Mutation Research 743 (2012) 1–9

Contents lists available at SciVerse ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

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

DNA damage in fish (*Anguilla anguilla*) exposed to a glyphosate-based herbicide – Elucidation of organ-specificity and the role of oxidative stress

S. Guilherme[a,*], I. Gaivão[b], M.A. Santos[a], M. Pacheco[a]

[a] Department of Biology and CESAM, University of Aveiro, 3810-193 Aveiro, Portugal
[b] CECAV and Department of Genetics and Biotechnology, Trás-os-Montes and Alto Douro University, Vila Real, Portugal

ARTICLE INFO

Article history:
Received 9 February 2011
Received in revised form
14 September 2011
Accepted 20 October 2011
Available online 14 January 2012

Keywords:
Glyphosate
Genotoxicity
Oxidative stress
Fish

ABSTRACT

Organophosphate herbicides are among the most dangerous agrochemicals for the aquatic environment. In this context, Roundup®, a glyphosate-based herbicide, has been widely detected in natural water bodies, representing a potential threat to non-target organisms, namely fish. Thus, the main goal of the present study was to evaluate the genotoxic potential of Roundup® in the teleost fish *Anguilla anguilla*, addressing the possible causative involvement of oxidative stress. Fish were exposed to environmentally realistic concentrations of this herbicide (58 and 116 $\mu g\,L^{-1}$) during one or three days. The standard procedure of the comet assay was applied to gill and liver cells in order to determine organ-specific genetic damage. Since liver is a central organ in xenobiotic metabolism, nucleoids of hepatic cells were also incubated with a lesion-specific repair enzyme (formamidopyrimidine DNA glycosylase – FPG), in order to recognise oxidised purines. Antioxidants were determined in both organs as indicators of pro-oxidant state. In general, both organs displayed an increase in DNA damage for the two Roundup® concentrations and exposure times, although liver showed to be less susceptible to the lower concentration. The enzyme-modified comet assay showed the occurrence of FPG-sensitive sites in liver only after a 3-day exposure to the higher Roundup® concentration. The antioxidant defences were in general unresponsive, despite a single increment of catalase activity in gills (116 $\mu g\,L^{-1}$, 3-day) and a decrease of superoxide dismutase activity in liver (58 $\mu g\,L^{-1}$, 3-day). Overall, the mechanisms involved in Roundup®-induced DNA strand-breaks showed to be similar in both organs. Nevertheless, it was demonstrated that the type of DNA damage varies with the concentration and exposure duration. Hence, after 1-day exposure, an increase on pro-oxidant state is not a necessary condition for the induction of DNA-damaging effects of Roundup®. By increasing the duration of exposure to three days, ROS-dependent processes gained preponderance as a mechanism of DNA-damage induction in the higher concentration.

1. Introduction

The increasing use of pesticides in contemporary agriculture is considered a major problem worldwide. Although the application of these agrochemicals is concentrated in terrestrial areas, they can reach the aquatic environment by drift, runoff, drainage and leaching [1], raising a number of environmental concerns especially in systems of shallow waters. Among pesticides, organophosphates constitute the predominant class [2]. In this context, the use of Roundup®, a glyphosate-based non-selective herbicide, has increased mainly due to the cultivation of genetically modified crops [3]. As a consequence of the extensive use of this commercial formulation, glyphosate has been widely detected in water bodies [4–7], increasing significantly the risks to non-target organisms, namely fish [8].

Although some studies have considered glyphosate to be only slightly toxic for aquatic animals [9,10] and with low potential to bio-accumulate [10], glyphosate-based formulations are generally more toxic than pure glyphosate [11,12], mainly due to the interference of surfactants [13]. Despite the existence of many studies concerning the deleterious effects of Roundup® on fish, only a few addressed its genotoxic potential. The available data showed genotoxicity of Roundup® to fish, expressed as cytogenetic and DNA-damaging effects [8,14,15]. Nevertheless, the concentrations tested in these studies were excessively high compared with the levels detected in natural water bodies. In addition, the mechanisms behind genetic damage and organ-specificities remain almost unexplored. Only recently, the association of Roundup® genotoxicity with oxidative stress was investigated for the first time in fish, following short-term exposure to environmentally realistic concentrations [16].

Elevated levels of reactive oxygen species (ROS) and/or depressed antioxidant defences may result in DNA oxidation and increased steady-state levels of unrepaired DNA, which is a

* Corresponding author.
E-mail address: sofia.g.guilherme@ua.pt (S. Guilherme).

1383-5718/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2011.10.017

well-known process underlying genotoxicity, in particular in the context of environmental genotoxicants [17,18]. Since organophosphate pesticides are known as inducers of oxidative stress [19], the hypothesis that DNA damage induced by Roundup® may also have an oxidative cause should be considered. This association has already been demonstrated in humans for organophosphate pesticides [20]. In relation to fish, the only available study demonstrated that DNA and chromosomal damage induced by Roundup® in blood cells was not paralleled by an increased pro-oxidant state, as evaluated by antioxidant responses [16]. This study also recommended the assessment of oxidation of DNA bases (for instance, by applying the comet assay with an extra digestion step of the nucleoids, with enzymes that specifically recognise oxidised bases) as a more straightforward strategy to obtain the required mechanistic knowledge.

Genotoxic studies in fish are frequently performed in erythrocytes, due to the ease of sampling and their adaptability to the most common methodologies [21,22]. However, according to Sharma et al. [23], other cell types should be used for monitoring genotoxic effects, thereby exploiting tissue-specific responses and acquiring a better perspective about the overall condition of the organisms. When waterborne contamination is considered, gills are the first target organ due to the large surface area in direct and continuous contact with the external medium, and its involvement in uptake [24,25]. Additionally, the liver is also of great interest for health assessment of individual fish in view of its multi-functionality and its primary role in the metabolism of xenobiotics, which is essential for activation and inactivation/detoxification of contaminants absorbed via different routes [26]. Moreover, exposure of fish to Roundup® induced histological injuries in both gills and liver [27], despite the fact that antioxidant alterations were only demonstrated in liver [28,29].

Considering that genotoxicity stands for a strongly adverse impact of chemicals on wild organisms and in view of the knowledge gaps previously recognised, the main goal of the present study was to evaluate the genotoxic potential of Roundup® to gill and liver cells of fish (*Anguilla anguilla*), following short-term exposure to environmentally realistic concentrations (58 and 116 $\mu g\,L^{-1}$), addressing the possible causative involvement of oxidative stress. The standard procedure of the comet assay was applied to gill and liver cells in order to reflect organ-specific genetic damage. Additionally, and considering the peculiarities of liver in fish physiology, the comet assay with an extra step where nucleoids are incubated with a DNA lesion-specific repair enzyme (formamidopyrimidine DNA glycosylase – FPG) was applied to hepatic cells in order to specifically target oxidised DNA bases. Superoxide dismutase (SOD), catalase (CAT), glutathione-*S*-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR) activities, as well as total glutathione (GSHt) content, were determined in both organs as indicators of pro-oxidant state.

2. Material and methods

2.1. Chemicals

A commercial formulation of glyphosate (Roundup® Ultra, distributed by Bayer CropScience, Portugal), containing isopropylammonium salt of glyphosate at 485 $g\,L^{-1}$ as the active ingredient (equivalent to 360 $g\,L^{-1}$ or 30.8% of glyphosate) and polyethoxylene amine (16%) as surfactant, was used. Formamidopyrimidine DNA glycosylase was purchased from Andrew Collins, University of Oslo, Norway. All the other chemicals required to perform the comet assay and to quantify antioxidants were obtained from Sigma–Aldrich Chemical Company (Spain).

2.2. Test animals and experimental design

European eel (*A. anguilla* L.) specimens with an average length of 25 ± 3 cm and weight of 32 ± 5 g (yellow eel stage) were captured from an unpolluted area of the Aveiro Lagoon – Murtosa, Portugal. Eels were acclimated to laboratory for 12 days and kept in 80-L aquaria under a natural photoperiod, in aerated, filtered, de-chlorinated and recirculating tap water, with the following physico-chemical conditions: salinity 0, temperature $20 \pm 1\,^{\circ}C$, pH 7.3 ± 0.2, ammonia <0.1 $mg\,L^{-1}$, nitrite 0.06 ± 0.03 $mg\,L^{-1}$, nitrate 25 ± 6.0 $mg\,L^{-1}$, dissolved oxygen 8.1 ± 0.5 $mg\,L^{-1}$. During this period, fish were fed every other day with fish roe.

The experiment was carried out in 20-L aquaria, in a static mode. Physical-chemical characteristics of the water during the experiment were daily monitored and fell in the intervals described above for the acclimation period. Fish were not fed one day before the experiment was started, or during the experimental period. Thirty-six eels were divided over six aquaria (six fish per dose per duration group; $n = 6$) and exposed to 58 $\mu g\,L^{-1}$ (two aquaria) and 116 $\mu g\,L^{-1}$ (two aquaria) of Roundup®, equivalent to 18 and 36 $\mu g\,L^{-1}$ of glyphosate, respectively. Another two aquaria were kept with clean water as negative control groups. For each pesticide concentration, 1- and 3-day exposures were tested, corresponding to the two different aquaria mentioned above. No mortality was observed during the whole experiment. After each exposure period, fish were sacrificed by cervical transection and bled. Liver and gills were collected and washed in ice-cold phosphate-buffered saline (PBS). A tissue portion of each organ was immediately processed for the comet assay and the remaining tissue was stored in micro-tubes, frozen in liquid nitrogen and kept at $-80\,^{\circ}C$ until further procedures for analysis of antioxidants.

2.3. Evaluation of genetic damage

2.3.1. Comet assay

Liver and gill cell suspensions were obtained by mincing briefly a part of the tissue with a pair of fine scissors in 1 mL of PBS and by pipetting up-and-down the finely minced tissue pieces [30]. The conventional alkaline version of the comet assay was performed according to the method of Collins [18] with slight modifications. Two gel replicates, containing each approximately 2×10^4 cells (cell suspension in PBS) in 70 μL of 1% low melting-point agarose in PBS, were placed on a glass microscope slide, pre-coated with 1% normal melting-point agarose. The gels were covered with glass coverslips and left for ±5 min at 4 °C to let the agarose solidify, and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) at 4 °C, for one hour. Then, slides were gently placed in the electrophoresis tank, immersed in electrophoresis solution (±20 min, 0.3 M NaOH, 1 mM EDTA, pH > 13) for alkaline treatment. Electrophoresis was performed at a fixed voltage of 25 V and a current of 300 mA, which results in 0.7 $V\,cm^{-1}$ (achieved by adjusting the buffer volume in the electrophoresis tank). The slides were stained with ethidium bromide (20 $\mu g\,L^{-1}$).

For the liver, an additional set of slides was prepared to apply the comet assay with an extra step of digesting the nucleoids with FPG. This lesion-specific endonuclease converts oxidised purines, including the major purine oxidation product 8-oxoguanine and other altered purines (ring-opened purines or formamido-pyrimidines) into DNA single-strand breaks [17]. Thus, after lysis of agarose-embedded cells, slides were washed three times with enzyme buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, 0.2 $mg\,mL^{-1}$ bovine serum albumin, pH 8) at 4 °C. Then, 50 μL of FPG in buffer was applied in the centre of each gel, along with a coverslip, prior to incubation at 37 °C for 45 min in a humidified atmosphere. Another set of slides was submitted to the same treatment, although incubated only with buffer. Subsequent steps – alkaline treatment, electrophoresis and staining – were as described above.

One slide with two gels each, and 100 nucleoids per gel, were observed for each fish and organ, with a Leica DMLS fluorescence microscope (400× magnification). The DNA damage was quantified by visual classification of nucleoids into five comet classes, according to the tail intensity and length, from 0 (no tail) to 4 (almost all DNA in tail). The total score expressed as a genetic damage indicator (GDI) was calculated multiplying the mean percentage of nucleoids in each class by the corresponding factor, according to the formula:

$$\begin{aligned}\text{GDI} = {} & [(\%\,\text{nucleoids class 0}) \times 0] + [(\%\,\text{nucleoids class 1}) \times 1] \\ & + [(\%\,\text{nucleoids class 2}) \times 2] + [(\%\,\text{nucleoids class 3}) \times 3] \\ & + [(\%\,\text{nucleoids class 4}) \times 4]\end{aligned}$$

GDI results were expressed as arbitrary units on a scale of 0–400 per 100 scored nucleoids (as average value for the two gels observed per fish). When the comet assay was performed with the additional FPG step (for liver), GDI values were calculated in the same way but the parameter designated GDI_{FPG}. Besides the GDI, the frequency of nucleoids observed in each comet class was also expressed, as recommended by Azqueta et al. [17]. In order to improve the expression of the extent of DNA damage, the sub-total frequency of nucleoids with medium (class 2), high (class 3) and complete (class 4) damaged DNA was also calculated [8,31].

As positive controls, both gill and liver cells were treated with 50 μM hydrogen peroxide (Sigma–Aldrich, Spain) for 5 min, according to Collins et al. [32], and the respective GDI values were scored.

2.4. Antioxidant system analyses

2.4.1. Tissue preparation and fractionation

Both organs (gills and liver) were homogenized in a 1:10 ratio (tissue volume: buffer volume) with a Potter-Elvehjem homogenizer, in chilled phosphate buffer