The cytogenetic effects of acrylamide on *Carassius auratus* periperial blood cells

Dehong Tan, Lingnan Li, Sha Wang, Baodong Wei, Xuan Zhang, Bingxin Sun, Shujuan Ji *

College of Food, Shenyang Agricultural University, 110866 Shenyang City, China

ARTICLE INFO

ABSTRACT

The cytogenetic effects of acrylamide on golden fish *Carassius auratus* peripheral blood cells were investigated *in vivo*. The fish were exposed to 5, 10, and 20 mg/L acrylamide in water for 96 h. Following exposure, peripheral blood samples were obtained to assay for nuclear anomalies and DNA damage. Acrylamide induced micronuclei and other nuclear anomalies including binuclei, nucleoplasm-bridged nuclei, and nuclear buds. The frequencies of these nuclear anomalies were significantly higher after treatment with 10 and 20 mg/L acrylamide ($p < 0.05$). DNA damage was assayed using the alkaline comet assay. Both olive tail moment and the percentage of tail DNA intensity significantly increased after treatment with 20 mg/L acrylamide ($p < 0.05$). In conclusion, acrylamide induces obvious genotoxicity in *C. auratus* peripheral blood cells.

1. Introduction

Acrylamide is a dietary contaminant and environmental toxicant (DeArmond and DiGoregorio, 2013). It is a vinyl monomer derived from a wide range of foods through the Maillard-Browning reaction during the cooking process (Postles et al., 2013). In industry, acrylamide is produced to generate polyacrylamide, which is extensively applied as a flocculant for clarifying drinking water and treating municipal waste waters, as a water-proofing agents in tunnel and dam construction, and for other uses (Carere, 2006). The annual industrial production of acrylamide reached nearly 100 million kg in recent years (Parzefall, 2008). The wide use and indiscriminate discharge of acrylamide has led to its presence in aquatic ecosystems (Buranasilp and Charoenpanich, 2011; Liu et al., 2013). Moreover, acrylamide is difficult to remove from water because of high water solubility, an extremely low 1-octanohater partition coefficient and a very low vapor pressure (Daughton, 1988). Acrylamide contamination poses a threat to aquatic life (Weideborg et al., 2001).

Many studies have examined the toxicological properties of acrylamide. Neurotoxicity was observed in laboratory animals over 30 years ago. More recent studies have shown genotoxic, reproductive, and carcinogenic effects (Daughton, 1988). Acrylamide has been widely investigated both *in vitro* and *in vivo* for genotoxicity; it can induce chromosomal aberrations, micronucleus (MN) formation, sister chromatin exchange, unscheduled DNA synthesis, single-strand DNA breaks, aneuploidy, polyploidy, and other mitotic disturbances (Taubert et al., 2006). Recent studies revealed that acrylamide induced mutations of *Hprt* in Big Blue rat lymphocyte (Mei et al., 2010) and gpt in rat testis (Koyama et al., 2011). In contrast, acrylamide did not cause genetic mutations in *Salmonella*, *Escherichia coli* (*E. coli*), or *Klebsiella pneumoniae* in the presence or the absence of an exogenous activation system (Besaratinia and Pfeifer, 2005). Acrylamide is a multi-organ carcinogen, causing tumor formation in many organs including the lungs, uterus, skin, mammary glands and brain (Capuano and Fogliano, 2011; Parzefall, 2008). Based on genotoxic and carcinogenic experimental findings, acrylamide has been classified by the International Agency for Research on Cancer (IARC) as a probable human carcinogen (Taubert et al., 2006). However, epidemiological studies have failed to provide evidence of a clear connection between the acrylamide consumption and cancer in humans (Arribas-Lorenzo and Morales, 2012).

The genotoxicological studies described above usually used terrestrial animals and microorganism to collect data for health concern; there are limited data regarding acrylamide toxicity to aquatic creatures. Fish are widely distributed aquatic vertebrates that bioaccumulate toxic substances and respond to low

---

concentrations of environmental pollutants (Dixon and Wilson, 2000; Klobučar et al., 2010). Genetic damage can disrupt the homeostasis of organisms and their offspring, and can also introduce genetic diversity (Bickham and Smolen, 1994). Hence, it is important to assess genotoxicity when determining consequences of pollution to living organisms. The present study focused on the genotoxic effects of acrylamide on *Carassius auratus* (golden fish), a species that may be bred easily and can be obtained in many areas.

2. Materials and methods

The experiment was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments.

2.1. Fish

C. auratus weighing 20.0 ± 2.0 g were purchased from a local pet fish market (Shenyang city, China). All fish were acclimated under laboratory conditions at $18 \pm 1\ ^\circ\text{C}$, 12/12 h dark/light cycle for 7 days before testing. During the acclimation period, all fish were placed in the same tank (200 L) with dechlorinated tap water, and fed commercial feed. One-fourth of the water in the fish tank was changed every day during acclimation, and air was bubbled continuously.

After acclimation, fish were randomly assigned to five 10-liter tanks (20 per tank), each corresponding to one treatment group. Three treatment groups were exposed to 5, 10, and 20 mg/L acrylamide (Sigma–Aldrich, China) in water, corresponding to approximately 1/16, 1/8, and 1/4 of the $LD_{50-96\ h}$ (83.3 mg/L, unpublished data), respectively. Intraperitoneal (i.p.) injection with 20 mg cyclophosphamide (Cytoxan, Bristol Meyers Squibb, USA) per kg of body weight was used as a positive control (Kumar et al., 2012). A negative control group was raised without any chemical treatment.

After 96 h of exposure with no water changes, peripheral blood was collected from each fish by cardiac puncture with a heparinized syringe. The blood samples were stored on ice. Blood from 10 randomly selected fish from each group was processed for the micronucleus and comet assays.

2.2. Nuclear anomalies assay

Peripheral blood samples were smeared on clean microscope slides, fixed with absolute methanol for 10 min, stained with 10% Giemsa solution (Sigma, USA) for 10 min, and then examined using an optical microscope with a 100X oil-immersion lens (Olympus BX41, Japan). Only intact cells with clear cellular and nuclear membranes were scored. Micronuclei (MN) were defined as small round structures in the cytoplasm that were similar in color and structure to chromatin, in the same optical plane as the main nucleus, between 1/5 and 1/100 the size of the main nucleus, lacking nuclear bridges or direct contact with the main nucleus (Minissi et al., 1996). Binuclei (BN) are two nuclei with approximately equal sizes in the same cell. Nucleoplasm-bridged nuclei (NPB) are two nuclei in a cell connected by a narrow or wide nucleoplasmic bridge. Nuclear buds (NBUD) have the same morphology as MN, but are connected to the nucleus by a narrow stalk of nucleoplasmic material (Çavaş, 2008).

Three slides were prepared for each sample; 1000 cells were scored by microscopy for each slide; and the frequencies of MN and other nuclear anomalies were quantified per 1000 cells (‰).

2.3. Alkaline comet assay

The assay was performed according to the basic procedure of Singh et al. (1988) as modified by Klobučar et al. (2010). Briefly, 10 µL of blood (10^4 cells, diluted in PBS) was mixed with 90 µl of 0.5% low melting point (LMP) agarose and placed on a slide coated with normal 1% agarose. The slide was placed into a $4\ ^\circ\text{C}$ refrigerator until the blood–agarose mixture solidified, and a third layer of 0.5% LMP agarose was added and allowed to solidify as mentioned above. The slides were immersed in freshly prepared lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 10% DMSO, 1% Triton X-100, pH 10) for 1 h at $4\ ^\circ\text{C}$ to lyse the cells. After rinsing with double-distilled water, the slides were placed on a horizontal gel electrophoresis platform, covered with a chilled alkaline electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH 13) and left in the dark for 20 min. The slides were electrophoresed for 30 min at 300 mA in an electrophresis apparatus (PowerPace Basic, Bio-Rad, USA), and then neutralized twice for 25 min in 0.4 M Tris–HCl, pH 7.5 at $4\ ^\circ\text{C}$. The slides were stained with 0.02 mg/mL of acridine orange in 10 mM sodium phosphate at pH 7.4, rinsed 3 times with the same buffer, and covered with coverslips. The slides were examined using a fluorescence microscope (Nikon TS-100, Japan) with a CCD camera. 50 cells per slide were analyzed with comet assay software project CASP 1.2.2 software. Cells with ⩾50% tail DNA, which may represent dead or dying cells, were excluded from the analysis.

2.4. Statistical analysis

The experiment was repeated 3 times. Statistical analyses were performed using the statistical software SPSS version 16.0. Results for both Alkaline comet assays and frequencies of nuclear anomaly cells were expressed as means ± standard deviation ($n = 10$). Significant differences between means were determined using a one-way analysis of variance and Duncan's test ($p < 0.05$).

3. Results

3.1. Micronuclei and other nucleus anomalies

Representative examples nuclear anomalies are shown in Fig. 1, and the frequencies of these abnormalities are shown in Fig. 2. Few nuclear abnormalities were observed in peripheral blood erythrocytes from fish in the untreated negative control group. As a positive control, fish were injected i.p. with cyclophosphamide. Peripheral blood erythrocytes had significantly more micronuclie (MN), binuclei (BN), nucleoplasm-bridged nuclei (NPB), and nuclear buds (NBUD) than the negative controls ($p < 0.05$). In addition, other kinds of nuclear anomalies such as notched nuclei and fragmented nuclei were also apparent in the positive control group, but statistical analyses were not completed for these anomalies.

In acrylamide treated *C. auratus* peripheral blood erythrocytes, the MN frequency showed a dose-dependent increase. The frequency of MN in the 10 and 20 mg/L acrylamide groups were $8.7 \pm 1.2‰$ and $11.4 \pm 1.5‰$, respectively, which were significantly higher than those in the negative control group ($3.1 \pm 0.9‰$) ($p < 0.05$). The MN frequency in the 5 mg/L group ($3.8 \pm 0.5‰$) was not significantly greater than the negative control group.

Similar to that of MN, frequencies of BN, NPB, and NBUD in acrylamide treatment groups also increased in a dose-dependent manner. For the negative control, 5, 10, and 20 mg/L acrylamide groups, the frequencies of BN were $2.3 \pm 0.3‰$, $1.8 \pm 0.3‰$, $3.7 \pm 0.5‰$, and $5.3 \pm 1.5‰$, respectively; the frequencies of NPB were $2.1 \pm 0.3‰$, $4.3 \pm 1.3‰$, $6.3 \pm 1.6‰$, and $7.6 \pm 1.5‰$, respectively; and the frequencies of NBUD were $1.6 \pm 0.3‰$, $2.2 \pm 0.3‰$, $4.5 \pm 0.6‰$, and $5.3 \pm 0.7‰$, respectively. Frequencies of these nuclear abnormalities in the 10 and 20 mg/L groups were significantly higher than those in the negative control group ($p < 0.05$). In the low concentration acrylamide (5 mg/L) group, only the frequency of NPB was significantly higher than the negative control group ($p < 0.05$).

3.2. DNA damage

In the alkaline comet assay, several endpoints including Olive tail moment (OTM) and the percentage of tail DNA can be effectively used as an index of DNA damage. OTM is determined by calculating the relative fluorescence intensity in the head and tail, while the percentage of tail DNA is determined as the extent of DNA migration (Wu et al., 2011). As shown in Fig. 3, the positive control group displayed significant increases in both OTM and percentage of tail DNA intensity when compared to the negative control group. The 10 and 20 mg/L acrylamide treatment groups showed increased intensification of both indices; however, the differences were only significant in the 20 mg/L acrylamide group, compared to negative control ($p < 0.05$).

4. Discussion

The MN and other nuclear aberration assay and alkaline comet assay are sensitive, rapid, and frequently-applied tools for detecting genotoxic effects of chemicals (Ali et al., 2009). The peripheral blood erythrocytes in most fish species are sizable and have a large nucleus, making them ideal for such investigations (Klobučar et al.,