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Sublethal toxicity of Roundup to immunological and molecular aspects of *Biomphalaria alexandrina* to *Schistosoma mansoni* infection

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ABSTRACT

The present study was performed to elucidate the cellular mechanisms of *Biomphalaria alexandrina* snails hemocytes against sublethal concentration (10 mg/L) of herbicide Roundup (48% Glyphosate) and/or *Schistosoma mansoni* infection during 7 days of exposure. Obtained results indicated that herbicide treatment and/or infection led to significant increase ($P < 0.05$) in total hemocytes count during exposure period. Examination of hemocytes monolayers resulted in observation of 3 morphologically different cell types, round small, hyalinocytes and spreading hemocytes. Spreading hemocytes are the dominant, more responsive and highly phagocytic cell type in all experimental groups. Moreover, the exposure to herbicide, infection or both together led to a significant increase ($P < 0.05$) of *in vitro* phagocytic activity against yeast cells during 7 days of exposure. In addition, flow cytometric analysis of cell cycle and comet assay, resulted in DNA damage in *B. alexandrina* hemocytes exposed to herbicide and/or *S. mansoni* infection when compared to control group. The immunological responses as well as molecular aspects in *B. alexandrina* snails have been proposed as biomarkers of exposure to environmental pollutants.

1. Introduction

Particular attention has been directed to immunological changes induced by environmental pollution in aquatic invertebrates (Livingstone et al., 2000). The defense mechanisms and immunological responses which consist of the immune system have been considered as biomarkers of pollution in aquatic invertebrates (Galloway and Depledge, 2001). Despite the lack of an adaptive immune system, invertebrates are able to survive among potential pathogens and respond to infection by activation of various defense mechanisms (Little et al., 2005). The immune system is likely to be one of the most sensitive physiological systems to pollutants (Fournier et al., 2000). Pollutants can interact with immune system components and interfere with protection function that induce immune suppression and decrease of disease resistance (Wong et al., 1992). Schistosomiasis is one of the major communicable diseases with socio-economic importance in the developing world. It was estimated that 800 million people in 74 countries are at risk while more than 200 million were infected (Steinmann et al., 2006). *Biomphalaria* snails have a great medical importance as intermediate hosts of *S. mansoni* (Paraense, 2001). In this respect, interactions between *Biomphalaria* snails and schistosomes have received much attention. Defense system of freshwater snails mainly depends on hemocytes, which are the circulating cells involved in mediating internal defense and immune functions (Yoshino et al., 1998). Phagocytosis is one of the various functions of hemocytes, which is a non-specific immune mechanism against non-self materials (Galloway and Depledge, 2001; Negrao-Correa et al., 2007). The response patterns, density and functions, of hemocytes can be affected by xenobiotics and parasites (Livingstone et al., 2000). Furthermore, the development of an infectious disease results from an imbalance between the host and the pathogen due to external factors, like pollutants, and/or internal factors, like susceptibility of the host (Snieszko, 1974).

Herbicides are distinctive group of pesticides and are considered as selective weed killers. Roundup is a commercial herbicide with active compound glyphosate used in a broad spectrum in agricultural applications for weed control (USDA, 1984; Williams et al., 2000; Cavas and Konen, 2007). Due to its high water solubility and extensive usage, especially in shallow water systems, the exposure of non-target aquatic organisms to this herbicide is a concern (Tsui and Chu, 2003). Results of previous studies showed that glyphosate may have effect on plants, fishes, amphibians, arthropods and snails by causing physiological, immunological and biochemical alterations (Glusczak et al., 2006; Achiorno et al., 2008; Schneider et al., 2009; Benamu et al., 2010).

Comet assay is becoming a major tool for environmental biomonitoring (Grazeffe et al., 2008) because of its sensitivity and determination of DNA strand breaks in individual cell (Lee and Steinert, 2003). Some studies reported that glyphosate treatment of human lymphocytes *in vitro* resulted in increased chromatid exchange (Bolognesi et al., 1997), chromosomal aberrations and

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indicators of oxidative stress (Lioi et al., 1998). Furthermore, Roundup proved to increase DNA adducts in mice (Peluso et al., 1998). Recently, Mladinic et al. (2009) used comet assay to measure glyphosate impact on DNA of human lymphocytes and the results revealed that glyphosate increased tail intensity.

It is hypothesized that immunological biomarkers in snails e.g. *Biomphalaria* would improve their use as diagnostic organisms in biomonitoring. Therefore, the present study was undertaken to evaluate the immunological and molecular responses of *B. alexandrina* snails to the effect of sublethal concentration of Roundup herbicide treatment and/or *S. mansoni* infection.

2. Materials and methods

2.1. Experimental animals and infection

Laboratory bred *Biomphalaria alexandrina* snails and *Schistosoma mansoni* ova were obtained from The Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute, Giza, Egypt. The adult snails (8–10 mm shell diameter) were maintained in 5 L capacity plastic aquaria under lab conditions at a density of 50 individual per tank. Water temperature was maintained at 25 ± 2 °C. The snails were fed dry lettuce leaves daily. For snail infection *B. alexandrina* snails were individually exposed to 7–9 freshly hatched miracidia from *S. mansoni* ova in glass test tubes for 2 h (Anderson et al., 1982).

2.2. Experimental material

Roundup herbicide was used in the liquid commercial form produced by Monsanto Agricultural Company, USA. It consists of 48% (480 g/L active ingredient "a.i.") of glyphosate (N-phosphonomethyl glycine). Sublethal concentration of Roundup (10 mg/L), equivalent to 0.02 mg/L a.i. glyphosate was prepared from stock solution (1000 mg/L). Fifty snails were placed in 5 L aerated plastic aquaria containing the used concentration according to Osman et al. (2008).

2.3. Experimental bioassays

Four treatments were employed in this study, (50 snails were used in each treatment):

1. Normal control group.
2. *S. mansoni*-infected group.
3. 10 mg/L Roundup-treated group.
4. Treated-infected group.

The evaluation of sublethal effect of Roundup and/or *S. mansoni* was done by: (1) total and differential hemocyte counts, (2) *in vitro* phagocytosis, (3) comet assay and (4) flow cytometric analysis of cell cycle. Each ones are detailed below.

2.3.1. Total and differential hemocyte counts

The hemolymph was collected from the four experimental groups as described by Sminia (1972). In brief, the headfoot was touched by a Pasteur pipette; as a result the snail was forced to retract deeply into its shell and extruded hemolymph. From each individual snail, ca.75 μL hemolymph was obtained and collected using a micropipette. Hemolymph samples were collected at intervals of 6 h, 1, 3 and 7 days post-exposure from the four experimental groups. The number of total hemocytes was counted in 10 snails individually from each experimental group using haemocytometer. For differential hemocytes count, hemolymph samples were placed onto glass slides and allowed to settle in moist chamber. The hemocytes monolayers were stained after methanol fixation with Giemsa's stain. Data were expressed as mean values of a proportion of each cell population from the original 100 cells.

2.3.2. In vitro phagocytosis

The suspension of freshly prepared yeast cells was diluted in PBS to get the ratio of 10,000 cell/mL for use and phagocytosis was carried out as described by Abdul-Salam and Michelson (1980). Freshly collected hemolymph from 10 individual snails was overlaid with an equal volume of yeast suspension on a clean glass slide. Reaction mixtures were incubated in moist chamber for 60 min. The phagocytic reaction was stopped using absolute methanol after washing with PBS (pH 7.4). For each slide 100 cells was examined and phagocytic activity was expressed as the mean values ± SE of positive phagocytized hemocyte during 7 days of exposure.

2.3.3. Comet assay

Comet assay was performed for measuring DNA damage after 24 h post-herbicide treatment and/or *S. mansoni* infection by single cell gel assay which permits the detection of single stranded DNA breaks (SSBs) in one cell according to Singh et al. (1988) and Grazeffe et al. (2008). For each experimental group, collected hemolymph from 5 individual snails was pooled in 1.5 Eppendorf tube. Three microscope slides were covered with 1.5% normal melting agarose dissolved in phosphate buffered saline (PBS) free of Ca^{2+} and Mg^{2+} and maintained overnight at room temperature (25 ± 2 °C). A volume of 100 μL of pooled hemolymph was dissolved in 300 μL of 0.7% of low-melting agarose dissolved in PBS Ca^{2+} and Mg^{2+} free and placed on the first gel layer at 37 °C. After solidification at 4 °C for 10 min, the slides were immersed in a jar containing cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium sarcozinate, 1% Triton X-100 and 10% dimethyl sulfoxide (DMSO)), pH 10 at 4 °C for 2 h. After lysis, the slides were placed in horizontal electrophoresis box. The unit was filled with a fresh alkaline electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH 13) to a level of 0.25 cm above the slides. The cells were exposed to alkali for 15 min to allow DNA unwinding and expression of alkali-labile sites. To electrophoresis the DNA, an electric current of 25 V (0.86 V/cm) and 300 mA was applied for 20 min. Alkali and electrophoresis treatments were performed in an ice bath. All these steps were conducted under dim light to prevent the occurrence of additional DNA damage. After electrophoresis, the slides were placed horizontally and Tris buffer (0.4 M Tris, pH 7.5) was added to neutralize the excess alkali. Finally, slides were fixed with absolute ethanol for 10 min and stained with 100 μL ethidium bromide (20 μg/mL). From each slide 100 cells were examined visually and the percentage of damaged hemocytes DNA was scored (Lee and Steinert, 2003). Examination of the slides was done under fluorescence microscope (Olympus BX 60, Japan) equipped with an excitation filter 510 nm and barrier filter of 590 nm (1000 × magnification). All chemicals were purchased from Sigma.

2.3.4. Flow cytometric analysis of cell cycle

Flow cytometric analysis was carried out for detecting apoptosis of *B. alexandrina* hemocytes isolated from pooled hemolymph samples from 15 snail in each experimental group 7 days post-herbicide treatment and/or *S. mansoni* infection. The flow cytometer used is FACS calibur flow cytometer (Becton Dickinson, Sunnyvale, CA, USA) equipped with a compact aircooked low power 15 mW argon ion laser beam (488 nm). The average number of evaluated nuclei per specimen was 20,000 and the number of nuclei scanned was 120 per second. DNA histogram derived from flow cytometry was obtained with a computer program for Dean and Jett mathematical analysis (Dean and Jett, 1974). Data analysis was conducted using DNA analysis program MODFIT (verity software house, ME 04086 USA, version, 2.0). Apoptosis was measured by using the sub G1 peak staining with propidium iodide (Cohen and Al-Rubeai, 1995).

2.3.5. Statistical analysis

Total and differential hemocyte counts and phagocytosis data are presented as mean ± standard error. The significance of difference between the means was calculated according to the way analysis (ANOVA) followed by Student's *t*-test (Sokal and Rohlf, 1981). Result was considered statistically significant at $P < 0.05$.

Table 1
Effect of Roundup on total hemocytes count/ml in hemolymph of non-infected and *S. mansoni*-infected *B. alexandrina* snails during 7 days of exposure.

Exposure period	Experimental groups			
	Control	Herbicide treated	Infected	Treated- infected
6 h	13.98 ± 1.17	15.3 ± 0.98	3.6 ± 0.5 *	8.2 ± 0.3*
1 day	11.46 ± 0.7	40.6 ± 2.1*	15.9 ± 1 *	12.9 ± 1
3 days	11.68 ± 1.3	39.3 ± 2.2 *	8.26 ± 0.5	40.8 ± 1.4 *
7 days	14.25 ± 1.2	39.8 ± 2.7 *	4.7 ± 0.4 *	37.9 ± 1.6 *

Data are presented as mean/10^4 ± standard error (n=10; 1 snail per replicate).

* Significant at $P < 0.05$.