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In vivo genotoxicity evaluation of atrazine and atrazine–based herbicide on fish *Carassius auratus* using the micronucleus test and the comet assay

Tolga Cavas *

Department of Biology, Faculty of Sciences and Letters, Uludag University, 16059 Gorukle, Bursa, Turkey

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ABSTRACT

Atrazine is a selective triazine herbicide used to control broadleaf and grassy weeds mainly in corn, sorghum, sugarcane, pineapple, and other crops, and in conifer reforestation planting fields. It has been showed that atrazine is one of the most frequently detected pesticides in agricultural streams and rivers, over the past two decades. Although the toxic properties of atrazine are well known, the data on the genotoxic effects of atrazine on aquatic organisms are rather scarce. Thus, in the present study we aimed to evaluate the genotoxic effects of atrazine and an atrazine–based herbicide (Gesaprim®) on a model fish species *Carassius auratus* L., 1758, (Pisces: Cyprinidae) using the micronucleus test and the comet assay in peripheral blood erythrocytes. Fish were exposed to 5, 10 and 15 μg/L atrazine and to its commercial formulation for 2, 4 and 6 days. Ethyl methane sulfonate (EMS) at a single dose of 5 mg/L was used as positive control. Our results revealed significant increases in the frequencies of micronuclei and DNA strand breaks in erythrocytes of *C. auratus*, following exposure to commercial formulation of atrazine and thus demonstrated the genotoxic potential of this pesticide on fish.

1. Introduction

To increase the production in agriculture, large amounts of chemicals are released into the environment, mainly on croplands and pastures. The chemical compounds get into aquatic ecosystems can cause hazardous effects on marine and freshwater organisms (Russo et al., 2004). Most of the contaminants in the aquatic environments exert their effects via genotoxic and metabolically toxic mechanisms simultaneously causing genotoxic damage/disease syndrome or carcinogenesis (Kurelec, 1993; Konen and Cavas, 2008). Thus, current awareness of the potential hazards of pollutants in the aquatic environment has stimulated much interest in the use of aquatic organisms as indicators for the monitoring of environmental genotoxicity.

Fish, as an aquatic vertebrate, become one of the most suitable models to estimate possible risks in the aquatic environment, due to their ability to efficiently metabolize and accumulate chemical pollutants. Among the three major cytogenetic endpoints (chromosomal aberrations, sister chromatid exchange and micronuclei) the micronucleus (MN) test is considered as the most suitable technique for fish species (Al-sabti and Metcalfe, 1995; Cavas and Ergene-Gozukara, 2005). The comet assay is also a sensitive genotoxicity test system which has been used for in the last two decade for assessing DNA damage in single cells (Tice et al., 2000; Collins, 2004). Since enucleated fish erythrocytes contains DNA, both MN test and comet assay have been successfully applied in red blood cells of fish (Cavas and Konen, 2007, 2008; Campos-Ventura et al., 2008).

Today, it is well known that pesticides not only affect target organisms but also have some side effects on non-target organisms (Joy et al., 2005). Atrazine is a selective triazine herbicide used to control broadleaf and grassy weeds in corn, sorghum, sugarcane, pineapple, and other crops, and in conifer reforestation plantings (Kiely et al., 2004). Results of studies over the past two decades showed that atrazine is one of the most frequently detected pesticides in agricultural streams and rivers (Fischer-Scherl et al., 1991; Comber, 1999; Vryzas et al., 2011). Furthermore, it was shown that the environmental concentrations of atrazine caused reduced reproduction and spawning, as well as tissue abnormalities in laboratory studies with fish (Bringolf et al., 2004; Tillitt et al., 2010). However, despite its widespread use and potential environmental toxicity, data on the genotoxic effects of atrazine and its commercial formulations on fish are scarce (Campos-Ventura et al., 2008). Thus, in the present study, it was aimed to comparatively evaluate the genotoxic effects of atrazine and an atrazine based herbicide (Gesaprim®) on a model fish species *Carassius auratus* L., 1758, (Pisces: Cyprinidae) using the micronucleus test and the comet assay in peripheral blood erythrocytes.

* Tel.: +90 2242941869; fax: +90 2242941899.
E-mail address: tcavas@uludag.edu.tr

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2. Materials and methods

2.1. Fish and chemicals

Goldfish, *C. auratus* (Linnaeus, 1758) belonging to the family Cyprinidae was chosen for this study due to its proven sensitivity to genotoxic chemicals and its common availability from breeders. Specimens of juvenile goldfish with average weight and length of 5 ± 1 g and 4 ± 1 cm, respectively, were purchased from a local breeder. Before the experiments, they were acclimated under laboratory conditions for 3 weeks at a population density of 15 specimens in 40 L aquaria containing aerated dechlorinated tap water with the following composition: temperature = 23 °C, calcium hardness = 54 mg/L, total hardness = 70 mg/L $CaCO_3$, pH 7.82, dissolved oxygen = 6.1 mg/L, conductivity = 0.209 mS/cm, ammonia = 0.001 mg/L [NH_3-N]. The photoperiod was 12:12 h. Fish were fed once a day with commercial fish pellets. Feces and pellet residues were removed daily by suction. Atrazine ($C_8H_{14}ClN_5$ – PESTANAL® 45330 98% purity) was purchased from Sigma. A commercial formulation of atrazine, Gesaprim® containing 500 mg/mL as the active ingredient was used for experiments. All the chemicals needed to perform the micronucleus test and the comet assays were obtained from the Sigma–Aldrich Chemical Company.

2.2. Experimental design

The herbicide Gesaprim® consisted of 500 mg of atrazine/mL as the active ingredient. The other ingredients of Gesaprim are proprietary information and not available to the author. Three different concentrations of atrazine (5, 10, and 15 μg/L) were selected based on previously detected aquatic environmental concentrations. Immediately prior to treatment the atrazine was dissolved in methanol to obtain three solutions of different concentrations and than added to aquaria to obtain final atrazine concentrations of 5, 10, and 15 μg/L. Gesaprim was diluted in various volumes obtain three different dilutions. Aquaria were than treated with each dilution to obtain final concentrations of Gesaprim® corresponding to 5, 10, and 15 μg/L atrazine.

Goldfish were placed in four different aquaria containing dechlorinated tap water (negative control), 5 mg/L EMS (positive control), methanol (solvent control), atrazine and Gesaprim. 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. The test water was renewed once at 2 days. Fish also were fed once every 2 days. A total of 75 goldfish (five fish for per dose per duration group) were used for the experiments.

2.3. Analysis of micronuclei and other nuclear abnormalities

Peripheral blood smears were air dried and after fixation in pure ethanol for 20 min was stained with 10% Giemsa solution for 25 min. All slides were coded and scored blind. Two slides were prepared for each fish, and 2000 cells were scored from each slide under 1000× magnification. Small, non-refractive, circular or ovoid chromatin bodies, displaying the same staining and focusing pattern as the main nucleus, were scored as micronuclei (Al-Sabti and Metcalfe, 1995). Nuclear abnormalities (NAs) other than micronuclei in erythrocytes were classified into four groups. Briefly, cells with two nuclei were considered as binucleated. Blebbed nuclei had a relatively small evagination of the nuclear membrane and contained euchromatin. Nuclei with evaginations larger than those in the blebbed nuclei, including those with several lobes, were classified as lobed nuclei. Nuclei with vacuoles or voids with appreciable depth into the nucleus were recorded as notched nuclei (Carrasco et al., 1990). Nuclear abnormality data was expressed as total nuclear abnormality calculated by the sum of all nuclear abnormalities.

2.4. The comet assay

The alkaline comet assay was performed according to the method of Tice et al. (2000) with some modifications. Blood samples collected from caudal veins of fish were diluted with 1 mL of PBS. About 60 μL of the diluted sample were mixed with 200 μL of 0.65% low-melting-point (LMP) agarose. A 75 μL of the mixture were than layered on the slides precoated with on 0.5% normal melting point (NMP) 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 low-melting-point 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% DMSO 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 approximately 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 25 V, 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. Two hundred cells (100 per replicate) were scored at 400× magnification. The DNA damage was quantified by visual classification of cells into five categories "comets" corresponding to the tail length (Anderson et al., 1994): undamaged Type 0, low-level damage: Type I, medium-level damage: Type II, high-level damage: Type III and complete damage: Type IV. 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 (Palus et al., 1999). 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 fish (Pitarque et al., 1999).

2.5. Statistical analyses

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. Comet assay data distributions are generally non-Gaussian, even after logarithmic transformation, which precludes the use of parametric tests. Thus we applied non-parametric Mann–Whitney *U*-test which is used for evaluation of visual comet data.

3. Results

Micronucleus frequencies in peripheral erythrocytes of *C. auratus* exposed to atrazine and Gesaprim as well as parallel, negative, solvent and positive controls are summarized in Table 1. As shown in the table, significant increases in the positive control group with respect to the negative control group were observed ($P < 0.001$). No significant increase in the solvent control group was observed ($P > 0.05$). Similarly atrazine treatment did not cause significant increases in frequencies of micronucleated erythrocytes. On the other hand Gesaprim treatments significantly induced the micronucleus frequency in all experimental groups with respect to the control group ($P < 0.05$) with the exceptions of 5 and μg/L at the 2nd day ($P > 0.05$).

Total nuclear abnormality frequencies in atrazine and Gesaprim exposed fish are demonstrated in Figs. 1 and 2, respectively. As can be seen in the figures, atrazine treatment did not cause significant increase in the frequencies of abnormal nucleated erythrocytes ($P > 0.05$), whereas Gesaprim treatment significantly increased the frequencies of nuclear alterations ($P < 0.05$). No significant increase in the solvent control group was observed ($P > 0.05$).

Results of comet assay experiments are summarized in Figs. 3 and 4 and in Table 2. As shown in Figs. 3 and 4, atrazine treatment did not increase the percentage of damaged cells ($P > 0.05$), where as Gesaprim treatment significantly increased the percentage of damages cells in peripheral erythrocytes of *C. auratus* ($P < 0.01$). Similarly, GDI values are not affected by atrazine treatment ($P > 0.05$). However, Gesaprim treatment significantly increased the GDI values indicating the DNA damaging potential of commercial formulation (Table 2). One again, no significant increase in DNA damage the solvent control group was observed ($P > 0.05$).

4. Discussion

Atrazine is a triazine herbicide registered for the control of broadleaf weeds and certain grassy weeds. Following its first introduction into the market, approximately 50 year ago, atrazine has become one of the most widely used agricultural herbicides in the world. The widespread use of atrazine has led to its presence in the environment, mostly in surface and groundwater (Goldman, 1994). Since the determination of the genotoxic effect of pollutants in the aquatic environment has become a major requirement for protection of the aquatic ecosystem, evaluation of toxic and genotoxic properties of atrazine on aquatic organisms is crucial. Based on inadequate data for humans and limited data for experimental animals, atrazine was classified as "possibly carcinogenic to humans" (Group 2B) by the International Agency for Research on