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Establishment of the comet assay in the freshwater snail *Biomphalaria glabrata* (Say, 1818)

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ABSTRACT

The single cell gel eletrophoresis or the comet assay was established in the freshwater snail *Biomphalaria glabrata*. For detecting DNA damage in circulating hemocytes, adult snails were irradiated with single doses of 2.5, 5, 10 and 20 Gy of ^{60}Co gamma radiation. Genotoxic effect of ionizing radiation was detected at all doses as a dose-related increase in DNA migration. Comet assay in *B. glabrata* demonstrated to be a simple, fast and reliable tool in the evaluation of genotoxic effects of environmental mutagens.

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1. Introduction

Surface waters are recipients of most substances generated by industrial, agricultural and domestic activities and released into the environment [1]. Despite the complex chemical nature of polluted waters, it was demonstrated that mutagenic compounds can enter surface waters from a wide range of industrial and municipal sources [2,3]. Although exposure of aquatic organisms to genotoxic contaminants can pose a risk to human health via the food chain, there is also an ecological risk that may lead to heritable mutations and loss in the total genetic diversity [4]. Mutagens can also directly affect the reproductive potential of populations by alterations in the fecundity and offspring viability caused by germ cell mutations [5].

The single cell gel/comet assay [6] is becoming a major tool for environmental biomonitoring [7]. The comet assay has advantages over other DNA damage methods, such as sister chromatid exchange, alkali elution and micronucleus assay, because of its high sensitivity and because DNA strand breaks are determined in individual cells [8].

Several species of mollusks [9–12], polichaetes [13], fishes [14–16] and other aquatic organisms have been used to monitor environmental mutagens by using the comet assay.

The choice of a freshwater snail as a model for environmental biomonitoring of chemical mutagens took into account that invertebrates represent more than 90% of aquatic species. The phylum Mollusca has the highest number of animal species after arthropods and 80% of molluscs species are represented by gastropods [17,18]. Although there is few data on metabolism, cell cycle and DNA repair processes in invertebrates, it is essential to detect mutagenic effects in these organisms in order to evaluate the impact of pollution in an aquatic ecosystem [4]. In addition, *B. glabrata* is a good model for laboratory testing. The snails are easy to breed, need little space, can reproduce throughout the year under controlled conditions and have a short life-span. Being a simultaneous hermaphrodite, different reactions in both sexes do not occur.

Recently, we have developed an assay to evaluate germ cell mutations in the freshwater snail *Biomphalaria glabrata*. First, mutagenic effects of reference agents were evaluated. The test mutagens included the direct acting agents ionizing radiation [19] and mitomycin C [20] and a compound that requires metabolic activation, cyclophosphamide [20]. The system was efficient, sensitive and capable of metabolically activating indirect mutagens. In a follow-up study, the dominant lethal effects of the herbicide 2,4-D were

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demonstrated [21]. In this study, we evaluated DNA damage in hemocytes of *B. glabrata* after irradiation with ^{60}Co gamma by using the single cell gel/comet assay.

2. Material and methods

2.1. Animals

A pigmented wild strain of *B. glabrata* obtained from Barreiro (state of Minas Gerais) and reared in the laboratory over the past 20 years was used. The colonies are maintained in plastic aquaria with filtered, dechlorinated and aerated water and fed fresh lettuce. We used adult snails at least two months old and with a minimal shell diameter of 10 mm.

2.2. Exposure

Snails were exposed to ionizing radiation in plastic tubes, individually separated by cotton pads. Irradiation was carried out in a ^{60}Co source—Gamma-Cell Atomic Energy of Canada Ltd., model GC-220 from Instituto de Pesquisas Energéticas e Nucleares/Comissão Nacional de Energia Nuclear, in the presence of molecular oxygen at room temperature.

Animals were irradiated with single doses of 2.5, 5, 10 and 20 Gy at 2.82 KGy/h of dose rate. One negative non-exposed control group was exposed to the same conditions of the exposed group.

2.3. Sampling of hemolymph

Each snail was wiped and 100 μl of hemolymph was collected by puncturing the foot with a micropipette. The blood was placed directly into 1.5 ml Eppendorf tubes. Twenty four hours before sampling, snails were stimulated by gently touching the foot with the tip of a micropipette.

2.4. Comet assay

The alkaline comet assay was performed as described by Singh et al. [6] with modifications.

For each animal, three microscope slides were covered with 1.5% normal melting agarose (Sigma) dissolved in PBS (Ca^{2+} and Mg^{2+} free) and maintained overnight at room temperature. A volume of 100 μl of hemolymph dissolved in 500 μl of 0.5% low melting agarose (Sigma, PBS Ca^{2+} and Mg^{2+} free) was placed on this gel layer at 37 °C. After solidification at 4 °C (5–10 min), the slides were placed vertically in a cuvette with lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100 and 10% DMSO, pH 10.0) overnight at 4 °C to remove proteins. After lysis, the slides were placed side by side in a horizontal electrophoresis tank (17 cm × 20 cm) (Permatron) and immersed in alkaline buffer, pH 13.0 (1 mM EDTA and 300 mM NaOH) for 30 min to allow DNA damage expression and submitted to electrophoresis (0.74 V/cm, 150 mA) (Pharmacia) for 30 min at 4 °C. The slides were then neutralized with 0.4 M Tris buffer, pH 7.5, fixed with absolute ethanol for 10 min, and stained with 50 μl ethidium bromide (20 μg/ml) (Sigma). The comets were analyzed under a fluorescence microscope (Carl Zeiss) at 400×, with an exciting filter of 515–560 nm and a barrier filter of 590 nm.

Slides were coded independently and scored blindly.

2.5. Data analysis

The extent of DNA migration was evaluated by visual scoring. Comets were classified and assigned to four categories (0–3) according to the extent of DNA migration. The classification was carried out on the basis of an appearance of comets, i.e. tail length, head diameter and intensity in the following way. Comets with bright heads and no apparent tails were assigned to category 0, comets with very little heads and long, diffused tails to category 3. Comets displaying features intermediary between categories 0 and 3 were divided and assigned to easily distinguishable categories 1 and 2 (Fig. 1). Comets with small or nonexistent head and large diffuse tails were not included in the analysis.

2.6. Statistical analysis

The probability model used was the multinomial distribution, as a consequence of the experiment design. For each radiation dose, the number of comets was fixed in 100 per animal. Hence, the vector of comet category frequencies is a multinomial vector with 4 categories. Having ordered samples according to radiation doses and multinomial distributions, the multinomial logistic regression statistical tech-

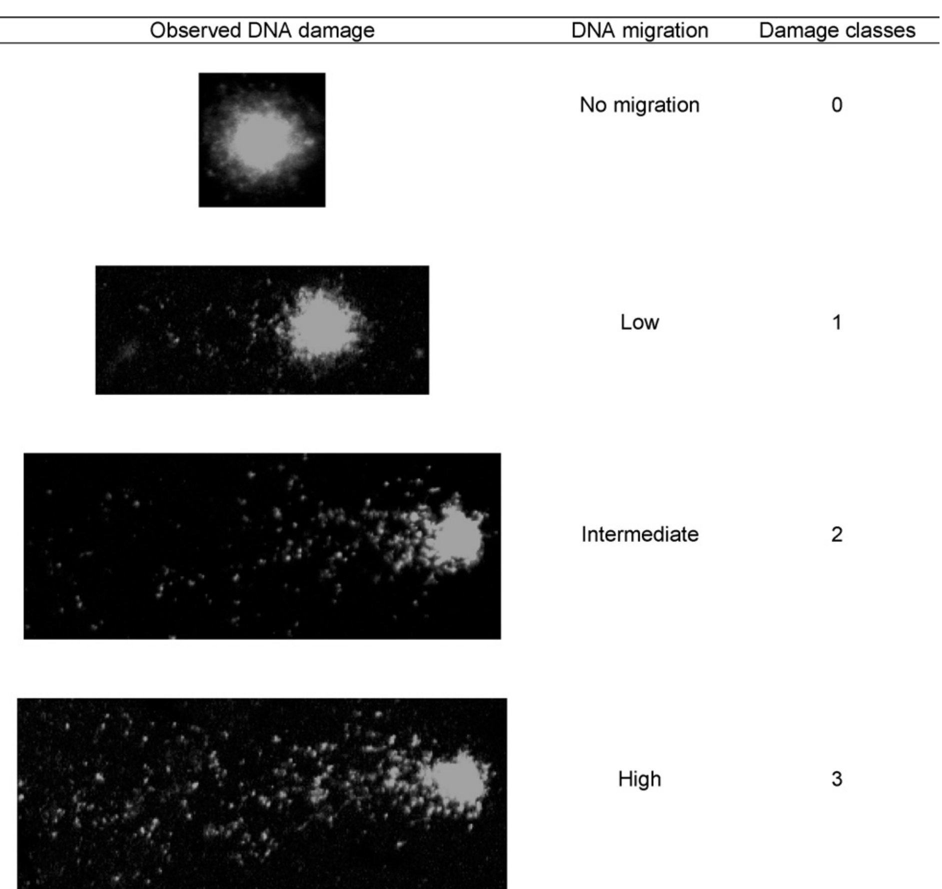

Fig. 1. Classes of DNA damage as detected by the comet assay in hemocytes of *Biomphalaria glabrata*.

Table 1
Comet assay: DNA damage in hemocytes of *B. glabrata* exposed to ^{60}Co gamma radiation

Dose(Gy)	Number of animals	DNA damage classes (%; S.E.)				Number of cells (%)
		C0	C1	C2	C3	
0	11	654 (59; 69)	125 (11; 17)	72 (7; 15)	249 (23; 61)	1100 (100)
2.5	9	442 (49; 64)	178 (20; 40)	105 (12; 21)	175 (19; 38)	900 (100)
5	9	197(22; 41)	253 (28; 27)	173 (19; 29)	277 (31; 52)	900 (100)
10	10	159 (16; 58)	296 (30; 34)	264 (26; 41)	281 (28; 49)	1000 (100)
20	9	58 (6; 46)	49 (5; 19)	133 (15; 22)	660 (73; 54)	900 (100)

S.E. = standard error for the total frequencies of DNA Damage classes.

nique was chosen. This statistical analysis was performed using the 'Multinomial Logistic Regression' module of Version 14.0 of the Statistical Package for Social Sciences (SPSS) for Windows. For details in this technique see, for instance, [22]. For additional applications see also [23]. In addition to the logistic regression, we used contingency tables with Chi-squared tests to measure the significances suggested from the logistic regression graph. For this we used the Chi-squared partitions [24].

3. Results

The data on DNA damage induced by ^{60}Co gamma radiation in *B. glabrata* is in Table 1. Table 2 shows the logistic regression coefficients values that were significantly different from zero. Table 3 shows the absolute and relative (%) frequencies of damage. Fig. 2 shows the logistic regression lines for each damage category.

Irradiated animals showed a dose dependent increase in the DNA migration. Considering the four different classes of DNA damage, the Chi-squared Homogeneity Test showed a highly significant difference among dose groups ($p < 0.0001$; Chi-square = 1534 with 12 degrees of freedom, d.f.). A strong dose–response relationship was observed for the classes 0 and 3. Classes 2 and 3 increased up to intermediate doses and then decreases to the high doses. A positive (negative) association was observed for the damage class 3 (class 0). The difference in animal responses to 5 and 10 Gy,

Table 2
Regression coefficients for the conditional probability of damage

Damage[a]	Coefficients	β	Wald Stat	d.f.	p
Damage 0	Constant	1.033000	240.963	1	0.000
	Dose	−0.188615	78.238	1	0.000
	Dose^2	0.000873	0.647	1	0.421
Damage 1	Constant	−0.653000	52.936	1	0.000
	Dose	0.228050	94.233	1	0.000
	Dose^2	−0.016270	190.381	1	0.000
Damage 2	Constant	−1.204000	130.023	1	0.000
	Dose	0.239692	89.811	1	0.000
	Dose^2	−0.012982	131.130	1	0.000

Damage type frequency as a function of dose: The logistic regression functions

$$\Pr(0\,\text{damage}) = \frac{e^{1.033-0.188615\times\text{dose}+0.000873\times\text{dose}^2}}{1+e^{1.033-0.188615\times\text{dose}+0.000873\times\text{dose}^2}+e^{-0.653+0.228050\times\text{dose}-0.016270\times\text{dose}^2}+e^{-1.204+0.239692\times\text{dose}-0.012982\times\text{dose}^2}}$$

$$\Pr(1\,\text{damage}) = \frac{e^{-0.653+0.228050\times\text{dose}-0.016270\times\text{dose}^2}}{1+e^{1.033-0.188615\times\text{dose}+0.000873\times\text{dose}^2}+e^{-0.653+0.228050\times\text{dose}-0.016270\times\text{dose}^2}+e^{-1.204+0.239692\times\text{dose}-0.012982\times\text{dose}^2}}$$

$$Pr(2\,\text{damage}) = \frac{e^{-1.204+0.239692\times\text{dose}-0.012982\times\text{dose}^2}}{1+e^{1.033-0.188615\times\text{dose}+0.000873\times\text{dose}^2}+e^{-0.653+0.228050\times\text{dose}-0.016270\times\text{dose}^2}+e^{-1.204+0.239692\times\text{dose}-0.012982\times\text{dose}^2}}$$

$$Pr(3\,\text{damage}) = \frac{1}{1+e^{1.033-0.188615\times\text{dose}+0.000873\times\text{dose}^2}+e^{-0.653+0.228050\times\text{dose}-0.016270\times\text{dose}^2}+e^{-1.204+0.239692\times\text{dose}-0.012982\times\text{dose}^2}}$$

[a] The reference category is damage 3.

Table 3
Absolute and relative frequencies of damage

Dose	Observed				Estimated[a]			
	Damage class				Damage class			
	0	1	2	3	0	1	2	3
0	0.595	0.114	0.065	0.226	0.607	0.112	0.065	0.216
2.5	0.491	0.198	0.117	0.194	0.430	0.203	0.123	0.244
5	0.214	0.281	0.192	0.308	0.285	0.276	0.183	0.255
10	0.159	0.296	0.264	0.281	0.138	0.297	0.267	0.297
20	0.064	0.054	0.148	0.733	0.067	0.054	0.147	0.731

[a] Estimated population frequencies from the logistic regression functions.

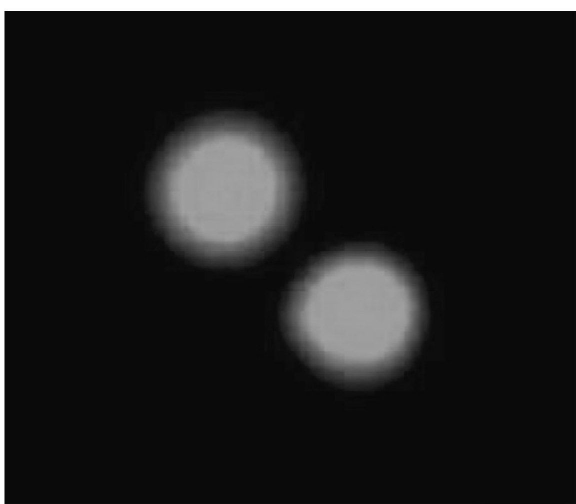

Fig. 2. Non-damaged HUVEC cells. From P. Sestili et al./Mutation Research 607 (2006) 205–214.

although lower than in other groups, was also statistically significant ($p = 0.01\%$; Chi-square = 21; 3 d.f.). However, damage classes 1 and 3 showed no statistical differences between doses 5 and 10 ($p = 43\%$; Chi-square = 1.7; 2 d.f.).

4. Discussion

B. glabrata was demonstrated to be a suitable organism for bioassays. These freshwater snails have been used for determination of various toxicological responses including acute toxicity, effects on embryonic development and germ cell mutagenicity [19–21,25–29].

In this study, genotoxic effects of ionizing radiation were evaluated in hemocytes of snails exposed to four single doses of ^{60}Co gamma radiation. Besides its high efficiency in inducing DNA strand breaks, ^{60}Co gamma radiation was selected as reference mutagen for this study because of some specific characteristics. Morales-Ramirez et al. [30] stated that ionizing radiation acts as a direct mutagen with minimal latency time of action, since it does not require absorption, distribution or metabolism; furthermore, the time of effective activity is practically zero. This can be advantageous in the establishment of a mutagencity test by minimizing variation in response due to interindividual differences in toxicokinetics.

Four dose levels of ^{60}Co gamma radiation were selected based on range-finding experiments (data not shown). 20 Gy was the lowest dose level producing lethality and nearly 100% of mortality was reached with 100 Gy. These results are in agreement with previous data on lethality of ^{60}Co gamma radiation in *B. glabrata* [31,32]. In the comet assay in *B. glabrata*, slides from animals irradiated with 50 and 100 Gy (data not shown), but not in the other groups, showed an excessively low number of cells preventing the analysis.

The comet assay was conducted according Singh et al. [6] with modifications. The assay was performed with cells from hemolymph of *B. glabrata*. Hemocytes from the hemolymph of mollusks and crustaceans and coelomocytes from the coelom of annelids have been used for the comet assay [8]. Hemolymph is suggested as the more appropriate test tissue for low cell manipulation and a shorter slide preparation time. Hemocytes are exposed to environmental agents through their physiological roles in the transport of toxicants and in various defense mechanisms [33]. An advantage of using hemocytes of *Biomphalaria* is its easy sampling through a non-invasive technique: hemolimph is released by simply touching the foot surface and a large number of cells can be obtained by previous estimulation through the same technique.

Different time intervals were tested for lysis. Negative results were obtained with 30 min up to 2 h of lysis. According recommendations for conducting the *in vivo* alkaline comet assay [34], the lysis duration can vary considerably, from less than 1 h to weeks if not months. There is a minimal time needed to appropriately liberate the DNA and this time might vary depending on the cell type. Overnight lysis in this work was based in the study of Bolognesi et al. [33] with hemocytes of *Dreissena polymorpha*.

Conditions of eletrophoresis used for *Biomphalaria* were determined with basis on previous experiments (data not shown). With 0.74 V/cm and 150 mA, low levels of DNA migration in control animals were obtained and the detection of effects with low doses of radiation was possible.

In this study, DNA migration was evaluated by visual scoring. Although image analysis is more accurate in the quantification of DNA damage, allowing to measure the fluorescence intensity and distribution of DNA throughout the comet and giving a quantitative measure of DNA migration, visual scoring has the advantage of technical simplicity and low equipment costs. According to international guidelines and recommendations for comet assay, image analysis is preferred, but it is not required and visual scoring of comets remains a well-validated evaluation method that can be used with high reliability [35].

In the visual scoring, a numeric index is usually employed to represent the average DNA migration, which may vary from 0 (all cells undamaged—0×100) to 400 (all cells damaged—4×100, for classification 0–4) [36]. As the usual index do not support standard topologies, we decided to use the whole frequencies for the statistical study.

In our study, the standard logistic multiple regression statistical technique was chosen. Contingency tables Chi-squared tests were additionally used to precisely measure the significances suggested from the logistic regression graph. This was the case of the comparison of G(5) and G(10), in which the difference in response was lower than in the other dose groups. To compare specifically these groups, the Chi-squared partitions were used [24]. We collapsed categories C(0) and C(2) in one category resulting now in 3 categories: [C(0) + C(2)], C(1), and C(3). Using these three modified categories, the resulting Chi-squared statistic (2 degrees of freedom) value is 1.7, showing non-significant difference among the two groups ($p = 43\%$). Completing the analysis, we consider only the two categories, C(0) and C(2), and perform the test to compare G(5) and G(10). The resulting statistic (1 degree of freedom) value for this test is 19.3, the complement of the reduced test (1.7) in relation to the global one (21).

A high interindividual variability was observed among the frequencies of comets in all groups due to, in some cases, few individuals. As these are the first results on DNA damage in the comet assay in *Biomphalaria* snails, we decided not to exclude any of the subjects, even the outliers. Background levels of damage in hemocytes of this species is not established, as well as the sensitivity of theses cells to any genotoxin. Taking into account this observed variability, the analysis has considered each dose group as a sample. It should be noticed that, for each group of radiation dose, we consider that all sample units are under the same conditions of stress. In such a way, all units inside a specific radiation group are considered to have the same value of the population parameter vector $P = (p0; p1; p2; p3)$ – the parameter of interest to be studied – that is the population relative frequency vector of the four kinds of damages. If, for instance, within the group we have 9 animals – i.e., 9 multinomial frequency vectors with the same parameter vector and same sample size (number of cells studied) equal to 100 – the

sum of all observed frequencies is a sufficient statistic distributed as multinomial with the same parameter of interest, P, and sample size 900. This experimental design turned the comparisons of the five radiation group parameter vectors, P, very precise.

Visual analysis in our study was effective in the detection of genotoxic effects of ^{60}Co. According criteria for determining a positive result [34], a significant increase or decrease in DNA migration at one or more dose groups should be obtained. In our study, a dose-dependent increase in DNA migration was observed. The lowest effective dose was 2.5 Gy, corresponding to approximately 3–4% of LD_{50} [31,32].

In irradiated snails, there was a significant increase in the frequencies of comets with small or nonexistent head and large diffuse tails (data not shown) usually classified in the comet assay as dead cells. We have excluded these cells from the analysis of DNA damage, since, according to comet assay guidelines, it could lead to a false positive result [34,35,37,38]. It was shown for *in vitro* tests that such cells can be present upon treatment with cytotoxic, non-genotoxic agents. However, such microscopic images can also be seen after treatment *in vitro* with high doses of radiation or high concentrations of strong mutagens, indicating that such comets are not uniquely diagnostic for apoptosis/necrosis [34,35,37,38]. In our study, the positive result obtained with ionizing radiation suggests a genotoxic effect instead of cytotocixity.

It is yet not clear whether increased DNA fragmentation due to cell death can result in the generation of false positive results in the comet assay. No data are yet available on whether cell death in tissues of experimental animals may also be associated with increased DNA migration patterns in the comet assay *in vivo*. There is consensus on the need to include measures of cytotoxicity and to address the possible effects of cytotoxicity in comet data interpretation. A dye exclusion test for membrane integrity and metabolic competency is one of the methods [34,35,37,38] suggested for processing additional comet assay slides under modified conditions. Due to the very low molecular weight of the DNA in apoptotic/necrotic cells, these cells may be lost under electrophoretic conditions and lead to an underestimate of the proportion of apoptotic/necrotic cells. By analysis of slides after lysis without further alkaline unwinding or electrophoresis or by omitting the electrophoresis step after alkaline unwinding, cells with low molecular weight DNA resulting from degradation due to double-strand breaks can be detected.

Fig. 4. HUVEC cells showing a wide halo. From P. Sestili et al./Mutation Research 607 (2006) 205–214.

This method might, therefore, provide valuable information for the interpretation of a positive response. In our study, additional slides were processed for DNA diffusion after lysis, but no clear distinction between cells with or without halo was possible (Figs. 3 and 4) and a higher frequency of cells with halo in control slides was observed (data not shown). Sestili et al. [38] have embedded human promonocytic cells in 2.0% of low-melting agarose to perform the halo assay. In our work, hemocytes of *B. glabrata* were suspended in 0.5% of LMP agarose, which could result in a high diffusion of non-damaged DNA and lead to a false positive result. In addition, at this LMP agarose concentration, boundaries of undamaged cells are not well delimited as in human promocytic cells (Figs. 5 and 6), which supports this assumption. Higher concentrations of LMP agarose could reduce DNA diffusion and the performance of the comet assay in *B. glabrata*. Considering the inconclusive results on cell death from the comet assay and to ascertain that the range of radiation doses was below cytotoxic levels, a trypan blue exclusion test was performed in 10 snails irradiated with 2.5 and 20 Gy. As the viabil-

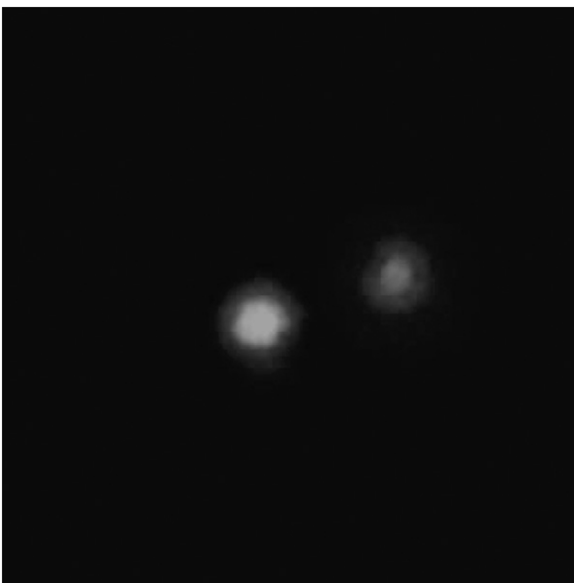

Fig. 3. Non-damaged hemocytes of *B. glabrata*.

Fig. 5. Hemocytes of *B. glabrata* showing a wide halo.

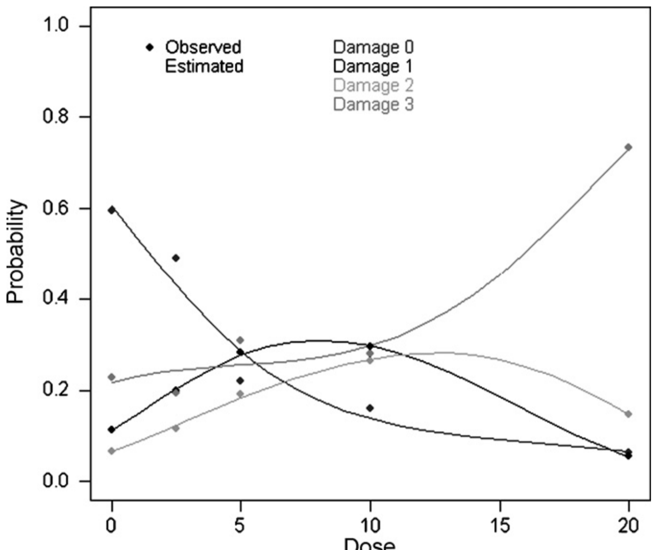

Fig. 6. Estimated population frequencies: Logistic Multiple Regression Model Damage 0, 1, 2 and 3 = classes of DNA damage.

ity of hemocytes was above 90% in both groups, we can attribute the presence of ghost cells in the comet assay to other factors than cytotoxicity.

In conclusion, our results showed that the single cell gel eletrophoresis assay in *B. glabrata* is a fast, simple and sensitive tool to detect DNA damage. Further experiments are being performed to adjust test parameters as the unwinding time and conditions of eletrophoresis in order to improve the performance and increase the sensitivity of the assay and data on additional reference mutagens will further validate this system to monitor environmental mutagens.

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