Effect of Sublethal Nickel Chloride Exposure on Crayfish, *Astacus leptodactylus* Ovary: An Ultrastructural, Autometallographic, and Electrophoretic Analyses

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Abstract: Cytological responses in different organs of sentinel organisms have proven to be useful tools for characterizing the health status of those organisms and assessing the impact of environmental contaminants. Our study shows that nickel (II) accumulated in both germ cells (oogonia and developing oocytes) and somatic cells (muscle cells, follicle cells) in the *Astacus leptodactylus* ovary. Muscle cells from ovarian wall show disorganization and the disruption of cytoplasmic microtubules and pyknosis of the cell nucleus. Follicle cells, both those that surround the developing oocytes and also those that are not associated with the oocytes contained within the cytoplasm vacuoles of different sizes, degenerated mitochondria, myelin bodies, disorganized microtubules, and pyknotic nuclei. The most evident pathological phenomenon was the alteration and disorganization of the basal matrix, which separates the ovarian interstitium from ovarian follicles compartment. Exposure to nickel induces cytoplasmic vacuolation in oogonia and developing oocytes, structural alteration of the developing yolk granules and condensation of the nucleoli. Ultrastructural autometallography has shown grains of silver-enhanced nickel inside the cytoplasm of the muscle cells with altered morphology, including the cytoplasm, nucleus, and basal matrix of the follicle cells, and in intracisternal granules and developing yolk granules of the oocytes.

Key words: crayfish, ovary, nickel, ultrastructure, autometallography

INTRODUCTION

Freshwater crayfish are prime candidates for indicator status among invertebrates because of their large size, longevity, and reliance on aquatic systems throughout their life (Reynolds & Souty-Grosset, 2012). Crayfish can be used to monitor the aquatic environments for heavy metal pollution because they are solitary bottom dwellers, which keep much of their bodies in contact with surrounding objects, can tolerate polluted environments and reflect pollution levels due to accumulation of respective metals in their tissues (Khan et al., 1995; Schilderman et al., 1999). Through their position in the food web, crayfish also have the potential for transferring toxins and contaminants to other organisms of higher trophic levels (Wigginton & Birge, 2007). Moreover, because of economic importance as commercial food for human consumption, accumulation of metals in crayfish tissues may represent a risk hazard to human health.

Nickel (Ni) is a chemical element found in the Earth’s crust that is ubiquitous in environmental compartments such as air, soil, and water (Laulicht et al., 2015). On the other hand, nickel and its compounds are of growing toxicological interest due to its large industrial application and the quantity released in the environment (Kienle et al., 2009; Vandenbrouck et al., 2009; Attig et al., 2010). Natural sources of Ni in the aquatic environment include erosion and weathering, whereas anthropogenic addition of Ni occurs from industrial practices such as alloy processing, smelting, and combustion of fossil fuels mining (Eisler, 1998; Blewett & Wood, 2015).

The toxicity of nickel compounds on female reproductive system has been studied in different biological systems, both in cell line systems (Sen & Costa, 1985; Shiao et al., 1998; Kročková et al., 2013) and different organisms, which include cockroach (Kindle et al., 1990), copepods (Mohammed et al., 2010), *Daphnia* (Pane et al., 2004; Taylor et al., 2016), fish (Nath & Kumar, 1990; Sioson & Herrera, 1995–1996; Brix et al., 2004; Alsop et al., 2014), mouse (Rao et al., 2009), and rats (Forgács et al., 1997; Kääkä et al., 1999; Kong et al., 2014).
Nickel accumulation in crayfish has been studied mostly by quantitative methods, which gave information on retention and elimination of the metal from different organs under different concentrations and times of exposure, in both the field (Alikhan & Zia, 1989; Alikhan et al., 1990; Bardeggia & Alikhan, 1991; Khan et al., 1995; Gherardi et al., 2002; Mackevičienė, 2002; Khan & Nugegoda, 2003; Kuklina et al., 2014) and the laboratory (Zia & Alikhan, 1989; Alikhan et al., 1990; Mwangi & Alikhan, 1993).

Cytological responses in different organs of vertebrates and invertebrates have proven to be useful tools for characterizing the health status of organisms and assessing the impact of environmental contaminants on exposed animals (Schramm et al., 2000; Gernhofer et al., 2001; Arockia Vasanthi et al., 2014). Microscopy remains an essential resource that provides direct and strongly supported data to explain and address safety concerns that arise in toxicity studies (Fagerland et al., 2012; Massar et al., 2012; Massar et al., 2014; Dey et al., 2015). Transmission electron microscopy (TEM) is an invaluable tool in the interpretation of the parameters used to measure toxicity, and contributes to the establishment of measures that aim to prevent or eliminate environmental contamination, a risk not only to ecosystems, but also to human health (Fontanetti et al., 2010).

Subcellular accumulations of heavy metals have not been available until the introduction of autometallography (AMG) (Timm, 1962; Danscher, 1984; Danscher & Møller-Madsen, 1985; Stoltenberg & Danscher, 2000). So far, AMG has been performed on crustacean tissues only to demonstrate mercury in gills, hepatopancreas, midgut and antennal glands of brown shrimp, Cragon crangon (Andersen & Baatrup, 1988), and shore crab Carcinus maenas (Laporte et al., 2002).

The present study aims to investigate at the ultrastructural level the effects of nickel chloride exposure on the ovary of Astacus leptodactylus, a crustacean representative for the monitoring of the freshwater environments, and to apply the AMG technique for localization of this heavy metal. Also, we correlate these findings with electrophoretic patterns in the ovary of crayfish exposed to nickel. To our knowledge neither nickel-induced ultrastructural changes and localization of this heavy metal by AMG nor electrophoretic patterns in the ovary of crayfish exposed to nickel have been reported on invertebrate ovary.

**Materials and Methods**

**Animals**

Females of narrow-clawed A. leptodactylus were collected, using a standard pond net, from ponds in the southern part of Romania and transported to the laboratory in plastic boxes. The specimens were acclimatized to laboratory conditions for 2 weeks, in aerated 180 L glass aquaria, with dechlorinated tap water, at room temperature (25°C).

All the experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee, in accordance with Romanian governmental guidelines for ethics in animal experiments.

**Nickel Exposure**

A quantity of 70 mg/L NiCl₂ (NiCl₂ × 6H₂O, 237.69 g/mol N6136-100G; Sigma-Aldrich, St. Louis, MO, USA) was added to aquarium water (20 L) as a nickel chloride solution, 30 min before addition of the crayfish (n = 15). The animals were sacrificed after 24 h, 3 and 7 days exposure. Five crayfish were kept in clean freshwater, serving as controls.

After beginning the nickel exposure, food was withheld (for both control and exposed crayfish), to minimize the effects of feeding on metabolic processes (Brett & Zala, 1975), until the end of the experiment (a total of 7 days).

The water in each aquarium was replenished every day to keep the metal concentrations constant.

**TEM**

Small ovary fragments were fixed in 2.5% glutaraldehyde buffered at pH 7.4 in 0.1 M sodium cacodylate, post-fixed in 1% OsO₄ in 0.1 M sodium cacodylate buffer, dehydrated and embedded in epoxy embedding medium (Fluka, 45345; Fluka, Ronkonkoma, NY, USA). Ultrathin sections were stained with uranyl acetate and lead citrate and then studied with a 208S Philips/FEI TEM (FEI Electron Optics B.V., Eindhoven, The Netherlands) operated at 80 kV.

**AMG**

For light microscopic autometallography fragments of crayfish ovaries were fixed in Bouin solution, dehydrated in ethanol, cleared in toluene, and embedded in paraffin. The 6-µm thick sections were silver-enhanced for microscopic analysis following the autometallographic method of Danscher (1984) and Danscher and Møller-Madsen (1985).

In brief, paraffin sections were incubated 1 h, at 26°C, in the dark, in AMG solution: 25% gum arabic, sodium citrate buffer (0.80 M sodium citrate in 1.27 M citric acid), 0.51 M hydroquinone, and 0.03 M silver lactate. All solutions were prepared with deionized water. After development, the sections were washed in running tap water at 40°C for 40 min and then incubated for 12 min in 5% sodium thiosulfate. The sections were counterstained with hematoxylin and examined with a Zeiss Axiosstar Plus microscope (Zeiss, Oberkochen, Germany). The photomicrographs were taken by digital camera (AxioCam MRc 5; Zeiss) driven by software AxioVision 4.6 (Zeiss).
Electrophoretic Analysis

Ovary fragments (0.1 g) were homogenized in 0.015 M Tris-HCl buffer, pH 6.8 containing 2 mM phenylmethanesulfonyl fluoride as protease inhibitor and 0.5 M NaCl, at 25°C, for 1 h (Zarnescu et al., 1997). Each homogenate was centrifuged at 11,000 x g, at 4°C, for 30 min. The surface lipid layer was removed, while the cloudy middle layer containing the yolk protein extract was separated in Eppendorf tubes, at −20°C, until analysis. Total protein content was assayed by the method of Bradford (1976).

Proteins were separated in a Biometra vertical slab gel apparatus (Biometra, Horsham, PA, USA), according to the method of Laemmli (1970), as previously described (Zarnescu et al., 1997). In brief, samples diluted with solubilization Laemmli buffer were denatured by heating at 100°C, for 5 min; 20 μL of each sample was loaded onto the gel and was separated in a 7.5% polyacrylamide gel. Electrophoresis was carried out at a constant current of 20 mA, for 1.5 h, until the tracking dye reached the bottom of the gel. Then, gels were stained with Roti-Blue 5 × solution, according to manufacturer’s instructions, and destained in 25% (v/v) methanol. Samples were co-migrated with high range molecular weight marker (36,000–200,000 Da) (Sigma-Aldrich).

For sample visualization and documentation, the gel was photographed under visible light illumination with a gel-densitometer with high-end camera (Vilber Lourmat, Cedex, France). Densitometric analysis of protein band intensity was performed using Gel Analyzer 2010 software. The results were reported as means of three replications.

The results were presented as mean values ± standard deviation for three independent samples (n = 3). Statistical analysis of the data was performed using the one-tailed paired Student’s t-test on each pair of interest. Differences were considered statistically significant at p < 0.05, as a minimal level of significance.

RESULTS AND DISCUSSION

The A. leptodactylus ovary is organized in three compartments: the ovarian wall, the ovarian follicles (ovarian sac),

Figure 1. Representative electron micrographs of ovarian walls of the control (a) and nickel-exposed (b–d) Astacus leptodactylus. b: At 3 days after nickel exposure the ovarian wall was disrupted showing wide spaces between the muscle cells (asterisks), and disorganization of cytoplasmic microtubules. The arrow indicates nucleus of muscle cell. c: Disorganized ovarian wall extensions (asterisks) inside the ovary at 7 days after nickel exposure. d: Pyknotic, fragmented muscle cell nucleus (arrow) at 7 days after nickel exposure. Mc, muscle cell.
and interstitial cells. The ovarian follicles include both germ cells (oogonia and developing oocytes) and somatic cells (follicle cells). The interstitial compartment situated between the inner muscular layer of ovarian wall and the basal matrix of ovarian follicle compartment contains muscle cells, hemal sinuses, blood vessels, and blood cells. Ultrastructure of the *A. leptodactylus* ovary has been described recently by our group (Petrescu et al., 2016).

### Ovarian Wall

In the control crayfish, the ovarian wall is composed of densely packed muscle cells, blood cells, blood vessels, and hemal sinuses. Also, the ovarian wall forms extensions which branch inside the ovary. Muscle cells are spindle shaped, have a central nucleus with invaginated nuclear envelope and differ from typical smooth muscle in that their cytoplasm contains numerous microtubules (Fig. 1a). In contrast, in the nickel-treated crayfish the ovarian wall was disrupted (Fig. 1b–1d), and abnormally large spaces were present between the muscle cells. Disorganization and the disruption of cytoplasmic microtubules also occurred in muscle cells from ovarian wall (Fig. 1b) and from ovarian wall extensions inside the ovary (Fig. 1c). Pyknosis and fragmentation of the nucleus was observed mainly after 7 days of *in vivo* exposure to nickel (Fig. 1d). Previous data indicated that nickel can affect the cytoskeletal protein sulphhydrils and the organization of the cytoskeletal elements, microtubules, and microfilaments in cultured 3T3 cells (Lin & Chou, 1990; Li et al., 1993). Few studies have been published on effects of nickel on muscle cells morphology. In fact we found only one light microscopy study (Jayaseelan et al., 2014), which showed that nickel nanoparticles caused degeneration in muscle bundles, focal area of necrosis, vacuolar degeneration, and splitting of muscle fibers in exposed skin of fish *Oreochromis mossambicus*.

In the light microscope, AMG revealed a deposition of metals in the ovarian wall muscle cells and in the intercellular spaces (Fig. 2a). Nickel grains were found intracellularly in the cytoplasm and nucleus of the muscle cells. Ultrastructural AMG have shown grains of silver-enhanced nickel inside the cytoplasm of the muscle cells with altered morphology (Fig. 2b). Despite the fact that there are no autometallographic studies on localization of nickel in muscle cells, quantitative studies performed on crustaceans have reported accumulation of nickel in the abdominal muscles of *Astacus astacus* (Mackevičienė, 2002; Kuklina et al., 2014) and *Cambarus bartoni* (Bagatto & Alikhan, 1987; Mwangi & Alikhan, 1993).

### Follicle Cells

In the crayfish ovary, the follicle cells were found as cellular cords continuous with follicular cells remaining in the ovary after ovulation, or as a simple follicular epithelium that surround the developing oocytes. In the control animals, follicle cell have nuclei with different sizes and shapes and the cytoplasm contains mainly mitochondria, Golgi apparatus, endoplasmic reticulum, and an extensive microtubule network (Fig. 3a). Nickel caused severe ultrastructural changes in follicle cells, the damage severity increased with treatment duration. Follicle cells, both those that surround the developing oocytes and also those that are not associated with the oocytes, contained within the cytoplasm vacuoles of different sizes, degenerated mitochondria and endoplasmic reticulum, myelin bodies, disorganized microtubules, and pyknotic nuclei (Fig. 3b-3c). The most evident pathological phenomenon was the alteration and disorganization of basal matrix which separates the ovarian interstitium from ovarian follicles compartment (Fig. 3d).

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**Figure 2.** Autometallographic staining of ovarian wall from nickel-exposed *Astacus leptodactylus*. **a:** Light micrograph demonstrating autometallography (AMG) deposits in the ovarian wall muscle cells (arrows) and in the intercellular spaces (arrowheads) at 24 h after nickel exposure. **b:** Electron micrograph showing AMG grains (arrows) inside the cytoplasm of the muscle cells with altered morphology (asterisk) at 3 days after nickel exposure. Ow, ovarian wall.
In the light microscope, nickel grains were mainly localized inside the follicular cell cords (Fig. 4a) and in the basal matrix that separate the ovarian follicles compartment from the ovarian interstitium (Fig. 4b). Ultrastructural AMG has shown grains of silver-enhanced nickel inside the nucleus and cytoplasm of the follicle cells (Fig. 4c). Moreover, nickel deposits were found in the basal matrix (Fig. 4d). The accumulation of nickel within basal matrix indicated the nickel is absorbed from the hemolymph. A number of AMG studies have shown that heavy metals as mercury (Baatrup et al., 1986; Andersen & Baatrup, 1988), zinc (Soto et al., 1996; Marigomez et al., 2002), and bismuth (Danscher et al., 2000) are trapped in the basement membrane. The presence of nickel in the basement membrane is attributed to its high affinity for anionic glycosaminoglycan sites of basement membranes (Templeton, 1987a, 1987b). Considerable evidence has been accumulated that nickel ions bind to and damage DNA (Nagy et al., 2011). Previous studies have shown that the primary target of Ni(II) is the nucleus, causing chromatin condensation through heterochromatization (Costa et al., 1994). Also, nickel replaces the natural divalent cation, magnesium ion (Mg$^{2+}$) (Ellen et al., 2009) and has an epigenetic carcinogenic effect by enhanced DNA methylation and compaction, rather than by mutagenic mechanism (Lee et al., 1995).

**Oocytes**

The ovary of the control crayfish used in the experiment was characterized by the presence of oogonia, previtellogenic and early vitellogenic oocytes. The cytoplasm of early vitellogenic oocytes contains numerous intracisternal granules and different sizes of developing yolk granules (Fig. 5c). Exposure to nickel induces different levels of cytoplasmic vacuolation in oogonia and developing oocytes (Fig. 5a, 5b). While in oogonia vacuoles are distributed throughout the cytoplasm (Fig. 5a), previtellogenic oocytes exhibit cytoplasmatic vacuolation mainly in the cortical cytoplasm (Fig. 5b). The results showed that when compared with controls (Fig. 5c) developing yolk granules have an altered structure (Fig. 5d). Nickel also induced nuclear morphological changes that includes irregular nuclear envelope, nuclear envelope...
fragmentation, and condensation of the nucleoli. Figures 5e, 5f show comparison between morphology of the nucleolus in the control (Fig. 5e) and nickel-treated crayfishes (Fig. 5f). Under the light microscope, AMG deposits were found in the cytoplasm (Fig. 6a), nucleus and nucleoli (Fig. 6b) of early previtellogenic oocytes. Moreover, at the ultrastructural level AMG grains were found in intracisternal granules (Fig. 6c) and different sizes of developing yolk granules (Fig. 6d). Yolk formation in crustacea involves both intra and extraovarian yolk synthesis (Adiyodi & Subramoniam, 1983). Primary proteic vitellus is generally synthesized by the ovary, while vitellogenin, a lipoprotein complex, is present in the hemolymph and internalized into oocyte through endocytosis (Meusy, 1980). Intracisternal granules are considered to be the sites of the endogenous precursors of proteinaceous yolk (Beams & Kessel, 1963). Previous studies have shown the presence of coated vesicles in previtellogenic oocytes of A. leptodactylus, suggesting that, before the onset of vitellogenesis, follicle cells can deliver other substance into oocytes by receptor-mediated endocytosis (Petrescu et al., 2016). In light of the findings in our study, we can therefore reasonably speculate that nickel may bind to various ligands in hemolymph or follicle cells and in this form is incorporated through endocytosis in the yolk.

Oocyte quality is a very important factor in reproductive success of a species, its impairment influencing reproductive functions and affecting the offspring quality. Recent studies have shown that Ni exposures affects fertilization success and early-life stages in a range of marine invertebrates like corals (Reichelt-Brushett, 1998; Howe et al., 2014a; Reichelt-Brushett & Hudspith, 2016), anemone (Howe et al., 2014a, 2014b), sea urchin (Novelli et al., 2003; Phillips et al., 2003), abalone (Hunt et al., 2002), polychaete (Gopalakrishnan et al., 2007; Gopalakrishnan et al., 2008), and copepod (Mohammed et al., 2010), Daphnia (Taylor et al., 2016).

Electrophoretic Analysis

Through sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with ovarian protein extract of control and nickel-treated crayfish we have observed a noticeable modification of 92 kDa band intensity (Fig. 7a). The control group had the highest 92 kDa protein level and, after 7 days exposure to nickel chloride, the intensity of ovarian 92 kDa protein subunit decreased compared with controls and with 24 h and 3 days after exposure (Fig. 7a). Densitometry using Gel Analyzer 2010 software has confirmed a significant decrease ($p < 0.05$) of the band intensity from

Figure 4. Autometallographic staining of follicle cells from nickel-exposed Astacus leptodactylus. Light micrograph showing autometallography (AMG) staining (arrows) inside the follicular cell cords (a) and in the basal matrix that separates the ovarian follicles compartment from the ovarian interstitium (b) at 24 h after nickel exposure. Electron micrograph showing AMG grains (arrows) of silver-enhanced nickel inside follicle cells nucleus (c) and basal matrix (d) at 7 days after nickel exposure. Bm, basal matrix; N, nucleus.
100% in the control to 65.43% after 24 h, and 61.73% after 3 days, to 5.5% in Ni-treated tissue for 7 days (Fig. 7b). It is possible that other proteic bands to suffer modifications of intensity, but they were not as visible as for 92 kDa protein band. In this regard, electrophoretic studies on crabs have shown that accumulation of vitellin and the percentage of ovary total protein decreased with the increase of cadmium concentration (Kang et al., 2012). Depletion in protein level in the ovary was also observed in freshwater prawn Macrobrachium kistnensis, exposed to tributyltin (Kharat et al., 2009).

Figure 5. Representative electron micrographs of oocytes of the control (c,e) and nickel-exposed (a,b,d,f) Astacus leptodactylus at 7 days after exposure. Cytoplasmic vacuolation (asterisks) in oogonia (a) and developing oocytes (b). c: Developing yolk granules (Y) in the control crayfish. d: Developing yolk granules with an altered structure in nickel-exposed crayfish. e: Morphology of nucleolus (Nu) in the control crayfish. f: Condensation of the nucleoli (arrows) in nickel-treated crayfishes. Ig, intracisternal granule; O, ooplasm; N, nucleus; Nu, nucleolus; Y, yolk granule.
Significant decline in protein content suggests intensive proteolysis or reduced protein synthesis due to the depletion of reserve proteins to overcome stress caused by nickel toxicity.

In conclusion, our study shows that nickel(II) accumulated in both germ cells (oogonia and developing oocytes) and somatic cells (muscle cells, follicle cells) in the *A. leptodactylus* ovary. Nickel exposure is associated with the loss of cellular architecture and depletion in protein level in the ovary. This study demonstrated the usefulness of microscopy and electrophoresis as suitable tools for detection of heavy metal-related cytotoxicity. Further studies using ultracentrifugation and Western blot must be accomplished in order to characterize the identity of ovarian proteins that are affected by nickel exposure.

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