Mutation Research 700 (2010) 32–38

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Genotoxicity assessment in the amphipod *Gammarus fossarum* by use of the alkaline Comet assay

Emilie Lacaze[a,b], Olivier Geffard[b], Sylvie Bony[a], Alain Devaux[a,*]

[a] Université de Lyon, ENTPE-INRA, Laboratoire des Sciences de l'Environnement, rue Maurice Audin, Vaulx en Velin, F-69518, France
[b] Cemagref, MALY, Laboratoire d'écotoxicologie, 3 bis quai Chauveau, 69336 Lyon, Cedex 9, France

ARTICLE INFO

Article history:
Received 15 December 2009
Received in revised form 30 March 2010
Accepted 19 April 2010
Available online 6 May 2010

Keywords:
Comet assay
Genotoxicity
Freshwater invertebrate
Germ cell
Haemocyte

ABSTRACT

Many xenobiotics and newly developed substances released in the aquatic environment have been found genotoxic for living organisms. There is interest in developing biomarkers of genotoxicity in different phyla and the need to increase our understanding of the impact of genotoxic insult on invertebrates, particularly on crustaceans. Freshwater invertebrates and particularly amphipods are highly relevant species ecologically. However, genotoxic responses of such species are rarely studied, whereas understanding these responses is becoming an urgent concern.

The aim of this study was to develop and optimize the Comet assay in the freshwater invertebrate *Gammarus fossarum* by use of different cell-types: haemocytes, oocytes and spermatozoa. In a first step, the Comet assay was performed on these three cell types after exposure to the model genotoxicant methyl methanesulfonate (MMS) *in vitro* and *in vivo*. Results showed a clear dose–response relationship for all tissues, a low variability and a high sensitivity of the response, demonstrating the effectiveness of the Comet assay to detect genotoxic insult in amphipods. In a second step, to explore the potential of this technique for use in ecotoxicological studies with amphipods, these organisms were exposed to five known or suspected genotoxic compounds. The results demonstrated the possibility to use the freshwater amphipod *G. fossarum* in environmental genotoxicity studies with the Comet assay.

1. Introduction

The European Water Framework Directive (WFD 2000/60/CE) specifies the need for monitoring programs in order to reach the good chemical and ecological status for all water bodies by 2015. To improve or maintain a stable health condition of aquatic ecosystems, early and robust tools such as biomarkers have to be developed and validated in ecologically relevant species (i.e. ecological scope biomarkers).

Many xenobiotics and newly developed substances released in the environment have been found genotoxic for living organisms [1]. Genotoxic agents may produce a cascade of adverse changes from the cellular to the individual levels [2]. A variety of methods have been developed for detecting DNA damage. Among them, the single-cell gel electrophoresis assay (also called the Comet assay) is a rapid, sensitive, relatively inexpensive method, requiring only a small number of cells and providing the opportunity to study DNA damage and repair in individual cells [3]. The Comet assay has been developed in a large range of aquatic species along a broad phylogenetic diversity, taking into account the species sensitivity towards genotoxic pressure. In the marine environment, attention has been essentially paid to invertebrates such as grass shrimp, sea urchin, sea anemone, polychaete and bivalve [4–8]. In contrast, in freshwater hydro-systems the assay has been mainly carried out in fish to assess the extent of DNA damage at polluted sites [9–11]. To our knowledge, no data concerning genotoxicity assessment are presently available for freshwater amphipods such as gammarids, even though they occupy key ecological niches and have been widely used as sentinel species in ecotoxicological studies [12–14]. In this study, *Gammarus fossarum* was selected because this species is currently used in ecotoxicological tests, widespread and abundant in Europe, and known to be sensitive to a wide range of stresses [15]. Moreover, this species constitutes an important food resource for macroinvertebrates, fish and amphibian species [16]. It also plays a major role in the leaf litter breakdown process and, consequently, in the entire food web [17].

In the majority of genotoxicity studies that use the Comet assay on aquatic species the assay is performed on fluent cells such as haemocytes or erythrocytes, rarely on spermatozoa and never on oocytes. Haemocytes are closely exposed to environmental agents through their role in the transport of toxicants and in var-

* Corresponding author. Tel.: +33 04 72 04 71 78; fax: +33 04 72 04 77 43.
E-mail addresses: emilie.lacaze@cemagref.fr (E. Lacaze), olivier.geffard@cemagref.fr (O. Geffard), sylvie.bony@entpe.fr (S. Bony), alain.devaux@entpe.fr (A. Devaux).

1383-5718/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2010.04.025

ious defence mechanisms. Spermatozoa and oocytes perform the unique and critical biological function to propagate the DNA used for the development of the next generation. While genotoxicity in somatic cells can lead to disorders at the individual level, genotoxic effects in germ cells can be passed on to future generations.

Thus, the present work deals with the development and validation of a genotoxicity biomarker by use of the Comet assay in three somatic and germ-cell types, respectively, haemocytes, oocytes and spermatozoa of the crustacean *G. fossarum* exposed to environmental contaminants. As it is not known to what extent the Comet assay can detect DNA damage in amphipod haemocytes, oocytes and spermatozoa, a positive control was required. For this purpose, the model genotoxicant methyl methanesulfonate (MMS) was selected, as recommended in the evaluation of new assays [18]. To test the feasibility and the sensitivity of the Comet assay on the three cell-types selected, *in vitro* MMS exposure was conducted. *In vivo* MMS exposure was then explored in order to take into account the overall metabolism of the organism in the various cell-type responses to the genotoxic insult. Finally, in order to assess the potential of this genotoxicity biomarker for application in ecotoxicological studies, gammarids were exposed to five industrial or agricultural chemicals, known or presumed to be genotoxic for aquatic species: the polycyclic aromatic hydrocarbon benzo[a]pyrene (BaP), the heavy metals cadmium and chromium, the pesticide paraquat and the pesticide metabolite aminomethyl phosphonic acid (AMPA, a breakdown product of glyphosate). BaP, forming bulky adducts and cadmium chloride, an inorganic carcinogen, are recommended as positive control chemicals in the report of the 2008 ECVAM workshop [18]. Potassium dichromate is considered as a reference mutagenic compound [19]. Paraquat has been one of the most widely used herbicides in the world for many decades. Although its use has been recently banned in the European Union, it still represents a known hazard to human and environment health. Glyphosate is also largely used in modern agriculture and raises some concerns regarding possible health and environmental hazards. AMPA, its main breakdown product, has a greater environmental persistence than glyphosate and poses a risk for aquatic pollution [20].

2. Materials and methods

2.1. Chemicals

LIVE/DEAD® BacLight™ Bacterial Viability Kit was purchased from Invitrogen Life Technologies. Phosphate-buffered saline (PBS), normal and low melting-point agarose, ethidium bromide, methyl methanesulfonate (MMS) [CAS number 66-27-3], BaP [CAS number 50-32-8], cadmium chloride [CAS number 10108-64-2], $K_2Cr_2O_7$ [CAS No. 7778-50-9], paraquat [CAS number 4685-14-7] and AMPA, Triton X-100, dimethyl sulfoxide (DMSO), and all other reagents of analytical grade were supplied by Sigma–Aldrich Chemicals (France).

2.2. Collection and maintenance of animals

Amphipods were collected in an upstream part of the Bourbre River (Isère, France), known to have a very low level of pollution. Sexually mature *G. fossarum* were collected with a hand-held net (2–2.5 mm) during March and April 2008 and quickly brought to the laboratory where they were transferred to 30-L aquarium tanks. Organisms were kept for 21 days at $12 \pm 0.5\,^{\circ}C$, with a 16/8 h light/dark cycle, continuously supplied with aerated uncontaminated groundwater (conductivity 600 μS/cm) before being used for experiments. They were fed *ad libitum* on alder leaves (*Alnus glutinosa*). These rearing conditions were applied during all further experiments. Organisms in precopula and with an average body length of 9 ± 1 mm were selected for the experiments in order to choose mature and same age-ranked organisms.

2.3. Cell isolation and cell-viability measurements

Samples of haemolymph fluid were collected with hand-made glass microcapillary tubes, as described by Felten et al. [21]. The cuticle was punctured between the cephalon and the first pereiomere, allowing to sample a haemolymph drop by capillarity. The haemolymph sample was then placed in a 200-μL Eppendorf tube containing 20 μL of chilled PBS. For each replicate (three replicates per condition) haemolymph collected from four gammarids were pooled in order to obtain a few thousand cells per tube for analysis in the Comet assay.

In order to collect mature spermatozoa and oocytes, only gammarids in precopula were chosen. Organisms were dissected under stereomicroscope magnification and tissues of interest were collected in 20 μL of chilled PBS following the procedure as described below. The cephalon was cut with microscissors and the four caecums were removed. Then, dorsal and ventral cuticles were excised. The male gonads including the seminal vesicle were gently removed with fine forceps. For genotoxicity measurement, the seminal vesicle from one male, which contains around 6000 mature spermatozoa (unpublished data from a previous study of *G. fossarum* spermatogenesis) was mixed with 20 μL chilled PBS with a micropipette. Three replicates corresponding to three males were processed. Mature females have two batches of up to 20 oocytes at the last stage of vitellogenesis (before the oocytes are deposited in the marsupium). These two batches of oocytes were collected at once in 20 μL of PBS. A pool of four females was required to collect enough mature oocytes per replicate. Three replicates were done.

The viability of the haemocytes and oocytes was assessed by the Trypan-blue exclusion method. The LIVE/DEAD® BacLight™ Bacterial Viability Kit was used to assess the viability of the spermatozoa. The test uses two nucleic acid stains, SYTO® 9 and propidium iodide, which differ in their ability to penetrate healthy cells. Contrary to SYTO® 9, propidium iodide penetrates only cells with damaged membranes. One hundred cells were examined by fluorescence microscopy. The ability of the test to distinguish between dead and viable gammarid cells was first validated by exposing the cells to ethanol at concentrations ranging from 3% to 30% in PBS. A significant dose–response relationship was established ($p < 0.001$) (data not shown).

2.4. Exposure conditions

2.4.1. In vitro exposure to MMS

Methyl methanesulfonate (MMS) was chosen as a direct-acting genotoxicant, in order to eliminate any confounding factors associated with route of uptake or biotransformation. Three replicates were performed for each cell type and each condition. For each replicate, 10 μL of the different cell-type suspensions previously prepared (corresponding to a pool of 4 organisms for haemocytes and oocytes, and to one organism for spermatozoa) were gently mixed with 10 μL of MMS solution at concentration of 1, 2, 10 and 20 mmol/L or 10 μL of PBS for the control. Stock solutions of MMS were prepared in PBS. The cells, kept on ice and in the dark, were thus exposed for 1 h to MMS at concentrations ranging from 0.5 to 10 mmol/L. The control cells were maintained under the same condition. Genotoxicity was further assessed in the different cell types by use of the alkaline Comet assay.

2.4.2. In vivo exposure to MMS

Adult *G. fossarum* specimens in precopula were exposed for 5 days to MMS (0, 4, 20 and 100 μmol/L) to study the dose–response relationship in the three cell types studied. MMS stock solutions were prepared in PBS at concentrations ranging from 1 to 100 mmol/L. Exposure media were obtained by adding 500 μL of the appropriate MMS stock solution to 500 mL of uncontaminated natural water. Three replicates of 14 gammarids were placed in glass flasks containing 500 mL of each test solution and media were renewed daily. At the end of the exposure period, living organisms were sacrificed and the three cell types were immediately collected for assessment of DNA damage.

2.4.3. In vivo exposure to environmental contaminants

Adult male *G. fossarum* specimens were first exposed to a concentration range of cadmium chloride, potassium dichromate, paraquat, AMPA and BaP for 5 days to determine the highest non-lethal concentration to be used for genotoxicity measurements. Stock solutions were prepared in distilled water except for BaP, which was prepared in dimethyl sulfoxide (DMSO). Test solutions were obtained by adding 500 μL of stock solution to 500 mL of uncontaminated natural water. Three groups of 10 mature male gammarids were placed in flasks containing 500 mL of each test solution. Glass flasks were used for BaP, AMPA and paraquat exposure, whereas polypropylene flasks were used for cadmium chloride and potassium dichromate exposure in order to limit adsorption of pollutants on flask surfaces. Two control conditions (each performed in triplicate) were tested: one in 500 mL uncontaminated natural water and another one obtained by adding 500 μL of DMSO to 500 mL of uncontaminated natural water. Media were renewed daily. At the end of the exposure period and for each compound, living organisms exposed to the highest non-lethal concentration in the concentration range of pollutants were sacrificed in order to assess cytotoxicity prior to evaluation of genotoxicity by the Comet assay.

2.5. Comet-assay procedure

The alkaline version of this technique, introduced by Singh et al. in 1988 and recommended by international expert groups for genotoxicity testing, allows evaluation of DNA damage including single- and double-strand breaks, DNA cross-links, alkali-labile sites and incomplete repair sites [22].

Only cell suspensions with viability >90% were used, unless otherwise stated.

Frosted microscope slides were first covered with melted normal agarose in PBS (0.8%) and dried overnight at $20\,^{\circ}C$. After collection of the cells, 20 μL of 1%