



Effects of 5-Fluorouracil, Etoposide and CdCl₂ in Aquatic Oligochaeta *Limnodrilus udekemianus* Claparede (Tubificidae) Measured by Comet Assay

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Received: 1 March 2015 / Accepted: 22 June 2015 / Published online: 7 July 2015
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Abstract Genotoxicity of 5-fluorouracil (5-FU), etoposide (ET) and cadmium chloride (CdCl₂) was evaluated in *Limnodrilus udekemianus*, cosmopolitan tubificid species, by alkaline single-cell gel electrophoresis (comet assay). Groups of 50 individuals were exposed in vivo in water-only short-term (96 h) tests to 5-FU (0.004, 0.04, 0.4, 4 and 40 μM), ET (0.004, 0.04, 0.4 and 4 μM) and CdCl₂ (0.004, 0.04, 0.4, 4 and 40 μM). Mortality of

worms was observed only for CdCl₂ (4 and 40 μM). Cell viability lower than 70 % was detected for 5-FU (0.4, 4 and 40 μM), ET (4 μM) and CdCl₂ (0.4 and 4 μM). All tested substances induced significant increase of DNA damage except 0.004 μM of ET. *L. udekemianus* being sensitive to all tested substances indicates that it can be used in ecogenotoxicology studies. Concern should be raised to cytostatics, especially to 5-FU, since concentration of 0.004 μM induced DNA damage is similar to ones detected in wastewaters.

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Keywords *Limnodrilus udekemianus* · 5-Fluorouracil · Etoposide · CdCl₂ · Genotoxicity · Comet assay

1 Introduction

It is evident that aquatic ecosystems are under strong anthropogenic pressure since surface waters receive large quantities of wastewater discharges from industrial, domestic and agricultural sources (Rajaguru et al. 2003). Although, wastewater treatment plants manage to deal with many environmental toxicants, they are inefficient in elimination of many pharmaceuticals which, in the end, reach the surface waters. Today, pharmaceuticals are designed to be more potent and degradation resistant. Many of them are not selective and are designed to affect protein targets at relatively low doses with impact on non-target organisms at low concentrations (Gunnarsson et al. 2008). Presence of genotoxic and potentially carcinogenic compounds in the aquatic environment is of major concern with respect

to the health of aquatic media biota and humans (Houk and Waters 1996; Ohe et al. 2004; Nehls and Segner 2005; Park et al. 2006). At current environmental concentrations, some active pharmaceutical ingredients can be associated with adverse developmental effects in aquatic organisms (Jobling et al. 2002; Khetan and Collins 2007). Among pharmaceuticals, cytostatics are of particular importance in respect to environment protection. They have carcinogenic, mutagenic, cytotoxic, genotoxic or embryotoxic potential (Kümmerer 2001). Cytostatics can enter surface water as metabolites or unchanged via urine and faeces of patients, through treated or untreated wastewater effluents (Brezovšek et al. 2014). Thus, special attention should be given to investigate potential negative impacts of cytostatics on aquatic biota.

Antimetabolite 5-fluorouracil (5-FU) is one of the most commonly used antitumour agents. It leads to inhibition of essential biosynthetic processes, and its metabolites incorporate into DNA and RNA (Longley et al. 2003). Since 5-FU is widely used and not easily biodegradable (Kummerer and Al-Ahmad 1997), the effect of this anticancer drug on non-target aquatic organism should be thoroughly investigated. Plant alkaloid etoposide (ET) is an anticancer agent which acts as topoisomerase II inhibitor leading to DNA breaks (Liu 1989). There is no data about biodegradability of ET in the environment, but since alkaloids of vinca (vincristine, vinblastine and vindesine) are slow in biodegradability in the environment, it can be assumed that ET has similar properties (Kosjek and Heath 2011).

Data regarding concentrations of cytostatics in the environment are scarce. Study of Kosjek et al. (2013) reported that 5-FU can be found in municipal and in hospital wastewaters in Slovenia in concentrations of 4.7–14 ng/l (median, 9.35 ng/l) and 35–92 ng/l (median, 63.5 ng/l), respectively. As expected, concentrations are much higher in sources of pollution such as hospital wastewaters. Regarding ET, the study conducted in France by Catastini et al. (2008) indicated presence of ET in wastewaters of two hospitals where concentrations of this anticancer drug ranged from 110 to 600 ng/l (median, 205 ng/l). Effluents from 21 hospitals in Beijing, China, were analyzed, and ET was detected in 15 effluent samples with concentrations ranging from 6 to 380 ng/l (median, 42 ng/l) (Yin et al. 2010). In Spain, ET was detected in wastewater effluent in concentration of 3.4 ng/l and in wastewater influent in concentration of 15 ng/l (Martin et al. 2011).

Cosmopolitan aquatic tubificid *Limnodrilus udekemianus* (Claparede) is one of the major components of the benthic communities especially those that inhabit organic-rich sediment (Lazim and Learner 1986; Deeds and Klerks 1999) and is an important part of the food chain. *L. udekemianus* lives in the water/sediment interface, the anterior part of the body is located in the sediment, and the posterior part is located in the water. Due to their way of life, this species is under the influence of variety of pollutants via sediment (through ingestion) and via water column (epidermal contact). *L. udekemianus* is sediment-dwelling detritivores characterized by low mobility. These features make this species easily affected by the presence of xenobiotics in aquatic environment.

Aquatic worms (Annelida: Oligochaeta) especially species belonging to the most diversified family Tubificidae are often used in ecotoxicology (Rodriguez and Reynoldson 2011). Many of these studies deal with bioaccumulation of metals, polycyclic aromatic hydrocarbons, herbicide and insecticides; with bioturbation of metals; or with behavioural changes (Keilty et al. 1988; Bouche et al. 2000; Millward et al. 2001; O'Gara et al. 2004; Ciutat et al. 2005; Steen Redeker et al. 2007; Paris-Palacios et al. 2010). To our knowledge, there is no study dealing with effects of different pollutants found in aquatic environment on DNA integrity of *L. udekemianus*.

The aim of this study was to investigate the impact of exposure to 5-FU and ET on the level of DNA damage in freshwater worm *L. udekemianus*. These two anticancer drugs were chosen to be investigated based on their different modes of action.

The level of DNA damage was assessed by comet assay (single-cell gel electrophoresis) applied on cell suspension of haemocytes and coelomocytes of *L. udekemianus*. One of the characteristics of Oligochaeta is the presence of two compartments containing free cells: the blood system with haemocytes and the coelom that includes several coelomocytes populations playing a role in the immune defence (Salzet et al. 2006). Being a sensitive method for assessing DNA damage, comet assay is commonly used in various areas, and lately, it is one of the standard tools in the pharmaceutical industry for the assessment of genotoxicity of new drugs (Wiklund and Agurell 2003). Although there are papers dealing with assessment of genotoxicity by comet assay on coelomocytes of earthworm *Eisenia fetida* (Salagovic et al. 1996; Zang

et al. 2000; Di Marzio et al. 2005), there is no data regarding comet assay on freshwater worm *L. udekemianus*.

In this study, CdCl₂ was used as a positive control since its toxic (Bouche et al. 2000; Steen Redeker et al. 2007; Maestre et al. 2009) and genotoxic (Juhel et al. 2007; Slobodskova et al. 2010; Vincent-Hubert et al. 2011; Filipič 2012) potentials were shown in various vertebrates and invertebrates.

2 Materials and Methods

2.1 Experimental Animals

Laboratory culture of freshwater worms *L. udekemianus* was established during 2012. Experimental animals were maintained in aerated aquaria with medical clay ("prirodna glina"TM, Riznica Prirode) and regional bottled springwater (RosaTM, Serbia: Ca²⁺, 10 mg/l; Na⁺, 2.7 mg/l; K⁺, <1 mg/l; Mg²⁺, 0.91 mg/l; HCO₃⁻, 42.7 mg/l; SiO₂, 13.7 mg/l; SO₄²⁻, 5.4 mg/l; NO₃⁻, 1.3 mg/l; Cl⁻, <1 mg/l) on 22±1 °C under 12-h dark/12-h light regime. Culture was fed food for fish (TetraMin, Germany) once a week. Water was replaced weekly.

2.2 Exposure Test

In vivo treatments were performed as water-only (without sediment) short-term (96 h) tests. Experiments were conducted in static system in 100 ml of solution of selected concentrations prepared in springwater. Treatments were performed as two independent experiments for each tested substances, 5-FU (CAS number 51-21-8, Sigma-Aldrich, Germany, ≥99 % HPLC; stock solution was prepared in distilled water—1 mg/ml), ET (CAS number 33419-42-0; Sigma-Aldrich, Germany; ≥98 % HPLC; stock solution was prepared in DMSO—50 mM) and CdCl₂ (CAS number 10108-64-2, Sigma-Aldrich, Germany; stock solution was prepared in distilled water—1 mg/ml).

Three days before and during each experiment, the worms were not fed, in order to empty their gut content and to avoid interaction between tested substances and food. Cultivated worms were carefully washed several times with distilled water to remove sediment particles from their bodies, and 300 or 350 non-fragmented worms of similar size were chosen under binocular magnifier (Zeiss, Stemi 2000-C; Carl Zeiss

Microscopy, GmbH, 37081 Gottingen, Germany) per experiment. From these groups of worms, 50 worms per each concentration and controls were placed 12 h before experiment in 100 ml of springwater with slight aeration in glass jars.

Worms were exposed to nominal concentrations of 5-FU: 0.004, 0.04, 0.4, 4 and 40 μM and ET: 0.004, 0.04, 0.4 and 4 μM. For positive control, treatment with CdCl₂ was used (nominal concentrations: 0.004, 0.04, 0.4, 4 and 40 μM). Since there are no data about basic level of DNA damage for *L. udekemianus*, for each experiment, negative control was done in duplicate in 100 ml of springwater. While CdCl₂ and 5-FU were dissolved in water, DMSO was used as solvent for ET. Therefore, 0.008 % DMSO (corresponding to concentration 4 μM ET) was used as solvent control. Concentrations of DMSO corresponding to lower concentrations of ET were not tested considering extremely low fraction of DMSO in solutions (80 ppb–8 ppm of DMSO).

2.3 Viability of Adults

After 96 h, worms were taken out from solution and placed in clean jars with 20 ml of springwater and survival of adults was determinate under binocular magnifier. Individuals that did not reacted with movement to a physical stimulation (touch with forceps) were considered dead.

2.4 Cell Collection and Preparation

In this work, collection of coelomocytes with the non-invasive ultrasonic bath extrusion and electrical stimulation (6 V) methods (Sauve et al. 2002; Hendawi et al. 2004) resulted in the high cell mortality and insufficient number of cells, while maceration of individuals with more viable cells but with many impurities. Finally, we decided to collect cells by cutting worms into pieces. There are no referent papers with the information of how many freshwater worms are needed to gain adequate number of cell for the comet assay. Therefore, in preliminary work, we started with different numbers of individuals to gain enough cells (at the beginning with 15 and 30 and then with 50 individuals). Based on our empirical experience, satisfactory number of cells (5×10⁴ cells/ml) was reached from 50 individuals. The cell suspensions (consisting of coelomocytes from coelom and haemocytes from interrupted blood vessels) were

obtained after cutting of viable worms/treatment (50 worms in the case of 100 % viability of adults or less if viability of adults was reduced) in 200 μ l of Lumbricus Balanced Salt Solution (LBSS) (Brousseau et al. 1997). Carefully, avoiding collecting body fragments, cell suspensions were transferred into 2-ml tubes filled with LBSS, centrifuged (3000 rpm, 10 min, 4 °C) and resuspended in 80 μ l of LBSS.

2.5 Cell Viability

Cell viability was assessed by differential acridine orange/ethidium bromide (AO/EB) staining (Squier and Cohen 2001). For this purpose, 20 μ l of cell suspension (prepared as described earlier) was mixed with 2 μ l AO/EB stain (2 μ g/ml) and viability was evaluated under fluorescence microscope (Leica, DMLS, Austria, under magnification \times 400, excitation filter 510–560 nm, barrier filter 590 nm). During evaluation of cell viability, different sizes and types of cells were observed which indicated their different origins (coelom and blood system). To avoid interference of apoptosis or necrosis in means of false-positive DNA damage increase, threshold level of cell viability was set at 70 % (Tice et al. 2000).

2.6 Comet Assay

To assess the level of DNA damage, we performed modified alkaline comet assay described by Singh et al. (1988). All steps of the alkaline comet assay were performed under indirect yellow light. The gels were composed of three layers of agarose. A day before, microscopic slides were pre-coated with 1 % normal melting point (NMP) agarose and left to dry for 24 h at room temperature. For the second, supportive layer, 80 μ l of 1 % NMP agarose was placed on two sites on top of the earlier prepared microscopic slides. By coverslips, second layer of 1 % NMP was spread over the slides and the day slides were placed on 4 °C for 5 min to allow complete polymerization of agarose after which the coverslips were removed. For the third layer, 70 μ l of 1 % low melting point (LMP 37 °C) agarose was mixed with 30 μ l of cell suspension (prepared as described earlier) and placed onto supportive layers on microscopic slides, covered with a coverslips and placed on 4 °C for 5 min. After removing the coverslips, the slides were immersed in freshly prepared cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris,

1.5 % Triton X-100, 10 % DMSO, pH 10, 4 °C) for 1 h. The slides were then placed in an electrophoresis chamber containing cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min at 4 °C in order to allow DNA unwinding. After DNA denaturation, electrophoresis was performed in the same buffer (0.5 V/cm, 300 mA, 20 min, 4 °C). After 20 min, the slides were taken out and placed into freshly made cold (4 °C) neutralizing buffer (0.4 M Tris, pH 7.5) for 15 min. Before analysis, staining was performed with 20 μ l per slide of acridine orange (2 μ g/ml). The slides were analyzed with a fluorescence microscope (Leica, DMLS, Austria, under magnification \times 400, excitation filter 510–560 nm, barrier filter 590 nm) linked to an image analysis Comet IV Computer Software (Perceptive Instruments, UK). Images of 50 nuclei were analyzed per slide. The nuclei excessively damaged (hedgehogs) were not scored. As a measure of the level of DNA damage, the percent of the fluorescence in the comet tail (tail intensity (TI)) was chosen and scored.

2.7 Statistical Analysis

Statistical analysis in this study was carried out using Statistica 6.0 (2001). Kolmogorov-Smirnov test for normality of distribution was used prior to statistical analysis. The normality of distribution was tested in each sample independently, and the data were in line with the requirements for the application of parametric test. Pairwise comparisons of the level of DNA damage in cells of worms exposed to different concentrations of test substances and controls were done using Student test (*t* test) with 95 % confidence limit ($p < 0.05$).

3 Results

3.1 The Effects of CdCl₂, 5-FU and ET on Survival of Adults

Among tested concentrations of CdCl₂ (0.004, 0.04, 0.4, 4 and 40 μ M), the survival of adults was less than 50 % (average 46 %) at 4 μ M, and at 40 μ M, the mortality was 100 %. During experiments, morphological alterations of worms were observed at 4 μ M CdCl₂. For this concentration, autotomy of the worms was detected.

In the case of 5-FU (0.004, 0.04, 0.4, 4 and 40 μ M) and ET (0.004, 0.04, 0.4 and 4 μ M), at all tested concentrations, survival of adults was 100 % (data not

shown). Also, visible morphological alterations on tested individuals were not recorded after exposure to these two cytostatics.

3.2 The Effects of CdCl₂, 5-FU and ET on Cell Viability

Cell viability lower than 70 % was detected for CdCl₂, at 0.4 and 4 μM (57.65 and 50.35 %, respectively), 5-FU at 0.4, 4 and 40 μM (49.68, 56.47 and 37.48 %, respectively) and ET at 4 μM (56.77 %) (Fig. 1). Although the concentration of 0.4 μM was found to be cytotoxic for CdCl₂ and 5-FU, and to be at the lower limit for cell viability in the case of ET, this concentration was included in genotoxicity assessment considering that this was the first concentration which reduced cell viability below the threshold value while concentration 4 μM for all three substances was excluded from genotoxicity assessment.

3.3 The Effects of CdCl₂, 5-FU and ET on DNA Damage Level

Values for negative controls were in range from 6.87 to 15.92 with average of 10.33±2.77 (mean±SD). Significantly higher values for negative control were detected only for ET treatment experiment comparing to negative controls in other experiments ($p<0.05$).

All tested concentrations of CdCl₂ and 5-FU led to significant increase ($p<0.05$) of DNA damage in both independent experiments (Figs. 2 and 3).

ET concentrations 0.04 and 0.4 induced significant increase ($p<0.05$) of DNA damage comparing to negative control. The lowest concentration of ET (0.004 μM)

did not resulted in significant damage comparing with control (Fig. 4).

4 Discussion

In this study, we have applied comet assay on freshwater worm *L. udekemianus*. By our knowledge, there is no data regarding comet assay on this species. To analyze data properly, it is essential to have knowledge of the basic level of DNA damage and physiological status of animals. With exception of the experiment with ET, we have observed insignificant variations in DNA level in negative controls (with average value of TI±SD: 10.33±2.77). The reason for high baseline level of DNA damage in control group of worms in the first ET treatment can be attributed to physiological status of animals. Two weeks before treatment with ET, many cocoons were observed in the laboratory culture. Based on available literature data (Kennedy 1966), breeding can lead to poor physiological status. The second experiment with ET was done a month later, and the base level of DNA damage was lower, but yet higher than for the two other tested substances.

To test model system (exposure of *L. udekemianus* in 96-h water-only test to 5-FU and ET), treatment with CdCl₂ was used as positive control. Cadmium can be found naturally in the environment in different concentrations. Concentration of this heavy metal depends on mineral composition of rocks and of surrounding environment, of abiotic factors (weathering, climate, soil type, pH, dilution) and of anthropogenic activity (industrial use for batteries, anticorrosive coatings of metals, pigment, etc.) (CCME 2014). Concentrations 40 and

Fig. 1 Viability of cells of *L. udekemianus* after exposure to CdCl₂, 5-FU and ET. Viability of cells was obtained by AO/EB differential staining. Values represent mean±SD of two independent experiments

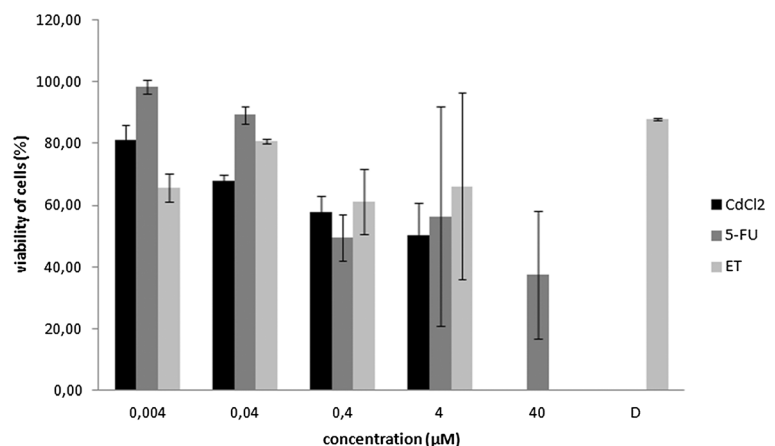
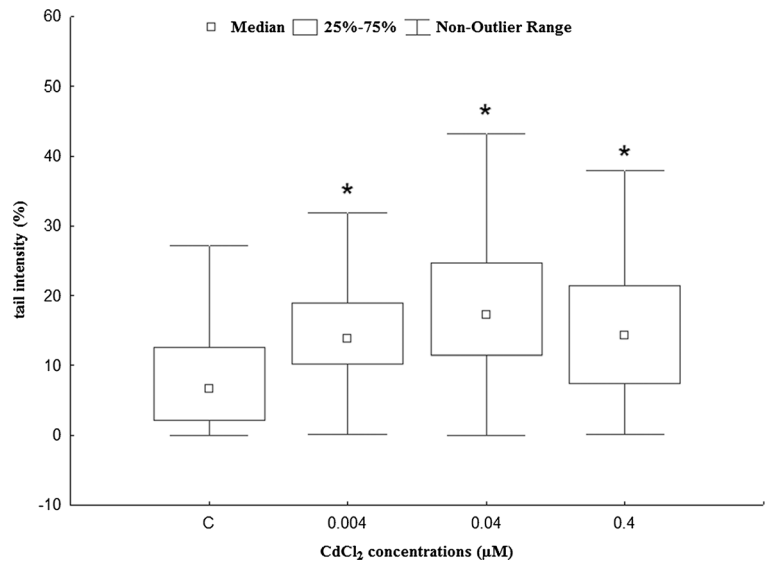


Fig. 2 Effect of CdCl₂ on DNA damage in cells of *L. udekemianus*. For each group, 100 nuclei were scored (50 per experiment). *Statistical significance using Student's *t* test ($p < 0.05$)



4 μM of CdCl₂ can be attributed to toxic concentrations, since total mortality of adults was obtained for the highest concentration (40 μM), and viability of adults for 4 μM was below 46 %. In our study, survival of adults treated with cadmium was higher than in study of Maestre et al. (2009) on *Tubifex tubifex*. At 4 μM, integrity of worm's body was severely affected; namely, majority of individuals were fragmented. Process of autotomy (loss of the caudal region of the worms, reaction to stress and mechanism of detoxification) is well described in the study of Bouche et al. (2000). In our

study, autotomy was only noticed not evaluated. Due to recorded toxic effect for viability of adults and cells at the concentration 4 μM, this concentration cannot be considered for assessment of genotoxicity. At concentration 0.4 μM, reduced cell viability was detected (average for two experiments 57.65 %), and therefore, this concentration should be also excluded for genotoxicity assessment. This indicates that for assessment of genotoxic potential of this mutagen agent on *L. udekemianus*, concentrations lower than 0.4 μM should be applied. As it can be seen from the results,

Fig. 3 Effect of 5-FU on DNA damage in cells of *L. udekemianus*. For each group, 100 nuclei were scored (50 per experiment). *Statistical significance using Student's *t* test ($p < 0.05$)

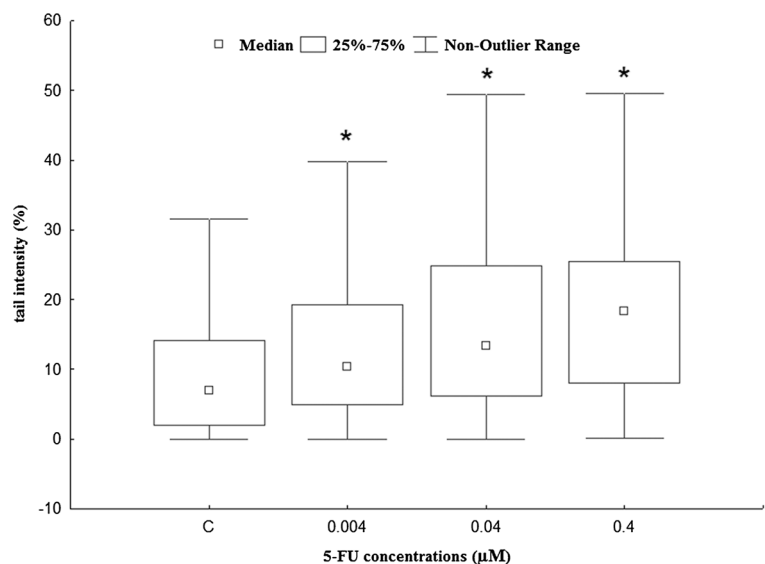
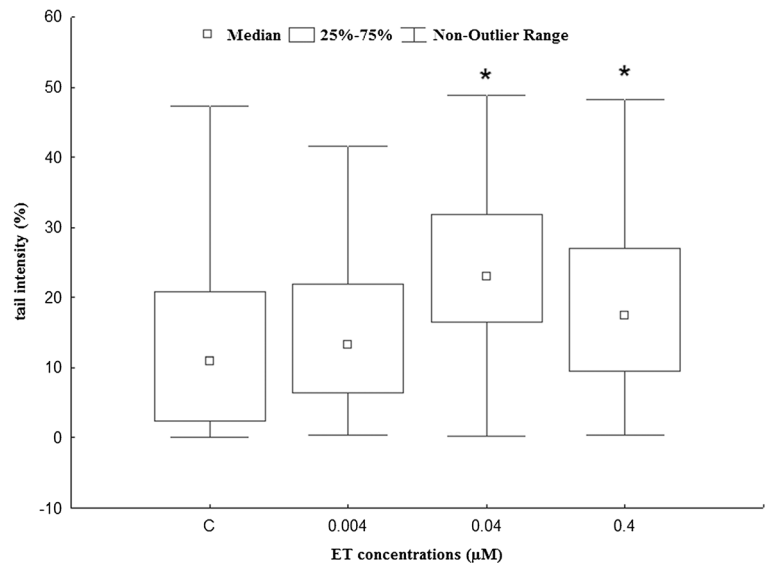


Fig. 4 Effect of ET on DNA damage in cells of *L. udekemianus*. For each group, 100 nuclei were scored (50 per experiment). *Statistical significance using Student's *t* test ($p < 0.05$)



concentrations of CdCl₂ 0.004 and 0.04 µM had significant impact on DNA integrity. This indicates that even lower concentrations which do not lead to observable morphological changes in this organism could cause health hazards to this worm.

In the case of 5-FU, frequently used anticancer drug, toxic effects on viability of adults were not registered in both independent experiments. External morphological changes of worms were not observed. For three higher concentrations of 5-FU, cytotoxicity was obtained: 40 µM/37.48 %, 4 µM/56.47 % and 0.4 µM/49.68 %. As these concentrations exhibited cytotoxic effects, they cannot be estimated as genotoxic ones. Results gained by comet assay show that all tested concentrations of 5-FU led to significant increase in DNA damage. Regarding environmental concern, it should be mentioned that the lowest concentration of 5-FU (0.004 µM/0.52 µg/l) is higher than the predicted environmental concentration (PEC < 0.54 ng/l) (Kosjek et al. 2013). Nevertheless, it must be taken into consideration that these experiments were conducted as short-term treatments (96 h). In the case when these drugs are present in the aquatic environment, organisms may be exposed to these substances for longer period of time.

Toxic effect of ET on survival of adults of *L. udekemianus* was not detected; namely, survival was 100 % for all tested concentrations. As in the case of 5-FU, for ET, morphological alterations of worms were not detected. Regarding cytotoxicity, low level of cell viability was detected for the highest concentration (average cell viability for two treatments at 4 µM was

56.76 %). For the other three concentrations (0.4, 0.04 and 0.004 µM), toxic effect was not detected (survival of adults 100 % and viability of cell were in range of 70 to 75 %); therefore, they were taken into consideration for assessment of genotoxicity. Regarding the level of DNA damage, all concentrations of ET with the exception of the lowest concentration of ET led to significant increase (Fig. 4). Concentrations found in hospital effluents (Catastini et al. 2008; Yin et al. 2010; Martin et al. 2011) are far below the effective concentrations in this study (the lowest concentration which led to a significant increase in DNA damage was 0.04 µM–23.54 µg/l; therefore, it can be concluded that environmentally relevant concentrations of this anticancer drug cannot induce significant increase in DNA damage in *L. udekemianus*.

To conclude, 96-h exposure *L. udekemianus* has shown to be sensitive to all tested substances. Concern should be raised about the presence of cytostatics in the environment, especially to 5-FU, considering that the lowest concentration which induced significant increase of DNA damage is similar to the ones detected in wastewaters. Although the PEC values for tested cytostatics are lower than the ones used in our study, it must be emphasized that in the environment, organisms are under constant influence of these pollutants. In the environment, organisms are dealing with the effects of mixture of pharmaceuticals and mixture of different pollutants. Impacts of these mixtures on the aquatic organisms are still unknown, and therefore, further research should consider this fact and the studies should

be organized in this direction. This study indicates that *L. udekemianus* can be proposed as model organism in ecogenotoxicology to assess the effects of aquatic pollutants on DNA integrity.

Acknowledgments This study is financially supported by the European Community's Seventh Framework Programme (FP7/2007–2013), Grant agreement no. 265264: CytoThreat: Fate and effects of cytostatic pharmaceuticals in the environment and the identification of biomarkers for improved risk assessment on environmental exposure and no. 603629-ENV-2013-6.2.1.-Globaqua: Managing the effects of multiple stressors on aquatic ecosystems under water scarcity and by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Projects No. TR 037009.

Conflict of Interests The authors declare that there is no conflict of interests regarding the publication of this paper.

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