Short-term immunotoxic effects of an anti-cancer drug (Etoposide) on the freshwater pond snail Lymnaea stagnalis

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Introduction

A growing body of evidences indicates the rise in pharmaceutical contamination of aquatic ecosystems. Anti-cancer drugs (AKs) are very special because they may avoid some toxicological studies, clinical trials or environmental toxicity assessments imposed for other pharmaceuticals (EMEA, FDA). In parallel, these drugs require high dosage. It generates high amounts of biologically active molecules into hospital effluents and by extension into surface waters. Both parent-compounds and metabolites are being encountered at high concentrations (ng/L to μg/L). The original molecules may be generated from the fraction non-metabolized by humans (≈0.40 for etoposide). Eventually, it may be the result of accidental spillages or lack of environmental good practices.

Among AKs, Etoposide is widely used in chemotherapy (total amount consumed in France in 2004 was 332.84 kg). It is prescribed worldwide against lung, testicles and blood cells (lymphoma) cancers in humans. This plant-derived alkaloid is an anti-neoplasmic, cytotoxic molecule reported to cause single DNA breaks with topoisomerase II inhibiting effects. It has been detected at concentrations (ng/L to μg/L). The highest therapeutic-like concentrations were chosen according to the environmentally-realistic concentrations in hospital effluents (5 μg/L). The lowest concentrations were chosen according to the cytotoxic effects observed in human plasma concentrations at 0.7–2 μg/mL. Exposure temperature was set at 20°C, constantly recorded and a neon light (1000 lux) allowed a photoperiod of 16 h/8 h (light/dark). Organisms were fed ad libitum with ground TetraPhyl® (30 mg/snail). During the test, mortality and water parameters (dissolved oxygen, ammoniac, pH, conductivity) were daily measured. The exposure lasted for three days (72 h).

Hemolymph collection and flow cytometry analysis

Hemolymph samples were collected with a micropipette by gently tickling the snail foot sole. Hemocyte density and viability were immediately measured. Acquisitions were performed on a Guava easyCyte™ 6-HT multicolor flow cytometer (Merck Millipore) using the GuavaSoft 2.7 software. The ViaCount module was used for cell density and viability determination, whereas the Incyte module was used for the phagocytosis assessment. Total hemocyte density (THD) and viability were measured using ViaCount reagent. Briefly, hemolymph was diluted (1:4) with the reagent into 96-well plates (Costar®, sterile, U-bottom, non-treated, polystyrene) and incubated 10 min (in the dark and under slow agitation). In total, 1000 events per sample were acquired. Regarding the phagocytosis, 200 μL of hemolymph collected from each individual were disposed onto a 96-well plate (Greiner® bio one, for cell cultures, sterile, F-bottom, polystyrene). An excess amount of 7.5×10² latex microbeads (polysciences YG 2.0 μm latex microsphere) were gently mixed with each sample. Plates were incubated for 24 h in the dark under gentle agitation. The supernatant was removed and cells were fixed (PBS containing 0.5% of formaldehyde (v/v) and 0.2% (w/v) of sodium azide). 20,000 events/sample were acquired and read in the YELLOW photomultiplier (583±26 nm). Calculations were performed as follows: active hemocytes were the proportion of cells that engulfed at least one microbead, whereas the mean number of microbeads per active hemocytes reflects the activity of phagocytosis.

Statistical analysis

Statistical analyses were performed by running a Kruskal-Wallis Test followed by repeated pairwise Wilcoxon post hoc tests when necessary to compare exposed groups with the control group. The result was considered statistically significant when P<0.05 (**P<0.001 while *P<0.05). Beforehand, the Shapiro test for normality distribution and Bartlett test for homogeneity of variances were run for each group. All statistical analysis were realized with the open source R software.

Results and Discussion

At therapeutic concentrations (100-1000 μg/L), a sharp drop of total hemocyte density (THD) was observed (Figure 1A). It is consistent with previous studies reporting cytotoxic effects on human blood cells at such concen-
trations. At the environmentally relevant concentrations a significant increase in hemocyte density was observed compared to controls (Figure 1A). Similar results have already been observed in L. stagnalis at environmentally realistic concentrations of several xenobiotics. Hemocytes viability significantly decreased with increasing drug concentration (Figure 1B). No significant effects were measured on phagocytosis activity. The proportion of active hemocytes is similar under all exposure conditions (approximately 20%, Figure 1C). Phagocytosis activity was around a mean of 2.9 microbeads per hemocyte in all treatments (Figure 1D).

Conclusions

Our study highlights the immune disruptive effects of etoposide on the pond snail L. stagnalis. At realistic concentrations found in effluents of oncology-specialized hospitals, it exerts immunomodulation effects, whereas at therapeutic-like concentrations the effects become immunosuppressive (cytotoxicity). In addition to this reduced number of hemocytes, a high cellular mortality rate was observed (up to 40%). However, it seems that phagocytosis carried out by the remaining viable cells was unaffected. It indicates that etoposide is rather cytotoxic than blocks phagocytosis pathways. This experimentation constitutes a first acute approach and further studies are required to clarify: i) the underlying mechanism of actions that lead to the observed increase in hemocytes density at low concentrations; ii) the effects of a chronic exposure; and iii) the further consequences on physiological functions such as growth and reproduction.

References

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