Mutagenesis vol. 22 no. 4 pp. 263–268, 2007
Advance Access Publication 10 April 2007
doi:10.1093/mutage/gem012

Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay

Tolga Çavaş* and Serpil Könen

Department of Biology, Faculty of Sciences and Letters, Mersin University, Mersin 33342, Turkey

Glyphosate is a widely used broad-spectrum weed control agent. In the present study, an *in vivo* study on the genotoxic effects of a technical herbicide (Roundup®) containing isopropylamine salt of glyphosate was carried out on freshwater goldfish *Carassius auratus*. The fish were exposed to three doses of glyphosate formulation (5, 10 and 15 ppm). Cyclophosphamide at a single dose of 5 mg/l was used as positive control. Analysis of micronuclei, nuclear abnormalities and DNA damage were performed on peripheral erythrocytes sampled at intervals of 48, 96 and 144 h posttreatment. Our results revealed significant dose-dependent increases in the frequencies of micronuclei, nuclear abnormalities as well as DNA strand breaks. Our findings also confirmed that the alkaline comet assay and nuclear deformations in addition to micronucleus test on fish erythrocytes in vivo are useful tools in determining the potential genotoxicity of commercial herbicides.

Introduction

To increase the production in agriculture, large amounts of chemicals are daily released into the environment, mainly on croplands and pastures. Today, it is well known that pesticides not only affect target organisms but also have some side effects on nontarget organisms (1). In spite of these inconveniences, it is not possible for many countries to reduce the use of pesticides without reducing crop yields (2). Herbicides used to control weeds accounted for the largest portion of total use, followed by pesticides, insecticides and fungicides.

Many scientific experiments have examined the use of herbicides in terrestrial ecosystems and the toxicity of herbicides to animals (3,4). However, less is known about their toxicity to aquatic ecosystems, including fish (5). The presence of herbicides in water is a consequence of weed control in terrestrial ecosystems and water reservoirs. Since there is growing concern over the presence of genotoxins in the aquatic environment, it is important to develop methods for detection of genotoxic effects in aquatic organisms (6). The genotoxic effects of environmental pollutants can be monitored using a broad range of both *in vitro* and *in vivo* biomarker assays but the micronucleus test and the comet assay are gaining popularity over other assays due to their sensitivity for detecting cytogenetic and DNA damage and the short time needed to complete a study (7–13). The formation of morphological nuclear abnormalities (NAs) was first described in fish erythrocytes by Carrrasco *et al.* (14). NAs, including blebbed nuclei (BL), lobed nuclei (LB) and notched nuclei (NT) and binucleated cells (BN), have been used by several investigators as possible indicators of genotoxicity (10,12,15,16). Although the mechanisms responsible for NAs have not been fully explained, these abnormalities are considered to be indicators of genotoxic damage and, therefore, they may complement the scoring of micronuclei in routine genotoxicity surveys.

Glyphosate is a broad-spectrum, nonselective herbicide used for inhibition of unwanted weeds and grasses in agricultural, industrial, urban, forestry and aquatic landscapes. The major formulation is Roundup® in which glyphosate is formulated as isopropylamine (IPA) salt and a surfactant, polyoxyethylene amine (POEA), is added to enhance the efficacy of the herbicide (17). Owing to its high water solubility and extensive usage in the environment, the exposure of nontarget aquatic organisms to this herbicide is a concern of ecotoxicologists (18). Results of studies dealing with the cytogenetic and DNA-damaging effects of glyphosate yielded controversial results in relation to the tested formulation, application dose, used test methodology and organism (19–27).

It has been noted that the relatively small toxicity of glyphosate contributes little to the total toxicity of the formulated product. Consequently, in some cases, the formulated product is found to be more toxic than the active ingredient, particularly to aquatic organisms that are more sensitive to surface-active substances (18,28). Studies on the genotoxicity evaluation of glyphosate formulations in fish are extremely scarce (29). Therefore, attempts have been made in the present study to determine the genotoxic effects of Roundup®, a glyphosate-based herbicide, on the goldfish *Carassius auratus* using the micronucleus test and the comet assay.

Materials and methods

Fish and chemicals

Goldfish, *C. auratus* (Linnaeus, 1758) belonging to the family *Cyprinidae* was chosen for this study because of its common availability in most fish markets in Turkey and also due to proven sensitivity to genotoxic chemicals (30,31,32). Specimens of juvenile goldfish with average weight and length of 5 ± 1 g and 6 ± 1 cm, respectively, were purchased from a local market. Before the experiments, they were acclimated under laboratory conditions for 3 weeks at a population density of 15 specimens in 20 l aquaria, and 25°C 12:12 h dark–light modes fish were fed once a day with commercial fish pellets. Feces and pellet residues were removed daily by suction.

A commercial formulation of glyphosate (Roundup©) containing isopropylammonium salt of glyphosate at 480 g/l as the active ingredient (equivalent to 360 g glyphosate per liter) and inert components, such as POEA surfactant and water, was used. All the chemicals needed to perform the micronucleus test and the comet assays were obtained from the Sigma–Aldrich Chemical Company.

Experimental design

Goldfish were placed in four different aquaria containing dechlorinated tap water (negative control) and three different concentrations of Roundup®

*To whom correspondence should be addressed. Tel: +90 324 3610001; Fax: +90 324 3610047; Email: tcavas@mersin.edu.tr

corresponding to 5, 10 and 15 ppm glyphosate obtained by serial dilutions of Roundup®. Cyclophosphamide (CP) was used as positive control at a concentration of 5 mg/l in another aquarium. For the micronucleus test, nuclear abnormality test and the comet assay, blood samples were obtained at the end of second, fourth and sixth days from the caudal vein of specimens following the exposure to Roundup®. The test water was renewed once at 2 days. Fish also were fed once per 2 days. Animals were killed by cervical dislocation. A total of 75 goldfish (five fish for per dose per duration group) were used for the experiments.

Analysis of micronuclei and other nuclear abnormalities

Peripheral blood samples were obtained from the caudal vein of the specimens and smeared onto precleaned slides. After fixation in pure ethanol for 20 min, the slides were allowed to air-dry and then the smears were stained with 10% Giemsa solution for 25 min. All slides were coded and scored blind. Five slides were prepared for each fish, and 1500 cells were scored from each slide under ×100 magnification. Nonrefractive, circular or ovoid chromatin bodies, smaller than the one-third of the main nucleus and displaying the same staining and focusing pattern as the main nucleus, were scored as micronuclei (9).

Nuclear abnormalities (NAs) other than micronuclei in erythrocytes were classified into five groups. Briefly, cells with two nuclei were considered as binucleated. BL had a relatively small evagination of the nuclear membrane and contained chromatin. Nuclei with evaginations larger than those in the BL, including those with several lobes, were classified as LB. Nuclei with vacuoles or voids with appreciable depth into the nucleus were recorded as NT (14,33).

The comet assay

The alkaline comet assay was performed according to the method of Tice *et al.* (34) with some modification. About 0.5 ml of blood sample collected from caudal veins of goldfish was diluted with 1 ml of phosphate-buffered saline. Sixty microliters of the diluted sample were mixed with 200 µl of 0.65% low melting point (LMP) agarose. Seventy-five microliters of the mixture were than layered on the slides precoated with a 0.5% normal melting point agarose and immediately covered with a coverslip and then kept for 10 min in a refrigerator to solidify. After gently removing the coverslips, the slides were covered with a third layer of 90 µl LMP agarose and covered with coverslips again. After solidification of the gel, coverslips were removed and the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, pH 10, with 10% dimethyl sulfoxide and 1% Triton X-100 added fresh) and refrigerated at 4°C for 2 h. After lysis, the slides were placed on a horizontal electrophoresis box side by side. The tank was filled with fresh electrophoresis solution (1 mM Na EDTA, 300 mM NaOH and pH 13.5) to a level ~0.25 cm above the slides. The slides were left in the solution for 20 min to allow the unwinding. Electrophoresis was performed using the same solution at 0.66 V/cm and 300 mA for 25 min. The slides were than neutralized gently with 0.4 M Tris buffer at pH 7.5 and stained with 75 µl ethidium bromide (20 µg/ml).

Slides were examined using an Olympus BX40 fluorescence microscope equipped with a wide band excitation filter of 330–385 nm and a barrier filter of 420 nm. From each fish, five slides were prepared and from each slide 200 cells were scored at ×400 magnification. The DNA damage was quantified by visual classification of cells into five categories 'comets' corresponding to the tail length: undamaged (35), Type 0; low-level damage, Type 1; medium-level damage, Type 2; high-level damage, Type 3 and complete damage, Type 4, as demonstrated in Figure 1. The extent of DNA damage was expressed as the mean percentage of cells with medium, high and complete damaged DNA, which was calculated as the sum of cells with damage Types II, III and IV (36). From the arbitrary values assigned to the different categories (from Type 0 = 0 to Type IV = 4), a genetic damage index (GDI) was calculated for each subject (37).

Statistical analysis

After assessing the normality of distribution of the data, both parametric and nonparametric tests were used in order to detect differences at the 0.05 level of significance. Differences between mean values were compared using the Student's *t*-test and least significant difference test for the micronuclei data and the Mann–Whitney *U*-test for the comet assay data.

Results

Micronucleus frequencies in erythrocytes of *C. auratus* exposed to glyphosate as well as parallel negative and positive controls are summarized in Table I. As can be seen in the table, MN frequencies in erythrocytes significantly increased following treatment ($P > 0.001$) with the exceptions of the two lowest doses of glyphosate (5 ppm and 10 ppm) at the second day ($P > 0.05$). Examples of micronucleated erythrocytes are presented in Figure 2.

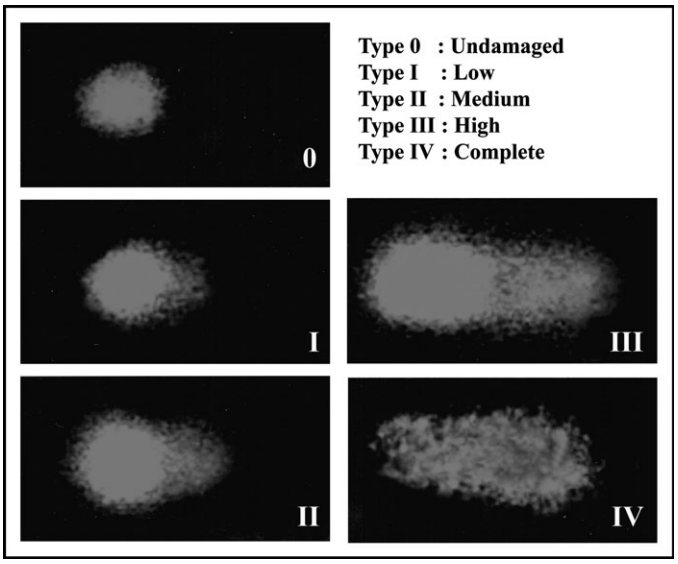

Fig. 1. Classification of the comet formations in fish erythrocytes. 0, Class 0 (undamaged); I, Class 1 (low damage); II, Class 2 (medium damage); III, Class 3 (large damage) and IV, Class 4 (complete damage).

In general, frequencies of NAs were found in the following order: BN = NT > LB = BL. When NAs were analyzed separately, the frequency of each NA did not showed significant differences ($P > 0.05$) with the exception of positive control CP ($P < 0.01$). On the other hand, analysis of total nuclear abnormalities showed significant dose- and duration-dependent increases following treatment with glyphosate ($P > 0.01$). Results of total NA analyses are given in the Figure 3.

Table II shows the proportion of damaged nuclei and the GDI as measured in the comet assay. A clear and statistically significant increase in DNA migration was found in the glyphosate exposed group compared to the control fish with the only exception of 5 ppm—second day group ($P > 0.05$). The differences in GDI between exposed and control groups reached statistical significance, mainly by the increased percentage of type B, C and D cells in the exposed group, compared with the control group. These increases were most evident at the two highest concentrations (10 and 15 ppm) of glyphosate ($P < 0.001$).

Discussion

Glyphosate is the most widely used herbicide in the world, being routinely applied to control weeds in both agricultural and urban settings (38). The most widely used formulation of glyphosate is Roundup and related products. Roundup is a combination of an active ingredient the IPA salt of glyphosate, and a surface-active agent POEA that enhances the spreading of droplets when they contact foliage. Glyphosate inhibits the enzyme 5-enolpyruvyl shikimate-3-P synthetase that controls aromatic amino acid synthesis in plants and is relatively nontoxic to animals (17). Although the herbicide is believed to be environmentally safe (39,40), some evidence of glyphosate's environmental toxicity has been demonstrated (41). Surfactants such as POEA and other inert compounds

Table I. MN frequencies (‰) in peripheral blood erythrocytes of *C. auratus* exposed to glyphosate (mean ± SE)

Treatment groups	Negative control	Positive control	Glyphosate		
			5 ppm	10 ppm	15 ppm
Second day	3.17 ± 0.48	19.8 ± 3.64***	4.5 ± 1.23	5.15 ± 3.01	12.2 ± 1.26***
Fourth day	2.88 ± 0.56	23.2 ± 2.89***	5.8 ± 1.45*	10.7 ± 2.29***	16.5 ± 1.18***
Sixth day	3.00 ± 0.86	26.7 ± 2.25***	8.3 ± 2.01**	13.5 ± 1.12***	18.7 ± 0.56***

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

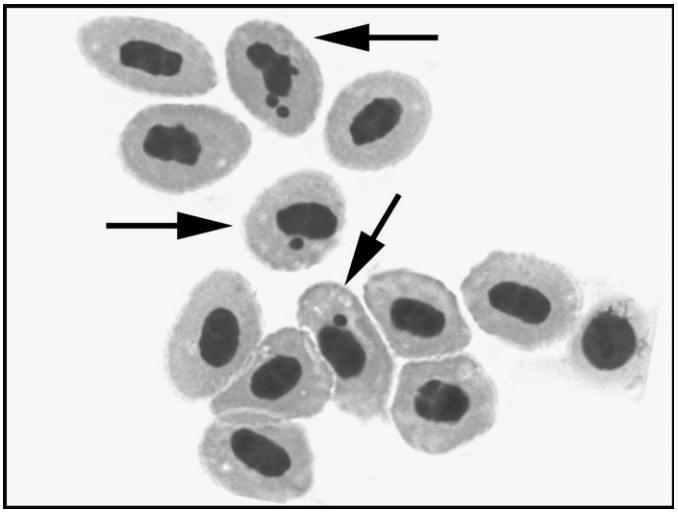

Fig. 2. Photomicrograph of the micronucleated erythrocytes in *C. auratus* exposed to glyphosate.

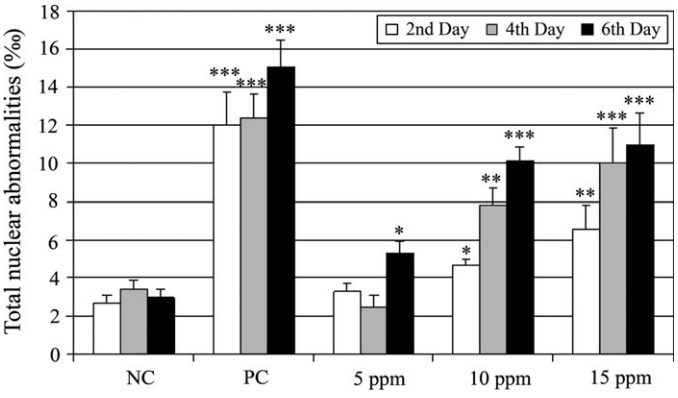

Fig. 3. Total NA frequencies in peripheral blood erythrocytes of *C. auratus* exposed to glyophosate.

were also previously suggested to increase the toxicity of this herbicide (42). As mentioned by Bolognesi *et al.* (25), the higher activity of technical formulations in inducing toxic and genotoxic damage in different experimental systems suggests a role of the surface-active agents and/or coformulants in the potentiation of the effects of the active ingredient. For example, according to Giesy *et al.* (43), the LC50 values (mg/l) for rainbow trout are between 8.2 and 27 for Roundup, between 0.65 and 7.4 for POEA and between 140 and 240 for glyphosate alone, showing that the POEA used in the glyphosate formulation is the most toxic component of the herbicide Roundup®. Because glyphosate is not applied as a pure active ingredient but in the form of technical formulations in field, it was necessary to evaluate the genotoxicity of the commercial form i.e. Roundup®.

Results of the genotoxicity studies on glyphosate products are conflicting depending on purity of the active agent, nature of inert components, type of the test method applied as well as test organisms used. For example, Li and Long (44) evaluated the genotoxicity of glyphosate (had a purity of 98%) in a set of *in vitro* and *in vivo* assays including *Salmonella thyphimurium* and *Escherichia coli* reversion assays, recombination with *Bacillus subtilis*, Chinese hamster ovary cells gene mutation assay, hepatocyte primary cultured/DNA repair assay and *in vivo* cytogenetic assay in rat bone marrow, and reported that no genotoxic activity was observed in the assays. In mammalian cells, glyphosate was also not mutagenic (45). No positive clastogenic and/or aneugenic effects were described in rat and mouse bone marrow *in vivo* both after injecting glyphosate and Roundup® (27,44).

On the other hand, roundup caused DNA damage in erythrocytes of *Rana catesbeiana* (13) and produced an increase in the percentage of aberrant cells and a weak induction of sister chromatid exchanges (SCEs) in human lymphocytes exposed *in vitro* (23). Kale *et al.* (26) reported that Roundup® treatment increased the frequency of sex-linked, recessive lethal mutations in *Drosophila*. Genotoxic effects of glyphosate were also demonstrated by wing spot test on *Drosophila* by Kaya *et al.* (22). Furthermore, Bolognesi *et al.* (25) documented DNA-damaging activity by alkaline elution technique and 8-hydroxydeoxyguanosine quantification in the mouse liver and kidney, and also a significant increase in micronucleus frequency in the mouse bone marrow *in vivo* with both substances. In mice injected with Roundup®, the frequency of DNA adducts in the liver and kidney increased at all the doses tested (24). An increase in the frequency of SCEs in human lymphocytes *in vitro* following exposure to Roundup was demonstrated by Vigfusson and Vyse (46). Lioi *et al.* (47) also reported a weak induction of SCE and a significant increase of chromosomal aberrations in cultured human lymphocytes following exposure to pure glyphosate.

Although toxicity studies of the effects of glyphosate on fish have been performed earlier (5,48,49), there was only one study that examined the cytogenetic effects of roundup and no study with the estimation of DNA damage in fish (29). In the study performed by Grisolia (29), it was reported that on the fourth day following a single intra-abdominal injection of roundup equivalent to 42, 85, 170 mg/kg doses of glyphosate significantly increased the micronuclei frequencies on erythrocytes of fish *Tilapia rendalli*. In our study, we observed that treatment with Roundup®, equivalent to 5, 10 and 15 ppm concentrations of glyphosate, induced significant increases in frequencies of micronuclei as well as DNA damage as revealed

Table II. Analysis of DNA damage as measured by comet assay in peripheral erythrocytes of *C. auratus* exposed to glyphosate

Treatment groups	Duration (days)	Proportion of damaged nuclei (%)[a]					% of damaged cells (II + III + IV)[b]	GDI[c]
		Type 0	Type I	Type II	Type III	Type IV		
Negative control	2	75.67	17.00	4.67	1.67	1.00	7.33 ± 1.20	0.35 ± 0.04
	4	69.65	25.33	4.36	0.33	0.33	5.00 ± 2.00	0.36 ± 0.06
	6	71.00	23.00	4.00	0.68	1.32	6.00 ± 1.00	0.38 ± 0.04
Positive control	2	56.33	19.67	10.37	6.63	7.00	24.00 ± 2.08	0.88 ± 0.04***
	4	50.00	21.23	12.00	8.10	8.67	28.64 ± 4.10	1.04 ± 0.09***
	6	44.67	17.33	20.00	9.33	8.67	38.00 ± 1.73	1.20 ± 0.06***
5 ppm glyphosate	2	64.33	25.00	7.00	2.67	1.00	10.67 ± 0.88	0.51 ± 0.04*
	4	62.67	26.00	6.00	3.00	2.33	11.33 ± 1.86	0.56 ± 0.07*
	6	60.67	25.67	8.00	4.00	1.67	13.67 ± 3.18	0.60 ± 0.08**
10 ppm glyphosate	2	56.67	24.33	12.67	3.67	2.67	19.00 ± 2.65	0.71 ± 0.05***
	4	45.00	24.67	18.00	8.00	4.33	30.33 ± 0.88	1.02 ± 0.03***
	6	40.00	24.67	20.00	9.33	6.00	35.33 ± 0.88	1.17 ± 0.01***
15 ppm glyphosate	2	46.33	23.33	17.67	6.00	3.33	27.00 ± 0.58	0.93 ± 0.01***
	4	44.33	19.67	21.33	10.00	4.67	36.00 ± 2.65	1.11 ± 0.07***
	6	35.67	17.00	31.00	10.33	6.00	47.33 ± 1.67	1.34 ± 0.04***

[a]0–IV indicate grades of DNA damage [after Anderson *et al.* (35)].
[b]Percentage of damaged cells = Type II + III + IV [after Palus *et al.* (36)].
[c]GDI = (Type I + 2 × Type II + 3 × Type III + 4 × Type IV)/(Type 0 + I + II + III + IV) [after Pitarque *et al.* (37)].
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

in peripheral erythrocytes of *C. auratus*. Similar doses of glyphosate were previously shown to cause toxic effects in fish. For example, treatment with 4, 6 and 8 mg/l doses of glyphosate caused toxic effects on carps as revealed by the reduction of the phagocytic activity of blood and kidney neutrophils and macrophages and histopathological effects in liver characterized with edema cells, vacuolar degeneration and focal fibrosis (50). Furthermore, Glusczak *et al.* (51) reported that treatment with Roundup® equivalent to 3, 6, 10 and 20 mg/l doses of glyphosate for 96 h significantly decreased the acetylcholinesterase activity in brain and glycogen and glucose in muscle tissues of *Leporinus obtusidens*. Studies concerning small ponds with static water showed that the rate of glyphosate dissipation/degradation ranged from 1.5 to 3.5 days, with first-order half-lives (52). Therefore, toxicity studies at relatively high glyphosate concentrations are environmentally relevant, particularly when fish are acutely exposed immediately after glyphosate application (5).

Accumulation of DNA damage may occur either through an increase in the number of DNA-damaging events or a decrease in DNA repair. Although the DNA repair mechanisms in fish are not as efficient as those in mammals (53,54), they do exist. For example, Saleha Banu *et al.* (55) reported that, in erythrocytes of *Tilapia mosambica* treated with the pesticide Monocrotophos, the maximum increase in mean comet tail length was observed at 24 h and reductions in mean comet tail length were seen at 48 and 72 h. They also observed that by 96 h, values had returned to control levels at all doses, indicating repair of the damaged DNA and/or loss of heavily damaged cells. Due to blood cell kinetics, erythrocytes are in continuous replacement. According to a few available studies, the erythrocyte's life span in fish varies among species over a range of 80–500 days (56,57) with an erythrocyte maturation period of 17–23 days. (58). It was shown that maturation of *C. auratus* erythrocytes required ~16 to 20 days (59) and the life span or erythrocytes may up to 270 days as demonstrated in *C. auratus langsdorfi* (57). In our study, treatment with Roundup© significantly increased the DNA damage following 2 days exposure and gradual increases in GDI values were noticed at the fourth and sixth days, indicating inhibition of DNA repair during the exposure period. It was also shown that Roundup 3 plus, a glyphosate-based herbicide, inhibited the synthesis of DNA occurring in the S phase of the cell cycle in sea urchin embryos (60). Considering the life span of fish erythrocytes, longer exposure periods could provide more information on the situation of DNA repair in goldfish exposed to glyphosate-based herbicides such as Roundup®.

In conclusion, our data indicate that fish are among the sensitive aquatic organisms tested for some chemicals commonly used in lawn care and that measurements of cytogenetic and DNA damage are useful as screening tools to evaluate potential effects of chemicals on nontarget aquatic organisms and clearly, more research on roundup genotoxicity in fish is needed to adequately protect this group.

References

1. Joy, V. C., Pramanik, R. and Sarkar, K. (2005) Biomonitoring insecticide pollution using non-target soil microarthropods. *J. Environ. Biol.*, **26**, 571–577.
2. Pimentel, D., Mclaughlin, L., Zepp, A. *et al.* (1991) Environmental and economic effects of reducing pesticide use. *Bioscience*, **41**, 402–409.
3. Ujhazy, E., Sadlonova, I., Dubovicky, M., Mach, M., Muckova, M. and Flaskarova, E. (2006) Teratological study of the herbicide 4-chloro-2-methylphenoxyacetic acid in rabbits. *J. Appl. Toxicol.*, **26**, 368–373.
4. D'Souza, U. J., Narayana, K., Zain, A., Raju, S., Nizam, H. M. and Noriah, O. (2006) Dermal exposure to the herbicide-paraquat results in genotoxic and cytotoxic damage to germ cells in the male rat. *Folia Morphol.*, **65**, 6–10.
5. Szarek, J., Siwicki, A., Andrzejewska, A., Terech-Majewska, E. and Banaszkiewicz, T. (2000) Effects of the herbicide Roundup™ on the ultrastructural pattern of hepatocytes in carp (*Cyprinus carpio*). *Mar. Environ. Res.*, **50**, 263–266.
6. Hayashi, M., Ueda, T., Uyeno, K. *et al.* (1998) Development of genotoxicity assay systems that use aquatic organisms. *Mutat. Res.*, **399**, 125–133.
7. Andrade, V. M., de Freitas, T. R. and da Silva, J. (2004) Comet assay using mullet (Mugil sp.) and sea catfish (Netuma sp.) erythrocytes for the detection of genotoxic pollutants in aquatic environment. *Mutat. Res.*, **560**, 57–67.
8. Ateeq, B., Abul Farah, M. and Ahmad, W. (2005) Detection of DNA damage by alkaline single cell gel electrophoresis in 2,4-dichlorophenoxy-acetic-acid- and butachlor-exposed erythrocytes of *Clarias batrachus*. *Ecotoxicol. Environ. Saf.*, **62**, 348–354.

9. Al-Sabti, K. and Metcalfe, C. D. (1995) Fish micronuclei for assessing genotoxicity in water. *Mutat. Res*., **343**, 121–135.
10. Cavas, T. and Ergene-Gozukara, S. (2005) Micronucleus test in fish cells: a bioassay for in situ monitoring of genotoxic pollution in the marine environment. *Environ. Mol. Mutagen*., **46**, 64–70.
11. Cavas, T. and Ergene-Gozukara, S. (2003) Evaluation of the genotoxic potential of lambda-cyhalothrin using nuclear and nucleolar biomarkers on fish cells. *Mutat. Res*., **534**, 93–99.
12. Cavas, T. and Ergene-Gozukara, S. (2003) Micronuclei, nuclear lesions and interphase silver stained nucleolar organizer regions (AgNORs) as cyto-genotoxicity indicators in *Oreochromis niloticus* exposed to textile mill effluent. *Mutat. Res*., **538**, 81–91.
13. Clements, C., Ralph, S. and Pertas, M. (1997) Genotoxicity of select herbicides in *Rana catesbeiana* tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. *Environ. Mol. Mutagen*., **29**, 277–288.
14. Carrasco, K., Tilbury, K. L. and Myers, M. S. (1990) Assessment of the piscine micronucleus test as an in situ biological indicator of chemical contaminant effects. *Can. J. Fish Aquat. Sci*., **47**, 2123–2136.
15. Ayllon, F. and Garcia-Vazquez, E. (2000) Induction of micronuclei and other nuclear abnormalities in European minnow *Phoxinus phoxinus* and mollie *Poecilia latipinna*: an assessment of the fish micronucleus test. *Mutat. Res*., **467**, 177–186.
16. Da Silva Souza, T. and Fontanetti, C. S. (2006) Micronucleus test and observation of nuclear alterations in erythrocytes of Nile tilapia exposed to waters affected by refinery effluent. *Mutat. Res*., **605**, 87–93.
17. Solomon, K. R. and Thompson, D. G. (2003) Ecological risk assessment for aquatic organisms from over uses of glyphosate. *J. Toxicol. Environ. Health B Crit. Rev*., **6**, 289–324.
18. Tsui, M. K. and Chu, L. M. (2003) Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. *Chemosphere*., **52**, 1189–1197.
19. Sivikova, K. and Dianovsky, J. (2006) Cytogenetic effect of technical glyphosate on cultivated bovine peripheral lymphocytes. *Int. J. Hyg. Environ. Health*., **209**, 15–20.
20. Monroy, C. M., Cortes, A. C., Sicard, D. M. and de Restrepo, H. G. (2005) Cytotoxicity and genotoxicity of human cells exposed in vitro to glyphosate. *Biomedica*, **25**, 335–345.
21. Conners, D. E. and Black, M. C. (2004) Evaluation of lethality and genotoxicity in the freshwater mussel Utterbackia imbecillis (Bivalvia: Unionidae) exposed singly and in combination to chemicals used in lawn care. *Arch. Environ. Contam. Toxicol*., **46**, 362–371.
22. Kaya, B., Creus, A., Yanikoglu, A., Cabre, O. and Marcos, R. (2000) Use of the Drosophila wing spot test in the genotoxicity testing of different herbicides. *Environ. Mol. Mutagen*., **36**, 40–46.
23. Lioi, M. B., Scarfi, M. R., Santoro, A., Barbieri, R., Zeni, O., Di Berardino, D. and Ursini, M. V. (1998) Genotoxicity and oxidative stress induced by pesticide exposure in bovine lymphocyte cultures in vitro. *Mutat. Res*., **403**, 13–20.
24. Peluso, M., Munnia, A., Bolognesi, C. and Parodi, S. (1998) 32P-postlabeling detection of DNA adducts in mice treated with the herbicide Roundup. *Environ. Mol. Mutagen*., **31**, 55–59.
25. Bolognesi, C., Bonatti, S., Degan, P., Gallerani, E., Peluso, M., Rabbonu, R., Roggieri, P. and Abbondandolo, A. (1997) Genotoxic activity of glyphosate and its technical formulation Roundup. *J. Agric. Food Chem*., **45**, 1957–1962.
26. Kale, P. G., Petty, B. T., Jr, Walker, S., Ford, J. B., Dehkordi, N., Tarasia, S., Tasie, B. O., Kale, R. and Sohni, Y. R. (1995) Mutagenicity testing of nine herbicides and pesticides currently used in agriculture. *Environ. Mol. Mutagen*., **25**, 148–153.
27. Rank, J., Jensen, A. G., Skov, B., Pedersen, L. H. and Jensen, K. (1993) Genotoxicity testing of the herbicide Roundup and its active ingredient glyphosate isopropylamine using the mouse bone marrow micronucleus test, Salmonella mutagenicity test, and Allium anaphase-telophase test. *Mutat. Res*., **300**, 29–36.
28. WHO (1994) *Environmental Health Criteria: Glyphosate*. World Health Organization, Geneva.
29. Grisolia, C. K. (2002) A comparison between mouse and fish micronucleus test using cyclophosphamide, mitomycin C and various pesticides. *Mutat. Res*., **518**, 145–150.
30. Deguchi, Y., Toyoizumi, T., Masuda, S., Yasuhara, A., Mohri, S., Yamada, M., Inoue, Y. and Kinae, N. (2007) Evaluation of mutagenic activities of leachates in landfill sites by micronucleus test and comet assay using goldfish. *Mutat. Res*., **627**, 178–185.
31. Masuda, S., Deguchi, Y., Masuda, Y., Watanabe, T., Nukaya, H., Terao, Y., Takamura, T., Wakabayashi, K. and Kinae, N. (2004) Genotoxicity of 2-[2-(acetylamino)-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-6) and 4-amino-3,3′-dichloro-5,4′-dinitro-biphenyl (ADDB) in goldfish (*Carassius auratus*) using the micronucleus test and the comet assay. *Mutat. Res*., **560**, 33–40.
32. Anitha, B., Chandra, N., Gopinath, P. M. and Durairaj, G. (2000) Genotoxicity evaluation of heat shock in gold fish (*Carassius auratus*). *Mutat. Res*., **469**, 1–8.
33. Cavas, T. and Ergene-Gozukara, S. (2005) Induction of micronuclei and nuclear abnormalities in *Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant effluents. *Aquat. Toxicol*., **74**, 264–271.
34. Tice, R. R., Agurell, E., Anderson, D. *et al*. (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ. Mol. Mutagen*., **35**, 206–221.
35. Anderson, D., Yu, T. W., Phillips, B. J. and Schezer, P. (1994) The effect of various antioxidants and other modifying agents on oxygen-radical generated DNA damage in human lymphocytes in the comet assay. *Mutat. Res*., **307**, 261–271.
36. Palus, J., Dziubaltowska, E. and Rydzynski, K. (1999) DNA damage detected by the comet assay in the white blood cells of workers in a wooden furniture plant. *Mutat. Res*., **444**, 61–74.
37. Pitarque, M., Creus, A., Marcos, R., Hughes, J. A. and Anderson, D. (1999) Examination of various biomarkers measuring genotoxic endpoints from Barcelona airport personnel. *Mutat. Res*., **440**, 195–204.
38. Apselin, A. L. and Grube, A. H. (1999) *Pesticides Industry Sales and Usage 1996 and 1997 Market Estimates*. Biological and Economic Analysis Division, Office of Pesticide Programs, Office of Prevention, Pesticides, and Toxic Substances, US Environmental Protection Agency, Washington, DC.
39. Antón, A. F., Laborda, E. and De Ariz, M. (1994) Acute toxicity of the herbicide glyphosate to fish. *Chemosphere*, **28**, 745–753.
40. Franz, J. E., Mao, K. M. and Sikorski, J. A. (1997) *Glyphosate: A Unique Global Herbicide*. Washington, DC American Chemical Society Monograph, 189.
41. Relyea, R. A. (2005) The lethal impacts of Roundup and predatory stress on six species of North American tadpoles. *Arch. Environ. Contam. Toxicol*., **48**, 351–357.
42. Cox, C. (1999) Glyphosate (Roundup). In Hickey, E. (ed.), *Global Pesticide Campaigner*. Pesticide Action Network (PAN): North America. Vol. 9. pp. 12–19.
43. Giesy, J. P., Dobson, S. and Solomon, K. R. (2000) Ecotoxicological risk assessment for Roundup herbicide. *Rev. Contam. Toxicol*., **167**, 35–120.
44. Li, A. P. and Long, T. J. (1988) An evaluation of the genotoxic potential of glyphosate. *Fundam. Appl. Toxicol*., **10**, 537–546.
45. Williams, G. M., Kroes, R. and Munro, I. C. (2000) Safety evaluation and risk assessment of the herbicide roundup and its active ingredient, glyphosate, for humans. *Regul. Toxicol. Pharmacol*., **31**, 117–165.
46. Vigfusson, N. V. and Vyse, E. R. (1980) The effect of the pesticides, Dexon, Captan and Roundup, on sister-chromatid exchanges in human lymphocytes in vitro. *Mutat. Res*., **79**, 53–57.
47. Lioi, M. B., Scarfi, M. R., Santoro, A., Barbieri, R., Zeni, O., Salvemini, F., Di Berardino, D. and Ursini, M. V. (1998) Cytogenetic damage and induction of pro-oxidant state in human lymphocytes exposed in vitro to gliphosate, vinclozolin, atrazine, and DPX-E9636. *Environ. Mol. Mutagen*., **32**, 39–46.
48. Neskovic, N. K., Poleksic, V., Elezovic, I., Karan, V. and Budimir, M. (1996) Biochemical and histopathological effects of glyphosate on carp. *Cyprinus carpio* L. *Bull. Environ. Contam. Toxicol*., **56**, 295–302.
49. Jiraungkoorskul, W., Upatham, E. S., Kruatrachue, M., Sahaphong, S., Vichasri-Grams, S. and Pokethitiyook, P. (2003) Biochemical and histopathological effects of glyphosate herbicide on Nile tilapia (*Oreochromis niloticus*). *Environ. Toxicol*., **18**, 260–267.
50. Sopinska, A., Grochala, A. and Niezgoda, J. (2000) Influence of water polluted with herbicide Roundup on the organism of fish. *Med. Weter*., **56**, 593–597.
51. Glusczak, L., dos Santos Miron, D., Crestani, M., Braga da Fonseca, M., de Araujo Pedron, F., Duarte, M. F. and Vieira, V. L. (2006) Effect of glyphosate herbicide on acetylcholinesterase activity and metabolic and hematological parameters in piava (*Leporinus obtusidens*). *Ecotoxicol. Environ. Saf*., **65**, 237–241.
52. Goldsborough, L. G. and Beck, A. E. (1998) Rapid dissipation of glyphosate in small forest ponds. *Arch. Environ. Contam. Toxicol*., **18**, 537–544.
53. Espina, N. G. and Weis, P. (1995) DNA-repair in fish from polluted estuaries. *Mar. Environ. Res*., **39**, 309–312.

54. Wirgin, I. and Waldman, J. R. (1998) Altered gene expression and genetic damage in North American fish populations. *Mutat. Res.*, **399**, 193–219.
55. Saleha Banu, B., Danadevi, K., Rahman, M. F., Ahuja, Y. R. and Kaiser, J. (2001) Genotoxic effect of monocrotophos to sentinel species using comet assay. *Food Chem. Toxicol.*, **39**, 361–366.
56. Avery, E. H., Blaine, L. E., Freedland, R. A. and Comelius, C. E. (1992) Bile pigments in gallbladder and freshly-secreted hepatic duct bile from red fed and fasted rainbow trout, *Oncorhynchus mykiss*. *Comp. Biochem. Physiol.*, **101**, 857–861.
57. Fischer, U., Ototake, M. and Nakanishi, T. (1998) Life span of circulating blood cells in Ginbuna Crucian Carp (*Carassius auratus* Langsdorfi). *Fish Shellfish Immunol.*, **8**, 339–349.
58. Dinnen, R. D., Tomlionson, S. M., Hart, D., Chopra, C. and Hedde, J. A. (1987) Application of micronucleus assay to the peripheral blood cells of the rainbow trout *Salmo gairdneri*. *Can. Tech. Rep. Fish. Aquat. Sci.*, **1607**, 69–78.
59. Murad, A. and Houston, A. H. (1992) Maturation of the goldfish *(Carassius auratus)* erythrocyte. *Comp. Biochem. Physiol. Comp. Physiol.*, **102**, 107–110.
60. Marc, J., Belle, R., Morales, J., Cormier, P. and Mulner-Lorillon, O. (2004) Formulated glyphosate activates the DNA-response checkpoint of the cell cycle leading to the prevention of G2/M transition. *Toxicol. Sci.*, **82**, 436–442.

Received on December 12, 2006; revised on February 1, 2007; accepted on February 5, 2007