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LIGHT AND SCANNING-ELECTRON MICROSCOPIC STUDY OF EISENIA FETIDA COELOMOCYTES AFTER COPPER OXYCHLORIDE EXPOSURE

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ABSTRACT

Morphological alterations in *Eisenia fetida* coelomocytes are recommended biomarkers for soil contamination surveys. Copper oxychloride is a widely used agrochemical agent that causes excessive amounts of Cu accumulation in soils. The present study is a light and electron microscopic investigation of the morphological alterations in coelomocytes of *E. fetida* exposed to copper oxychloride. Exposure concentration was 350 mg/kg of artificial soil and exposure duration was 7 or 14 days. Colelomocytes were extrused into an ethanol and guaicol glyceryl (GGE) containing modified Hanks' balanced solution (M-HBSS-GGE) by a simple low vacuum holding device and processed for light and scanning electron microscopy. Lipid peroxidation (LP) in the earthworms was also measured to evaluate oxidative stress (OS). Swelling and vesicular deformation were the early signs of toxicity in eleocytes after 7 days. After 14 days, complete loss of vesicles and spongy surface were observed in eleocytes. Granulocytes showed more dramatical changes. Nucleus fragmentation and membrane rupture were observed after 14 days. Increased LP in the earthworms was accompanying these changes. As a result, along with biochemical markers such as LP, light and scanning electron microscopic changes in *E.fetida* eleocytes and granulocytes can be considered as valuable biomarkers of copper oxychloride toxicity. Evaluation of those markers in the future studies can constitute an important early warning in terms of soil accumulation.

Keywords: Copper oxychloride, *E. fetida*, Coelomocytes, Morphology, Lipid peroxidation

ÖZET

Eisenia fetida sölomositlerindeki morfolojik değişiklikler toprak kontaminasyon araştırmaları için önerilen biyolojik belirteçlerdir. Bakır oksiklorid toprakta aşırı miktarda bakır birikimine neden olabilen yaygın kullanılan bir agrokimyasal ajandır. Bu çalışma bakır oksiklorid'e maruz bırakılan *E. fetida* sölomositlerindeki morfolojik değişikliklerin ışık ve taramalı elektron mikroskobik bir araştırmasıdır. Maruz bırakılma konsantrasyonu 350 mg/kg/yapay toprak olup, maruz kalma süresi 7 veya 14 gün'dür. Sölomositler etanol ve guaikol gliseri eter (GGE) içeren Hank's balanced solüsyon (M-HBSS-GGE) içerisine basit bir düşük vakumlu tutma cihazı yardımıyla toplanmış, ışık ve taramalı elektron mikroskobi için işleme alınmıştır. Solucanlardaki lipid peroksidasyonu (LP), oksidatif stresi (OS) değerlendirmek amacıyla ölçülmüştür. 7 günün sonunda şişme ve veziküler deformasyon eleositlerdeki erken toksisite belirtileridir. 14 günün ardından, eleositlerde vezikküllerin tamamen kaybı ve süngerimsi yüzey gözlenmiştir. Granülositler ise daha dramatik değişimler göstermiştir. 14 günün sonunda çekirdek fragmentasyonu ve membran parçalanması görülmüştür. Artan LP tüm bu değişikliklere eşlik etmektedir. Sonuç olarak, *E.fetida* sölomositleri ve granülositlerindeki ışık ve taramalı elektron mikroskobik değişiklikler LP gibi biyokimyasal işaretlerle birlikte ele alındığına bakır oksiklorid toksisitesinin değerli bir belirteci olarak gözönüne alınabilir. Bu belirteçlerin ileriki çalışmalarda değerlendirilmesi topraktaki birikim açısından önemli bir erken uyarı sistemi oluşturabilir.

Anahtar Kelimeler: Bakır oksiklorid, *E. fetida*, Sölomosit, Morfoloji, Lipid peroksidasyonu

INTRODUCTION

Between 60-80% of the total soil biomass is consisted of earthworms which have significant impact on soil structure and function [1, 2]. While they are contributing to the aeration and nutrient cycle in this ecosystem, earthworms can be regularly exposed to the soil chemicals [3]. In recent years earthworms have been recognized as valuable bioindicators of soil pollution because of their capacity to reflect the results of these exposures as biomarker responses [4, 5, 6, 7]. Morphological and cytochemical changes in the earthworm coelomocytes have been investigated in previous studies as biomarker of different chemical exposures particularly including metals [8, 9, 10]. Among many other earthworm

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species, *Eisenia fetida* is the most commonly studied organism as an indicator of chemical exposures because it can be easily cultured in the laboratory and give response to a wide variety of toxic substances. In general, coelomocytes of the earthworms have been classified in three main types. Eleocytes which resemble the invertebrate liver possessing primarily nutritive function, and either hyaline or granular amoebocytes (granulocytes) involved in immune defense functions including phagocytosis. The use of morphologic alterations in *E. fetida* granulocytes and eleocytes have been recommended as sensitive, simple, and quick biomarkers for soil contamination [8, 11].

Copper oxychloride is a broad-spectrum fungicide applied to the foliage of a wide variety of fruits and vegetables [12]. It is sprayed directly on crops and in long term, can result in significant amount of copper (Cu) accumulation in soil. Similar to other metals, mechanism of copper oxychloride toxicity in the earthworms depends on the oxidative stress (OS) inducing effects of Cu. However copper oxychloride induced lipid peroxidation (LP) in *E. fetida* has not been assayed to date as an index of OS.

Effects of copper oxychloride toxicity to *E. fetida* has been investigated by different study groups including changes in growth and reproduction parameters or lysosomal membrane stability in coelomocytes. It is reported that copper oxychloride exposure caused significant reduction in the growth and cocoon production in *E. fetida* [13]. Reinecke et a.l (2002) suggested that neutral red retention assay (NRR) in coelomocytes can be an indicator of Cu exposure stress at an early stage [14]. However, Svendsen et al (2004) caveats the use of NRR assay as an earthworm biomarker of pollutant exposure, depending on the inconsistency in parameters such as dose–response relationship, sensitivity, and ecological relevance [15]. Therefore, the present study aims to investigate the morphologic alterations in the characteristics of *E. fetida* coelomocytes as biomarkers of copper oxychloride toxicity. LP in the whole body of the earthworms was also measured to evaluate OS.

1. MATERIALS AND METHODS

1.1. Animals and Experimental Design

Adult *E. fetida* weighing between 300-400 g were the kind gift of a local producer. Animals were utilized for the laboratory exposures at least two weeks before the experiments in a medium prepared according to OECD guidelines (1984) [16]. 500 g of artificial soil was added to the plastic containers (22 × 35 cm bottom, 20cm height). Control and experimental groups were consisted of three containers in each group (n=10). Artifical soil was consisted of 10 sphagnum peat, 20 % kaolinite, 1.0 % calcium carbonate, 70% quartz sand, 50 ± 10 % moisture dry mass. pH was between 5-9 and temprature was between 15-24 ºC. The study was designed as 4 groups. Control group of animals were maintained in mentioned conditions during 7 or 14 days of periods (7 days of control group and 14 days of control group). Copper oxychloride was applied to the soil in a single application by mixing 500 g of artificial soil with 250 ml of copper oxychloride solution in a concentration corresponding to 350 mg of Cu to the kg of artificial soil [17]. Experimental group animals were exposed to copper oxychloride during 7 or 14 days of periods (350 mg/kg/art. soil 7 days group and 350 mg/kg/art. soil 14 days group). At the end of 7 or 14 days, both control or experimental group animals were then processed for the extrusion of coelomocytes or measurement of lipid peroxidation.

1.2. Extrusion of Coelomocytes

Figure 1 shows the low vacuum holding device which was used for the aspiration of animals before extrusion [18].

Figure 1. Low vacuum holding device for the aspiration of animals before the extrusion of coelomocytes according to Diogene et al., 1997.

Extrusion solution was containing 71.2 mM NaCI , 6.7 mM ethylene diamine tetra acetic acid disodium salt (EDTA), 50.4 mM guaicol glyceryl ether (GGE), 2 % ethanol and a supplement of antibiotic and antimycotic agents in Hank's balanced salt solution (HBSS). Ethanol was added to the solution immediately before cell extrusion. Animals were maintained in 8 ml of extrusion solution durin 3 min. After extrusion, cells of each individual animal were centrifuged at 500 g 7 min 4 C and resuspended in a fix volüme of HBSS. Cells were examined under a Leica DM750 brightfield microscope.

1.3. Light Microscopic Analysis of Coelomocytes

Slides from 8 earthworms in each group were prepared by 96 % ethanol fixation and coelomocytes were stained with Giemsa stain. Light microscopic analysis were performed under a Leica DM 750 bright field microscope.

1.4. Scanning Electron Microscopic Analysis of Coelomocytes

The coelomic fluid from 8 earthworms in each group were fixed with 2.5 % glutaraldehyde (v/v) in 0.1 M PBS (pH 7.4). Samples were dehydrated in a graded series of ethanol. After critical point drying with CO_2, samples were mounted on aluminium stubs and sputter-coated with 20 nm of gold (EMS K550). Samples were observed under a Zeiss Ultra Plus Scanning Electron Microscope at 10 kV [19].

1.5. Semi-Quantitative Scoring of the Coelomocyte Pathology

Vesicular deformation and spongy surface were the observation criteria for eleocytes and examined under SEM. Nuclear enlargement, nuclear fragmentation and membrane rupture were the observation criteria for granulocytes. Nuclear changes were observed under LM and membrane rupture was examined under SEM. A total number of 160 (80 cells under LM and 80 cells under SEM) eleocytes or granulocytes were examined in each group. Semi-quantitative scoring of the pathological lesions were expressed as -: none, +: mild, ++: moderate and +++: severe according to Gibson-Corley (2013) [20].

1.6. Measurement of Lipid Peroxidation

Thiobarbituric acid reactive substances (TBARS) were measured according to Ohkawa et al. (1979) [21]. 0.1 g dry weight of earthworm samples were homogenized in 1.15% KCl solution 10% (w/v). An aliquot of the homogenate was added to a reaction mixture containing 200 µl of 8.1% sodium dodecyl-sulfate, 1500 µl 20% acetic acid, 1500 µl 0.8% thiobarbituric acid and 700 µl distilled water and heated at 95 C for 60 min. TBARS extracted with n-butanol and pyridine (15:1, v/v) was measured at 532 nm. Malonedialdehyde bis (dimethylacetal) was used as an external standard. Total protein content of the samples were measured by the method of Bradford (1976). Results were expressed as nmol TBARS per milligram of protein.

1.8. Statistical Analysis

All data were expressed as mean ± standard deviation. Statistical analyses of the groups were performed on SPSS package program by using One-way ANOVA following post hoc test. $P < 0.05$ was considered as statistically significant.

2. RESULTS

2.1. Light Microscopic Evaluation of Coelomocytes

Control group eleocytes were showing normal apearence characteristic with number of vesicles, small few lobopodia and small spherical nucleus (Figure 2.a). Copper oxychloride exposure resulted in a change in the nucleus-cytoplasm ratio of the cell after 7 days. Deformation of vesicles was observed. Surrounding membrane of the eleocytes was separated in some regions (Figure 2.b). After 14 days of exposure to copper oxychloride, nuclear deformation was accompanying cellular swelling. Outer membrane was disintegrated and vesicles were completely destroyed (Figure 2.c) (Table.1).

Granlocytes in control group were having many pseudopods and an eucentric nucleus. There were many granules spread through the cytoplasm (Figure 2.d). Pseudopods were shortened and decreased in number after 7 days of exposure. Nucleus was enlarged and heterochromatic (Figure 2.e). After 14 days of exposure, moderate number of the granulocytes were showing signs of necrosis such as nuclear and membranous fragmentation (Figure 2.e) (Table.1)

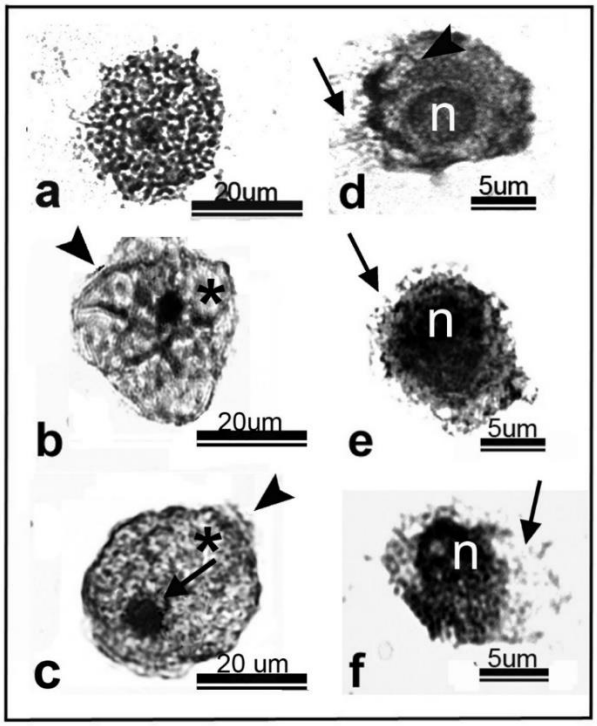

Figure 2. Light microscopic images of *E. fetida* coelomocytes in control and 350 mg/kg copper oxychloride exposed groups a) Control: Eleocyte with a small nucleus and numerous vesicles b) 350 mg/kg/7d group: Eleocyte showing vesicular deformation (*) and separation of the surrounding membrane (arrowhead) c) 350 mg/kg/14d group: Eleocyte with complete loss of vesicles (*), fragmentation of the surrounding membrane (arrowhead) and enlarged nucleus (arrow) d) Control: Granulocyte with prominent granules (arrowhead) in the cytoplasm, numerous pseudopods (arrow) and distinct nucleus (n) e) 350 mg/kg/7d group: Granulocyte with shortened pseudopods (arrowhead) and enlarged heterochromatic nucleus (n) e) 350 mg/kg/14d group: Granulocyte showing nuclear fragmentation (n) and membrane rupture (arrow) as signs of necrotic cell death

Table 1. Semi-quantitative scoring of the selected morphological criteria in microscopic observation of eleocytes and granulocytes. The pathological lesions were expressed as -: none, +: mild, ++: moderate and +++:severe according to Gibson-Corley (2013).

Coelomocytes	Criteria	Control	350 mg/kg/7d Copper oxychloride	350 mg/kg/14d Copper oxychloride
Eleocytes	Vesicular deformation	-	+++	++
	Spongy surface	-	+	+++
	Swelling	-	+++	+++
Granulocytes	Enlarged nucleus	-	++	++
	Nuclear fragmentation	-	+	++
	Membrane rupture	-	-	++
	Swelling	-	+++	++

2.2. Scannig Electron Microscopic Evaluation of Coelomocytes

Scanning electron microscopy of the control eleocytes were in normel appearance with distinct vesicles (Figure 3.a). 7 days of copper oxychloride exposure resulted in vesicular deformation, surface disorganization and separation of the surrounding membrane from the surface of damaged vesicles. Swelling was also observed (Figure 3.b). Eleocytes were possessing a spongy surface lack of vesicles at the end of 14 days. Fragmentation of the surrounding membrane was observed (Figure 3.c).

Control group granulocyte surface was showing regular small protrusions and many pseudopodia (Figure 3.d). Swelling and ballooning in surface protrusions and shortened pseudopods were observed

after 7 days of exposure (Figure 3.e). After 14 days, signs of necrosis were also clearly observed on the cell surface such as membrane rupture and discharge of the cytoplasmic content (Figure 3.f).

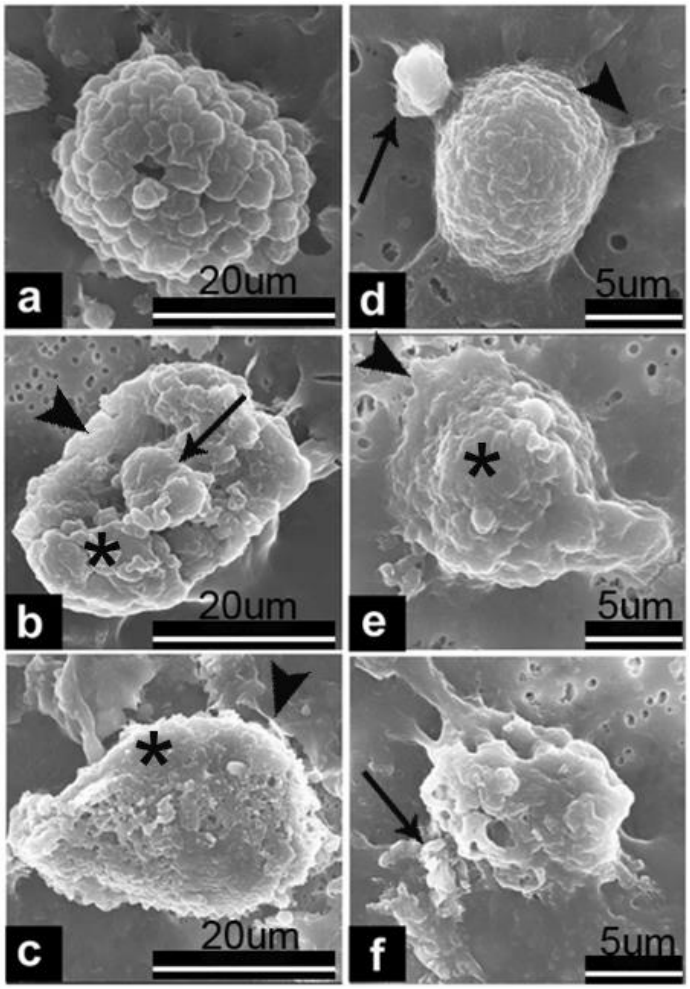

Figure 3. Scanning electron microscopic images of *E. fetida* coelomocytes in control and 350 mg/kg copper oxychloride exposed groups a) Control: Eleocyte with numerous vesicles b) 350 mg/kg/7d group: Eleocyte showing prominent vesicular deformation (arrowhead), irregular surface protursions (arrow) and swelling (*) separation of the surrounding membrane (arrowhead) c) 350 mg/kg/14d group: Eleocyte with complete loss of vesicles (*), spongy surface and fragmentation of the surrounding membrane (arrowhead) d) Control: Granulocyte showing phagocytosis (arrow) with numerous pseudopodia (arrowhead) and small surface protrusions e) 350 mg/kg/7d group: Granulocyte with swelling and surface deformation (*), ballooning protrusions and shortened pseudopodia (arrowhead) f) 350 mg/kg/14d group: Granulocyte showing necrotic cell death characteristic with the rupture of cell membrane (arrow)

2.3. Lipid Peroxidation

Figure 4 shows the results of LP. LP significantly increased in the earthworms' body both after 7 and 14 days of the exposure period. LP increased in the earthworms' body about 1.5 fold after 7 days when compared to control. Increase in LP was about 2 fold after 14 days when compared to control. Diffrence between 7 days and 14 days of experiental groups was about 1.3 fold.

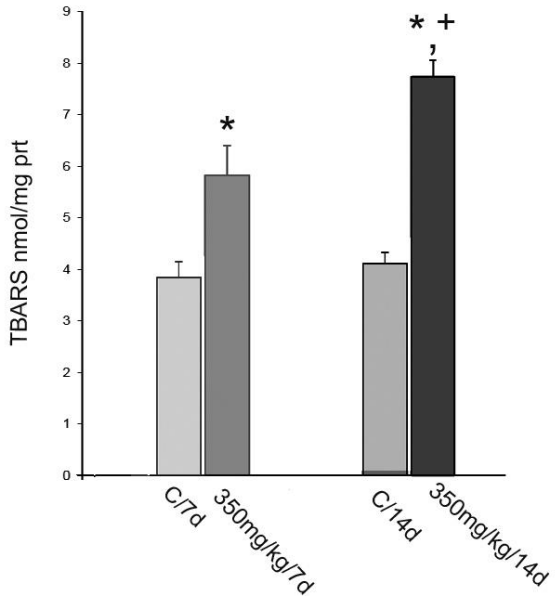

Figure 4. Lipid peroxidation in the earthworms' body. Values are mean ± SD, n = 6 (Student's t-test). * Represents statistically significant differences from control groups ($P < 0.05$). + Represents statistically significant differences between experimental groups ($P < 0.05$).

3. DISCUSSION

Coelomocytes are the cells with immune and other functions (such as blood clotting, wound healing and nutrition) in the earthworms and morphological changes in coelomocytes have been used particularly in *E. fetida* as biomarkers of toxicity [22, 23, 24]. Here we investigated the morphological changes in *E. fetida* coelomocytes and body LP occuring as a result of copper oxychloride exposure.

The first observable change was on the morphology of eleocytes. According to LM observations 7 days of exposure to copper oxychloride at the dose of 350 mg/kg/art.soil resulted in swelling in severe of the eleocytes accompanying vesicular shape deformations. SEM examinations revealed the details of vesicular change as irregular protrusions on cell surface. It is reported that organic pollutants can induce swelling in cells of the earthworm tissues such as the muscles or intestines [25, 26]. Cellular swelling is considered an early morphologic alteration of cellular injury associated with changes in the membrane potential [27]. Since Cu toxicity depends on the mechanisims related to OS, cellular swelling and vesicular changes in eleocytes are considered as a result of OS induced alterations in the membrane potential of eleocytes. In 7 days of exposure group LP was observed to be significantly induced. Granulocytes in 7 days of exposure group showed more dramatic changes mainly characterized with the nuclear enlargement. Disorganisation of the surface morphology was accompanying cellular swelling. Jevtic et al (2014) associated the changes in nuclear morphology with certain disease states, impact on chromatin organisation and altered gene expression [28].

After 14 days of exposure prominent swelling in eleocytes was observed. However more characteristic alterations were the spongy change and complete loss of vesicles in the surface of eleocytes that can be considered as signs of severe membrane damage. LP in this group of animals was about 2 fold when compared to control and about 1.3 fold when compared to 7 days exposure group.

Moderate number of granulocytes were showing nuclear fragmentation and membrane rupture that are considered as the signs of necrotic cell death. Necrotic cell death is a critical endpoint in high level OS. The degree of stimuli usually determines if cells die by apoptosis or necrosis [29]. The presence

of necrosis tells us that a cell has died but not necessarily how death occurred [30, 31]. Therefore, as it is shown with the LP results, OS level in the *E. fetida* tissues after 14 days of copper oxychloride exposure at 350 mg/kg/art. soil concentration was over the cells' tolerance capacity inducing the irreversible changes in the coelomocytes.

In their study investigating the copper sulfate and methiocarb induced alterations *on E. fetida* granulocytes, Calisi et al., (2009) reported enlargement of cells with both of the two chemicals [8]. Our results were in parallel with those by means of copper induced changes in granulocytes. However, the present study focused on the alterations in both eleocytes and granulocytes that can be considered as biomarkers of high dose copper oxychloride exposure at different durations. LP induction by copper oxychloride toxicity in *E. fetida* was also measured for the first time as sign of OS. The results suggested that copper oxychloride at the dose of 350 mg/kg/art.soil induced significant OS in *E. fetida* tissuses both after 7 days and 14 days of exposure. OS after 7 days was more tolerable by the organism associated with reversible changes such as swelling and vesicular deformation in eleocytes. In granulocytes changes in the nuclear morphology was accompanying cellular swelling. All those alterations are suggested as early biomarkers of copper oxychloride toxicity in *E. fetida.* After 14 days of exposure to copper oxychloride at the dose of 350 mg/kg/art.soil, spongy surface and complete loss of vesicles were the advanced alterations in eleocytes. However, moderate number of granulocytes showed irreversible changes such as nuclear fragmentation and membrane rupture as signs of the necrotic cell death which are suggested as the late biomarkers of copper oxychloride toxicity.

4. CONCLUSION

Morphological alterations in the eleocytes and granulocytes of *E. fetida* can be considered as useful biomarkers of copper oxychloride toxicity in both field and laboratory based investigations. Although these alterations are associated with the excess OS induced by copper oxychloride in the present study, future examinations will be helpful to elucidate the details of underlying mechanisms.

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