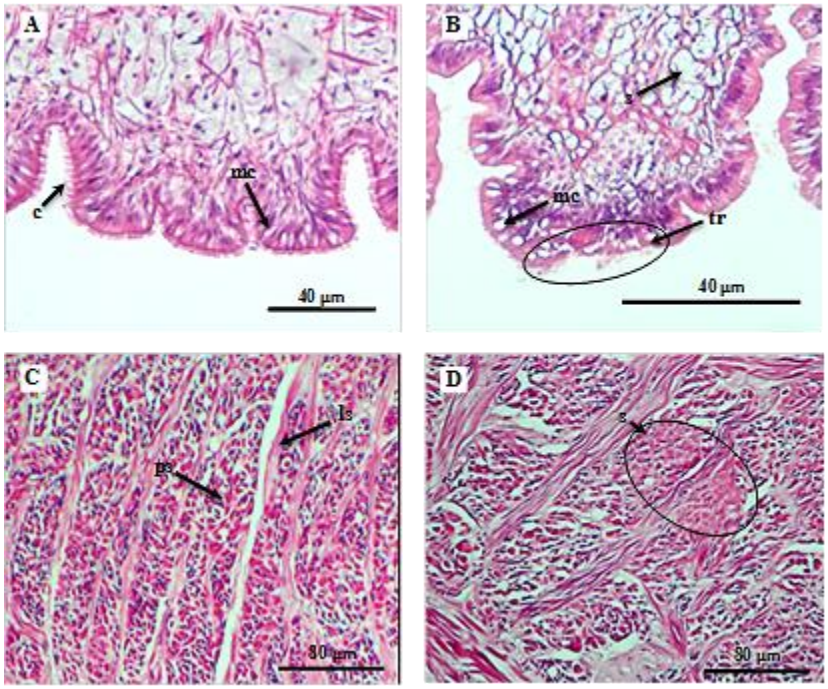

Figure 3. Light micrographs of transverse sections (H&E) of foot of control and metal-exposed specimens. (A and C) control and (B and D) exposed to Zn. mc: mucous cell; c: cilia; s: swelling; tr: tissue rupture; ls: longitudinal section of myocyte; ps: transverse section of myocyte.

aeration. During the experiment, dechlorinated tap water was used. Water temperature and pH were measured daily, which were 15-17°C and 6.8-7.4, respectively. Specimens were not fed through the experiment.

Exposure plan: Twenty four specimens (length: 12.7-13.3 cm) were introduced randomly into 3 experimental tanks (8 specimens per tank). There were 3 replicates in every sampling time and a control tank with 4 specimens. Considering natural concentrations of Zn in aquatic environments and based on previous studies (Naimo, 1995), bivalves were exposed to 125 µg l^{-1} of Zn for 18 days. At days

Table 1. Incidence time pattern of histopathological alterations in mantle, digestive gland and foot of Zn-exposed mussels during study period. Presented histopathological damages were observed in all 3 replicates of mussels in days 4, 9 and 18.

organ	Time		
	Day 4	Day 9	Day 18
Mantle	increase in mucous cells count	Haemocyte infiltration and aggregation in sub-epithelial connective tissues; granuloma; sub-epithelial hyperplasia;	Haemocyte infiltration and aggregation in connective and muscular tissues; tissue rupture
Digestive gland	Loss of digestive cells into tubules	Haemocyte aggregation in connective tissue; Atrophy of tubules; granuloma in interstitial space between tubules.	
Foot	Hypoplasia of external epithelium; increase in mucous cells count	Haemocyte infiltration and aggregation in sub-epithelial tissues; epithelium rupture	Myocyte inflammation (swelling); muscular tissue rupture.

4, 9 and 18, specimens of each tank were removed to collect obtaining tissue samples.

Tissue preparation: The valves of specimens were opened by cutting anterior and posterior abductor mussels using a scalpel. Samples of ≈ 0.5 cm^3 from each target organ (mantle, digestive gland and foot) were removed and fixed into Bouin's solution (saturated picric acid: 75 parts, 40% formaldehyde: 25 parts and glacial acetic acid: 5 parts) for 48h and then stored in 70% ethanol. The histological sections (5-7 µm thickness) were prepared based on Hewitson and Darby (2010) and stained using haematoxylin and eosin. Stained tissues were observed under a light microscope (Leica MS5).

Results

Histopathological observations:

Mantle: Histological sections of the mantle of control group had normal status with contiguous external epithelium with no sign of alteration in connective tissue of sub-epithelial (Fig. 1A, C and E). Mucous cells were seen sporadically in epithelium and sub-epithelium with equal size (Fig. 1A). The muscular cells were with conjunct structure (Fig. 1C) and the connective tissue was healthy (Fig. 1E).

Histological observations of treatments showed specific signs of tissue damage in mantle (Fig. 1B, D and F). The width of external epithelium was decreased and the number of the mucous cells in sub-epithelial layer were increased (Fig. 1B). Granuloma (pigmented cells with yellow to brown color) was found in sub-epithelial layers (Fig. 1D). Tissue rupture and dissociation of regular cellular structure in the muscular tissue observed (Fig. 1B, D and E). In addition, the haemocyte infiltration and aggregation of the myocytes was occurred (Fig. 1D).

Digestive gland: Figures 2A and C, display histological status of digestive gland and their junctions in control group with healthy basophilic columnar epithelial cells and digestive (dingy) cells in tubule structure. Also, connective tissue of interstitial space between tubules was normal with similar distribution of haemocytes (Fig. 2C).

Digestive gland exposed specimens showed atrophy of digestive tubules and loss of digestive and basophile cells into the tubule (Fig. 2B). Intensive haemocyte aggregations in connective tissue and interstitial space (Fig. 2B and D), as well as, granuloma was found in connective tissue observed (Fig. 2D).

Foot: In the control group, external epithelium with integrated ciliary structure was observed (Fig. 3.A). In subepithellial layer, the mucous cells had uniform distribution. In inner parts of foot, groups of myocytes were in various directions as their longitudinal and cross-sections were visible (Fig. 3C).

The foot of Zn-exposed specimens represents an increase in the number of mucous cells and swelling of connective tissue of subepithellial layers (Fig.3B). Hypoplasia of external epithelium was occurred. There were tissue rupture in epithelium and layers below that (Fig. 3B). Also, inflammatory myocytes were observed (Fig. 3D).

Incidence time pattern of changes: The chronological trends of histopathological effects of metal exposure are presented in Table 1. In the fourth day, histological changes were found in all organs. Intensity of damage in the mantle and foot was greater than the digestive gland. Haemocyte aggregation in the connective tissues was a common change in all studied organs in the ninth day. In day 18, haemocyte aggregation in muscular tissue of mantle was found, while in other two organs, such damage was not occurred. In day 18, an intense histological damage was observed in mantle and foot.

Discussion

Exposure to Zn is led to histopathological alterations in mantle, digestive gland and foot tissues. Haemocytes and other lipopigmented cells (garanulocytomes) are related to sorption and storing of toxic chemicals (Johansson and Söderhäll, 1992). These cells were found in mantle and digestive gland of studied organism. Occurrence of this type of cells due to metal exposure has been reported in previous studies (Neff et al., 1987; Wolfe, 1992; Chakraborty et al., 2012). Haemocyte infiltration and aggregation in damaged area of different tissues is a common defensive response of organism against toxic agents (Oliver and Fisher, 1999) which was found in this study in connective and muscular tissues of mantle and digestive gland.

Cilia of the external epithelial cells of mantle and foot are involved in dynamic activity of these organs. Exposure to Zn led to destruction of cilia. The loss of cilia (hypoplasia) of gill has been reported by Al-Subiai et al., (2011) as a result of Cu exposure. Damaging of ciliary structures of epithelial cells weakens the moving performance of these organs. Increase in number of mucous cells in mantle and foot was occurred in subepithellial regions after loss of cilia. Likely, increase in number of these cells was occurred to compensate attenuation of dynamic operation.

Our results showed apparent histopathological signs in different parts of digestive gland as result of exposure to Zn. Morphology of digestive tubules changed. Atrophy of digestive tubules in digestive gland in several studies reported in metal exposed organisms as a histopathological damage (Chakraborty et al., 2010; Sheir and Handy, 2010; Sheir et al., 2010; Al-Subiai et al., 2011). Digestive cells of tubules spilled into internal space of tubules. Usheva et al. (2006) reported such a histological damage in *Crenomytilus grayanus* in exposure to DDT.

As the results of this study, the regions where damages were greater, tissue rupture and disintegration including connective and muscular tissues (in mantle and foot) were found. Tissue rupture has been reported by Al-Subiai et al. (2011) in *Mytilus edulis*, due to Cu exposure. Besides, it has been reported that heavy metals can be stored in haemocytes and interstitial storage tissues in bivalve organs (Regoli and Orlando, 1994; Soto et al., 1996). Thus, tissue rupture can be assigned to Zn storage in connective tissue of the mantle.

Results showed swelling and inflammation of myocytes and mucous cells in foot in treatments. Previous studies reported swelling of myocytes in abductor muscles of *Mytilus edulis* (Al-Subiai et al., 2010) and swelling of pore cells in *Littorina litorea* (Watermann et al., 2008) due to exposure of heavy metals.

First signs of histopathological damages during experiment were appeared in mantle, digestive gland and foot in fourth day after exposure. Foot and mantle are directly contacted with pollutant, but exposure route of digestive gland is indirect. During experiment, by approaching to day 18, the extent of damages increased. In organs such as foot in which tissue mass intensity is greater, damages have been extended to inner parts at the end of experiment.

Based on finding of this study, exposure of *A. cygnea* to chronic concentrations of Zn is led to histopathological changes in their mantle, digestive gland and foot. Sensitivity of the mantle and foot are greater than digestive gland to Zn exposure. As a general conclusion, histopathological alterations in mantle, digestive gland and foot in *A. cygnea* are suggested as biomarkers for biomonitoring of heavy metal pollution in aquatic ecosystems.

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