In vitro effects of PCDDs/Fs on NK-like cell activity of *Eisenia andrei* earthworms

Hayet Belmeskine,[1] Sami Haddad,[2] Louise Vandelac,[3] Michel Fournier[1]

[1]INRS-Institut Armand Frappier, Laval; [2]Département de Santé Environnementale et Santé au Travail, Université de Montréal, Montréal; [3]Département des Sciences de l'Environnement de l'Université du Québec à Montréal, Montréal, Québec, Canada

Abstract

In this study, we assessed *in vitro* the effects of PCDD/Fs on the NK-like cell activity in *Eisenia andrei* earthworms using flow cytometry for analysis. NK-like cœlomocytes isolated from *E. andrei* and used as effectors were exposed to various concentrations of PCDDs/Fs mixture, C1 (6.25×10^{-3} ng 2378-TCDD/mL), C2 (12.5×10^{-3} ng 2378-TCDD/mL) and C3 (25×10^{-3} ng 2378-TCDD/mL), before adding them to human tumoral cells (K562) used as targets. We evaluated the percentage of targets lysed by Nk-like cells. The results showed a significant stimulation of the NK-like activity at C3 when PCDD/Fs were not removed from effectors before contact with targets, while no effects were noted when the effectors were washed (PCDD/Fs removed) or fixed. Assessment of the viability of the targets (K562), exposed alone and separately from effectors, to the three concentrations of PCDD/Fs, C1, C2 and C3, showed that all these concentrations were cytotoxic for K562. Results suggest that PCDD/Fs concentrations tested in this assay may be considered too low to induce suppressive effects on the immune function such as the NK-like activity in *E. andrei* earthworms.

Introduction

Earthworms play an important role in soil formation processes and in maintaining soil structure and fertility. They are interesting soil bio-indicators due to their ability to ingest organic and inorganic particles.[1] Currently, *Eisenia andrei* is recommended in standardized acute toxicity protocols[2] because of their sensitivity and ability to concentrate some insecticides,[3,4] heavy metals[5,6,7] and persistent organic pollutants (POPs)[8] in their body tissue. Moreover, this species was used to evaluate the sub-lethal effects of several xenobiotics.[9] In addition, numerous studies of the action of chemicals on a variety of acute toxic endpoints and subchronic-chronic processes, have demonstrated that a spectrum of chemicals alter the immune system of earthworms.[10,11,12,13,14] Thus, the immune system's potential was defined as a target organ system for use in assessing the toxicity of exposure to xenobiotics.[15] Because the immune responses are important host defense mechanisms, their modulation may result in an increased incidence of infections that could influence the survival of individuals and their populations.[15] Subcellular, cellular and organismal immunological indicators were previously used as assessment tools. Recently, there has been specific interest in effector molecules in antimicrobial and cytotoxic responses. Published data demonstrated cellular cytotoxic effects of cœlomocytes in xenogeneic and allogeneic culture of earthworm cœlomocytes, as well as cœlomocyte cytotoxicity against mammalian tumor cell lines.[16,17] Cell-mediated cytotoxicity studies also demonstrated that some persistent organic pollutants, such as PCBs[18] and PAHs,[19] exerted immunosuppressive effects on NK-like effector cells.

To date, there are few reports in literature describing the immunotoxicity of PCDDs/Fs in earthworms or in other invertebrate model; studies have only been carried out on mammalian models.[20] It is well recognized that PCDDs and PCDFs enter the environment as by-products of combustion processes, and represent the most toxic anthropogenic chemicals in the environment associated to their stability, lipophilicity, bioaccumulation and high persistence. In addition, numerous studies have reported that prolonged exposure to TCDD presents adverse health effects, including immunotoxicity, neurotoxicity, hepatotoxicity and carcinogenesis.[21,22,23,24,25] Moreover, the International Agency for Research on Cancer (IARC) evaluated TCDD as a Group 1 carcinogen, *i.e.* a human carcinogen.[26,27,28] Environmental exposure concentrations of 1 ng 2378-TCDD/g soil were considered *a level of concern* for causing cancer.[29] Thus, the presence of these toxic contaminants in soils may have direct harmful effects to the earth's ecosystem.

The objective of our study was to investigate, *in vitro*, the effects of a mixture of PCDDs/Fs on the NK-like cell activity of *E. andrei* earthworm cœlomocytes to lyse human tumor cells (K562), using flow cytometry for analysis.

Materials and Methods

Reagents

The mixture of PCDDs/Fs in nonane solution was obtained from Wellington laboratories (Ontario, Canada) and stored at 1°C. Dimethyl sulfoxide 99.5% (DMSO) was obtained from Sigma-Aldrich (USA). RPMI 1640, containing 25 mM HEPES buffer, L-glutamine and sodium bicarbonate, was supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. K562 (Sigma Aldrich) was cultured in this complete RPMI. The 3, 3'-dioctadecyloxacarbocyanine (DiO) was dissolved in DMSO to yield 3 mM. A stock solution of propidium iodide (PI) of 1 mg/mL in water was prepared. Purified water was obtained from a Milli-Q water purification system.

Earthworms

The *E. andrei* earthworms were initially purchased from the Carolina Biological Supply (Burlington, NC, USA) and were used to establish the laboratory culture. Prior to the experiment, the animals were maintained in earthworm bedding (Magic Products, Amherst Junction, WI, USA) at 20±1°C, 70-80% (w/w) moisture, a 16:8 h light:dark cycle, and fed once a week with cereal (Magic Worm Food, Magic Products).

Cell extrusion

Before the experiments, the earthworms

Correspondence: Hayet Belmeskine, INRS-Institut Armand Frappier, 531 boulevard des prairies, Laval, Québec, Canada H7V1B7.
Fax: +1.450.686.5801
E-mail: hayet.belmeskine@iaf.inrs.ca / hbelmeskine@hotmail.com

Key words: PCDD/Fs, immunosuppression, NK-like activity, flow cytometry, earthworms.

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were placed for 24 h on moist Whatman filter paper in Petri dishes[30] to extract ingested soil prior to cell extrusion. Six purged earthworms were used to measure the NK-like cell activity, and the assay was carried out in duplicate. A single worm was inserted into a 15 mL tube containing 3 mL of Lumbricus balanced salt solution (LBSS) composed of 1.5 mM NaCl, 4.8 mM KCL, 1.1 mM $MgSO_4.7H_2O$, 0.4 mM KH_2PO_4, 0.3 mM $Na_2PO_4.7H_2O$, 3.8 mM $CaCl_2$, and 4.3 mM $NaHCO_3$.[12,31,32] Cœlomocytes were extracted using an electrical extrusion method which consists of submitting the liquid medium to a 6 V current (lantern battery) for 20-30 s using aluminium wires.[13] The worm was then removed from the tube and the solution was gently shaken.

Cell yield and initial cell viability were determined by diluting 50 µL of cell suspension with 50 µL of 0.4% trypan blue (Sigma Chemical Co. St. Louis, MO, USA). This mixture was placed into an improved Neubauer hemacytometer and examined under the microscope (Table 1).[12,13]

Preparation of effector cells

Effector cells were prepared according to Brousseau *et al.*[32] The cœlomocyte concentration was adjusted in supplemented RPMI at 1×10^6 cells/mL and the dilutions were prepared according to the respective effector:target (E:T) ratios needed (Table 2).

Preparation of target cells

The target cells were prepared according to Brousseau *et al.*[32] Briefly, after 2-3 days culture, the concentration and viability of the K562, at exponential phase, were determined. The viability measured was greater than 95% (the condition in which the test could continue). The cells (1×10^7) were then centrifuged at 300×g for 10 min at room temperature to pellet. The supernatant and loosened pellets were gently discarded using a Pasteur pipette. Aliquots of 10 µL of 3 mM DiO were placed into polystyrene tubes. Then 1 mL of the K562 cells was added forcefully to disperse the dye (DiO). The tubes were incubated at 37°C in 5% CO_2 for 20 min, then washed twice with RPMI and the cells were resuspended in RPMI at a concentration of 1×10^6 cells/mL.

Exposure protocol

To establish the *in vitro* dose-response curves, three series of glass tubes containing the effector cell suspension (each ratio separately) were exposed to three different dilutions of a PCDDs/Fs mixture, C1, C2 and C3 (Table 3), respectively. These dilutions were made in DMSO (final concentration 0.004% per tube). Therefore, a set of tubes containing cœlomocytes with DMSO (vehicle) was added as control. Negative controls receiving no PCDD/Fs were included along the test with controls, which received DMSO (without PCDD/Fs) to evaluate any effect due to the solvent (vehicle). Effector cells were exposed for 3 h to PCDDs/Fs and then the target cells were added to measure the NK-like cell activity. Effectors and targets were incubated together in the dark for another 3 h at ratios of 40:1 and 20:1 (Table 2). After 3 h of incubation, 15 µL of PI (1 mg/mL) was added to each tube followed by a centrifugation at 1000×g for 30 s to pellet the cells. The tubes were then incubated in the dark for 1 h before flow cytometric analysis. To identify and understand the effects of the contaminated cell washing before contact with target cells, we carried out two experiments. In the first test, the contaminated effector cells were not washed (PCDD/Fs were not removed) and were put directly in contact with K562. The second test consisted of washing the effector cells twice with RPMI before contact with K562. Also, another test was added to understand whether chemical particles not eliminated by cells and present in cell suspension can exert effects on K562. So, effector cells were fixed with a fixation solution composed of 37% formaldehyde and sodium azide, before being added to K562. In parallel with these tests, the effects of PCDD/Fs mixtures on the viability of the target cells (K562) were studied separately.

Evaluation of NK-like activity

In order to evaluate the effects of PCDD/Fs on NK-like cell activity, flow cytometry was used to determine lysis of human erythroleukemic tumor cell line (K562). This method uses two fluorescent dyes to discriminate between effectors and target cells, and between live and dead target cells. DIO, green fluorescent dye, was used to label the membranes of K562, while PI (red fluorescent dye) was added during the assay when K562 membranes were disrupted by effectors. Data were acquired using a FACScalibur (Becton Dickinson, San José, USA) flow cytometer, and analyzed by the CellQuest Pro software (Becton Dickinson) to determine target cell death. A minimum of 5000 events was acquired for each sample.[32] Results were expressed as percentage of target cell (K562) lysis.

Statistical analysis

Data were expressed as arithmetic means±standard deviations. Statistical significances of differences among treatments were determined by ANOVA one-way followed by Tukey HSD post hoc test for specific comparison of means ($P<0.05$), significantly different from the vehicle control: *$P<0.05$, **$P<0.01$, and ***$P<0.001$. Statistica software, Version 6.0 (StatSoft, Tulsa, OK, USA) was used.

Table 1. Mean values of worm weights, cell yield and cell viability.

Weight of worms (g)	Cell yield	Cell viability (%)
0.43±0.09	(6.75±2.88)×10^6	85.03±5.41

Table 2. Effector and target cell ratios.

Ratio (E: T)	Number of effector cells	Number of target cells
40:1	4.0×10^5	1.0×10^4
	(400 µL)	(10 µL)
20:1	2.0×10^5	1.0×10^4
	(200 µL)	(10 µL)

Table 3. Final concentrations of PCDD/Fs in tubes before incubation for 3 h.

PCDDs/Fs	C1 (ng/mL)×10^{-3}	C2 (ng/mL)×10^{-3}	C3 (ng/mL)×10^{-3}
2378-TCDD	6.25	12.5	25
2378-TCDF	6.25	12.5	25
12378-PeCDD	15.625	31.25	62.5
12378-PeCDF	15.625	31.25	62.5
123678-HxCDD	15.625	31.25	62.5
123678-HxCDF	15.625	31.25	62.5
1234678-HpCDD	15.625	31.25	62.5
1234678-HpCDF	15.625	31.25	62.5
OCDD	31.25	62.5	125
OCDF	31.25	62.5	125

Results

Analyses of target cells are shown in Figure 1. In a Forward *versus* Side scatter dot plot, a gate (R1) was drawn around the target cells. A single parameter FL-1 histogram (gated on R1) was created. Figure 1A shows the negative control (the cells before labeling to DiO) (left peak), while Figure 1B represented DiO-labeled targets (right peak). After incubation of effector-DiO-labeled targets-PI, analysis of target cells was made using FL-1 (DiO) *versus* FL-3 (PI) dot plots gated on R1 (Figure 2). The right panel in Figure 2 represents DiO-labeled targets after exposure to contaminated effectors with PCDD/Fs. The upper right quadrant indicates that the targets were double positive, which means that targets were lysed. The lower right quadrant indicates DiO^+ representing intact target cells. Results from the first assay (PCDD/Fs not removed from effector cells before contact with targets) are shown in Figure 3. The profiles of NK-like cell function were similar between the 40:1 and 20:1 ratios. The results showed a significant increase ($P<0.01$) in the percentage of K562 lysis in cœlomocytes exposed to the higher PCDD/Fs concentrations C3 (containing 25×10^{-3} ng 2378-TCDD/ mL). In the second assay, performed with washed effector cells, only the 40:1 ratio was considered. No significant differences were observed at any of the PCDD/Fs mixture concentrations (Figure 4). In the third assay, where the effectors contaminated with PCDD/Fs were fixed before being put in contact with targets, the higher concentration C3 was tested (Figure 5). No significant difference was observed at C3 compared to controls. Parallel to these tests, the direct effects of the same concentrations of PCDD/Fs mixture (C1, C2 and C3) were tested on the target cells (K562) alone (1×10^6 cell/mL). The results shown in Figure 6 showed a significant decrease in the K562 viability at all concentrations, C1, C2 and C3, compared to controls ($P<0.001$).

Discussion

In the present work, the flow cytometry was used to study the cell-mediated cytotoxicity of earthworm NK-like cells isolated from the cœlomic cavity of *E. andrei* and exposed *in vitro* to various concentrations of PCDDs/Fs mixture. The target cells used were human tumor cells (K562).

Without washing the contaminated effector cells (cœlomocytes), the *in vitro* PCDDs/Fs exposure significantly stimulated NK-like cell activity at C3 (25x10-3 ng 2378-TCDD/mL) compared to controls, while no effect was shown at C1 and C2 (Figure 3). Generally, the

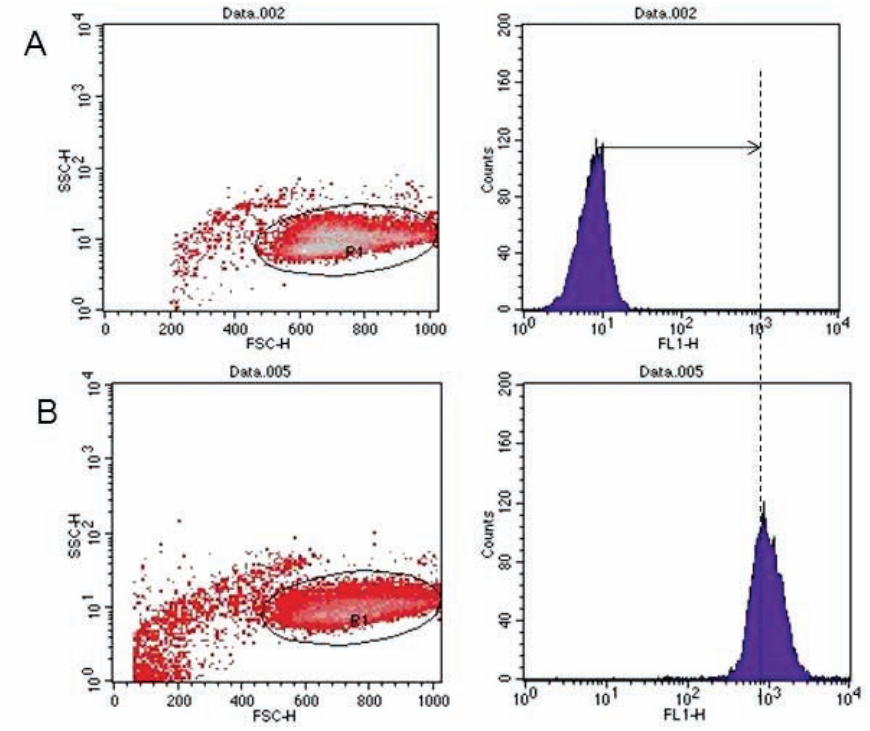


Figure 1. Flow cytometric analysis of DiO-labeled target cells. (A) Negative control (left peak). (B) DiO positive events identified (right peak).

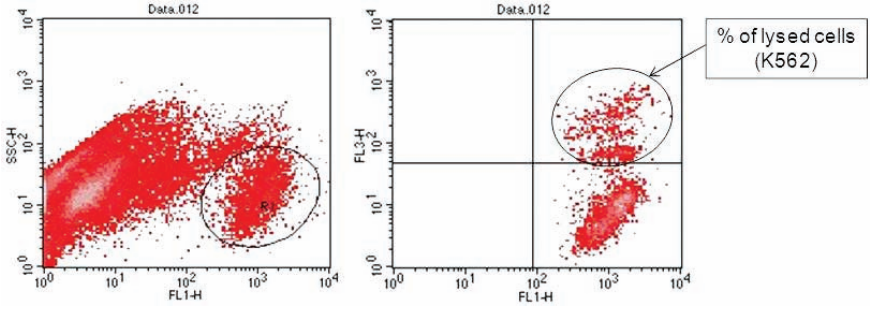


Figure 2. Flow cytometric analysis of target cells using FL-1 *versus* FL-3 dot plots gated on R1. The right panel showed the DiO-labeled targets after exposure to effectors. The upper right quadrant represents double positive events (targets). The lower right quadrant represents DiO-positive events (intact targets).

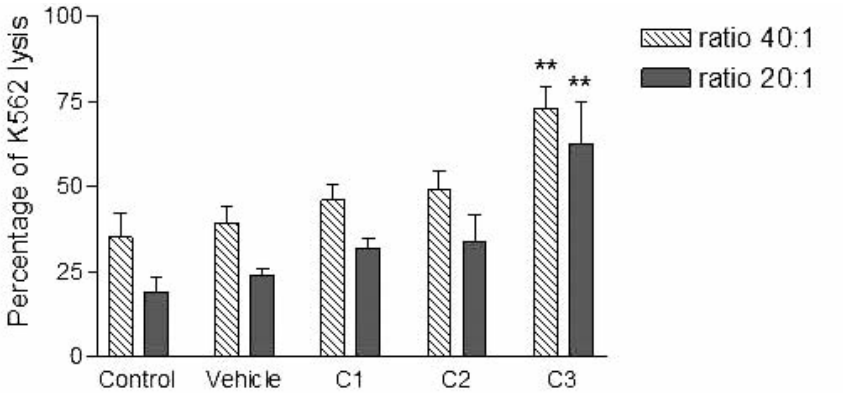


Figure 3. Effects of various concentrations of PCDD/Fs mixture on the *E. andrei* NK-like cell activity to lyse the human tumor cells (K562). The effectors were not washed to remove PCDD/Fs before contact with K562. Asterisks represent significant difference compared with vehicle controls (*P<0.05, **P<0.01).

enhancement of the NK cell activity was seen for low concentrations.[33] Comparable results have been reported with a mammalian laboratory model exposed to an endocrine disruptor and persistent contaminant, tributyltin chloride (TBTCl). This *in vivo* study observed enhancing effects of TBTCl on NK cell activity only in the low dose group (0.025 mg/kg/day) in old female rats.[33] Thus, the results concluded that the increase in NK cell activity was non-linear dose-response. The PCDD/Fs-associated immunostimulation observed in our study at concentrations of C3 was considered to be an immunotoxicological effect, since it may result in the loss of regulation within the immune system and can lead to adverse outcomes including autoimmune disease, anergy, and cancer.[34,35] In the second assay, it was noted that washing contaminated effectors before adding to DiO-labeled K562 targets did not have any effect and no statistically significant differences were observed between groups (Figure 4). Similar results were obtained for the third assay where the contaminated effectors were fixed (no active). So, the cell fixation did not present any effect on the percentage of K562 lysis (Figure 5). This cell fixation assay was conducted to verify if the PCDD/Fs were rejected by cells when fixed and could kill the targets. As shown in Figure 6, the PCDD/Fs alone were cytotoxic for K562 at the various concentrations used. In fact, significant inhibition was observed for all concentrations compared to controls.

To our knowledge, this is the first study of the immunotoxicological effects of a mixture of dioxins and furans on NK-like cell activity in earthworms. Therefore, the absence of data means we cannot compare our results. However, some studies on persistent organic pollutants (POPs) have been carried out. It was demonstrated that PCBs[18] and PAHs[19] exerted immunosuppressive effects on NK-like effector cells. It is important to note here that Patel *et al.*[19] have tested washed and non-washed effectors before adding DiO-labeled targets and they have demonstrated that in these two cases, exposure to the polycyclic aromatic hydrocarbon DMBA resulted in statistically significant immunosuppression of NK-like cell activity in earthworms. As far as mammalian laboratory models are concerned, different results were shown for dioxins depending on doses, species, and sex. For example, in mice, Wang *et al.*[36] have demonstrated that acute 30 μg TCDD/kg-treatment of female mice suppressed the NK cell activity. While, Funseth and Ilbäck[37] have shown that TCDD induces an increased activity of NK cells of male A/J mice after a loading dose of 5 μg TCDD/kg body weight, followed by 3 weekly maintenance doses of 1.42 μg TCDD/kg b.w. In humans, it has not yet been clarified whether dioxins have any effect on the NK cell activity.

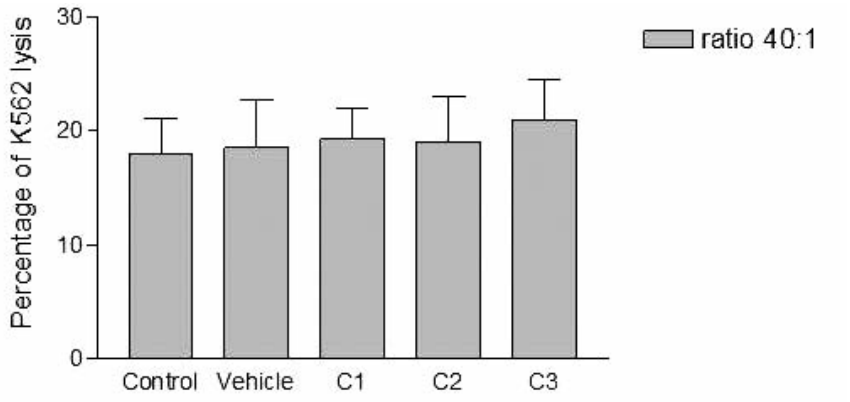


Figure 4. Effects of various concentrations of PCDD/Fs mixture on the *E. andrei* NK-like cell activity to lyse the human tumor cells (K562). The effectors were washed of the PCDD/Fs before contact with K562.

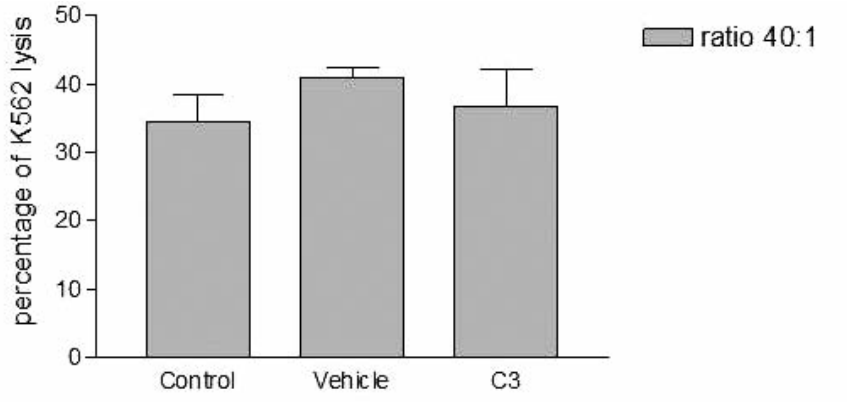


Figure 5. Effects of the higher concentration of PCDD/Fs mixture (C3) on the *E. andrei* NK-like cell activity to lyse the human tumor cells (K562). The effectors were fixed before contact with K562.

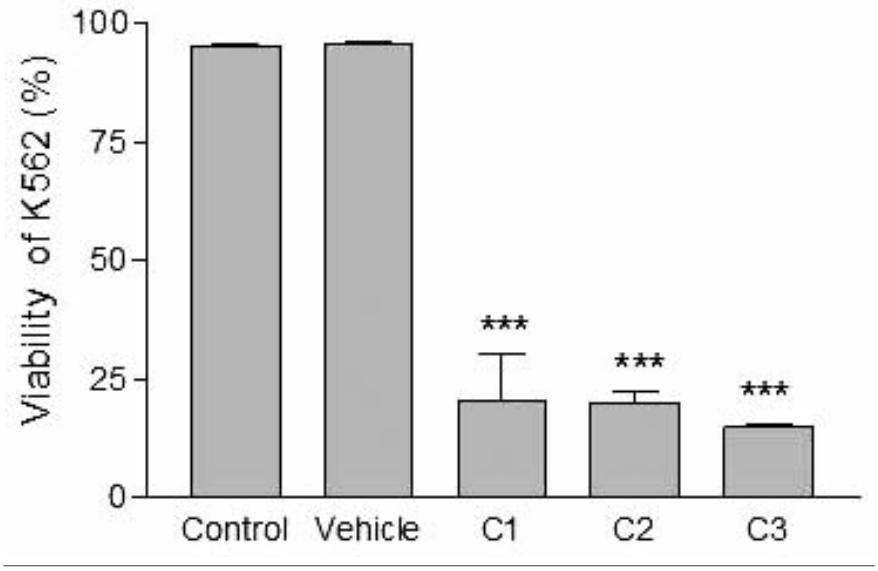


Figure 6. Effects of various concentrations of PCDD/Fs mixture on the human tumor cells (K562) after 3 h of *in vitro* exposure. Asterisks represent significant difference compared with vehicle controls (* P<0.001).**

Some studies have compared the quantitative levels of immune components such as NK cells (CD16+, CD56+) in breast-fed and bottle-fed infants whose mothers were exposed to PCDDs/Fs and PCBs. It was shown that the content of dioxins in breast milk was not enough to induce any change in theses-examined immunological parameters during the first year of life.[38, 39]

In conclusion, in our *in vitro* study, it would appear that the PCDDs/Fs presented enhancing effects on NK-like cell activity at the dose of 25×10^{-3} ng 2378-TCDD/mL in *E. andrei* earthworms. This effect may be explained as a compensatory activation of the body's defences brought about by disturbances in the function of other arms of the immune system.[37]

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