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Genotoxic effect of cadmium and zinc in the peripheral erythrocytes of Prussian carp (*Carassius gibelio* B.)

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Abstract

Introduction: Cadmium and zinc are often found in aquatic environment and may accumulate in living organisms. The aim of this study was to evaluate the genotoxic effect of Cd, Zn, and their binary mixture on the peripheral blood erythrocytes of Prussian carp (*Carassius gibelio* B.). **Material and Methods:** The fish were exposed to 4.0 mg/L Cd, 4.0 mg/L Zn or a mixture of 4.0 mg/L Cd and 4.0 mg/L Zn for a period of 14, 21 or 28 days. Genotoxic effects were investigated in peripheral blood cells using the comet assay and the erythrocyte micronucleus assay. **Results:** The results demonstrated that the frequencies of micronuclei (MN) and both nuclear and cellular abnormalities in erythrocytes were significantly higher in all exposure groups as compared to the control group. The fish exposed to the mixture of Cd and Zn presented the highest frequency of MN. Furthermore, there was a decrease in the frequency of MN and an increase in the occurrence of DNA integrity defects (DNA damage) with longer time of exposure to the metals studied. **Conclusion:** Erythrocyte micronucleus and comet assays confirmed the genotoxicity of Cd and Zn. The results of the tests applied (which showed considerable variability) suggest the involvement of various toxicity mechanisms. Therefore, an integrative and comprehensive approach, using a set of assays for toxicity profile determination, should be adopted during ecotoxicological studies and environmental risk assessment pertaining to these elements.

Keywords: genotoxicity, heavy metals, erythrocyte micronucleus assay, comet assay, Prussian carp.

Introduction

In the last decades, the quality of water and the biodiversity of aquatic ecosystems have considerably worsened because of excessive exploitation (4). Large amounts of chemicals are released into the environment as a result of intense urbanisation processes, industrial activities and agriculture contributing to surface water contamination (13, 31). Pollutants that are particularly harmful include heavy metals, *i.e.* lead, mercury, copper, zinc and cadmium, which may accumulate in living organisms (32). Aquatic environmental pollution with metals raises concerns all over the world because of the metals' persistence in the environment, potential toxicity and prevalence. There are reports indicating that various pollutants including heavy metals, which enter water together with sewage, damage the genomes of aquatic organisms, leading to genotoxic changes in their cells (8, 19).

DNA integrity defects caused by genotoxicants in the aquatic environment damage the genome at various levels of its organisation. These abnormalities include the induction of point mutations and karyotype defects and they induce nucleoplasmic processes and uncontrolled cellular proliferation; a genome made defective in these ways may also be inherited by subsequent generations (19).

Fish are excellent model animals for genotoxicological research and provide an early warning of toxicant-induced environmental changes and degradation caused by toxic substances (8, 19). Aquatic organisms are often exposed to a complex mixture of contaminants. Simultaneous exposure to both essential and non-essential metals may produce

effects that result from various types of interactions between these elements. Generally, the total impact of multiple toxicants on the organism may consist in an additive toxic effect, being the sum of the effects of individual substances. The toxic effect of a given mixture may be weaker (antagonism) or significantly stronger (synergism) than the sum of toxic effects of its individual components. Furthermore, one substance may have a protective effect against another (1). An example of heavy metals that often occur together in the aquatic environment would be cadmium and zinc**,** which are commonly used in industry and released into the environment as a by-product of zinc ore smelting (10)**.** Special focus is directed at toxic metals such as cadmium. This metal has no known essential role in living organisms and has been considered as the main threat to all living organisms among heavy metals (12). There are maximum permissible concentrations set down for this element in surface waters, bottom sediments, and fish flesh. Even low concentrations of Cd can cause DNA damage and this element has been classified by the International Agency for Research on Cancer (IARC) as a human carcinogen (2). On the other hand, zinc is an essential metal, which plays an important role in the biological function of numerous proteins and enzymes (17). However, like Cd, Zn can be toxic in excessive concentrations (24).

The effects of Cd and Zn known from the scientific literature are based on studies which analysed these metals separately. To date, there have been few studies analysing the combined effect of contamination with Zn and Cd, which occurs very often in surface waters (1). These elements are mainly found in drainage ditches in zinc ore mining areas, where cadmium occurs naturally, and in phosphate rocks used to manufacture fertilisers (14).

The Prussian carp (*Carassius gibelio*) is a good model for toxicological studies due to the ease of its breeding and maintenance in aquaria and wet labs, its high adaptability, and the ease of blood collection (38). Therefore, the present study was undertaken to assess the genotoxic effect of cadmium, zinc, and their binary mixture Cd + Zn on this fish species.

Methods used to determine the genotoxicity profile of aquatic pollution, for example with heavy metals, are the single cell gel electrophoresis (SCGE), otherwise known as the comet assay, and the erythrocyte micronucleus assay (EMN). These methods provide a means of assessing the extent of DNA integrity disorders in individual cells (SCGE) and nuclear chromatin damage in fish erythrocytes (EMN). These tests are commonly used as genotoxicity indicators of various agents in studies on aquatic organisms (36).

In this study, fish were exposed *in vivo* to sublethal doses of cadmium and zinc and their binary mixture for periods up to four weeks in length. Genotoxic effects were investigated in peripheral blood cells using the comet assay and the erythrocyte micronucleus assay.

Material and Methods

Experimental fish. The fish were obtained from the ponds of the Experimental Station of the Department of Animal Nutrition and Biotechnology, and Fisheries of the University of Agriculture in Krakow. A total of 84 three-year-old female Prussian carp (*Carassius gibelio* B.) were held in four 700 L tanks (21 fish per tank) in continuously aerated water. The physicochemical properties of the water in the tanks were as follows: pH 7.0, dissolved oxygen – 8.0 mg/L, and temperature 19°C. A 14/10 h light/dark photoperiod was maintained. The fish were fed 3% of their body weight daily with commercial dry pellets during a two-week acclimation period and throughout the experiment. The average weight of the fish was 160 ± 3.85 g. Cadmium chloride ($CdCl_2 \times 2.5H_2O$) and zinc sulphate ($ZnSO_4 \times 7H_2O$) were purchased from Avantor Performance Materials Poland S.A. (formerly POCH S.A.). A stock solution was made for each heavy metal by dissolving it in distilled water and adding it to the tank to obtain the desired final concentration.

After the two-week acclimation period, the fish were divided into four groups of 21 specimens each. These were the control group and three treatment groups exposed to 4.0 mg/L Cd, 4.0 mg/L Zn and a 4.0 mg/L Cd and 4.0 mg/L Zn mixture for up to 28 days. The water in the tanks was changed every other day to maintain constant concentrations of the metals. The concentrations of cadmium and zinc were selected taking into account the levels of these metals found in surface waters (ranging from 1 to over 16 mg/L) (27, 35).

After 14 and 21 days of exposure to the metals, blood samples of approximately 1 mL were drawn from the caudal vein with a sterile heparinised syringe from seven randomly selected fish from each group anaesthetised with 50 mg/L MS-222 (Sigma-Aldrich, St. Louis, MO, USA). After blood sampling, the fish were euthanised and then subjected to decapitation. The seven remaining fish on the 28th day were subjected to the same procedure. The experiments were performed in accordance with the research protocols approved by the Local Animal Ethics Committee in Kraków, Poland (approval no. 144/2019).

Comet assay–single-cell gel electrophoresis (SCGE) assay. The study material comprised blood diluted with phosphate-buffered saline (Sigma-Aldrich) at a 1:10 ratio. For the purpose of the SCGE assay, the cells were immobilised on a microscope slide between two layers: 0.5% low-melting-point agarose and 0.5% normal-melting-point agarose (both from Sigma-Aldrich). Next, the cells were incubated for 1 h in a lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 0.4 M

Tris-HCl, 1% sodium lauroyl sarcosinate, 10% Triton X-100, 1% DMSO, pH 10). Electrophoresis was performed in a TBE buffer (10 N NaOH and 200 mM EDTA, pH ≥ 13) at 25 V and 300 mA for 20 min, and neutralisation was carried out with 0.4 M Tris-HCl (Sigma-Aldrich). Detection was performed using 200 µg/mL ethidium bromide (37). DNA integrity was assessed in each fish based on the measurement of 100 cells. DNA integrity defects were assessed based on the percentage of DNA in the tail (% Tail DNA) and the tail moment values (TM) as % tail DNA multiplied by the tail length in the DNA studied.

The assessment was performed using a Zeiss Imager A2 epifluorescence microscope equipped with a Zeiss AxioCam MRc5 camera (Carl Zeiss Microscopy, Jena, Germany). Cellular damage was assessed using CASP 1.2.3b software (23).

Erythrocyte micronucleus assay (EMN). Blood smears in PBS at a 1:1 ratio were prepared for each fish in three repetitions. The preparations were dried at room temperature for 24 h and then fixed with methanol (Aldrich) for 10 min. The EMN assay was performed in accordance with the method described by Hussain *et al.* (19). For the purpose of microscopy, the preparations were stained in 10% aqueous Giemsa solution (Sigma-Aldrich) for 20 min. Microscopy analysis of two smears was performed for each fish, assessing 1,000 erythrocytes in each repetition. Chromatin damage was assessed based on the number of erythrocytes with micronuclei (1, 2 or 3 MN) or other nuclear or cellular defects. Erythrocyte nuclear abnormalities (ENA) were identified following the method used by Jindal and Verma (21). These abnormalities included nuclear buds (BUD), binuclear cells (BNC), abnormal shapes of the nucleus (AN), and different shapes of notched nuclei (NT). The preparations were also assessed in terms of erythrocyte cytoplasmic abnormalities (ECA) identified in accordance with the method described by Jindal and Verma (21). Erythrocyte cytoplasmic abnormalities included apoptotic cells (A) and abnormal shapes of cytoplasm (AC). The assessments and photographic documentation were performed under a Zeiss microscope with a Nikon DS-U1 sight controller microscope digital camera coupled with NIS-Elements F 2.30 software (Nikon, Tokyo, Japan).

Statistical analysis. The results of the comet assay were presented as the mean percentage of DNA in the comet tail and as TM values. The results of the analysis of erythrocytes with MN, BUD, BNC, A, AN, AC and NT incidence were presented as the frequency of these abnormalities (as a percentage of erythrocytes displaying the genotoxic abnormalities studied). The statistical analysis of the data was performed with Student's *t*-test. The level of significance was set at 0.05. Statistica 12 software (StatSoft, Tulsa, OK, USA) was used for the statistical analysis.

Results

Comet assay. The comparison of DNA damage in the erythrocytes of fish exposed to heavy metals (Zn, Cd and Cd + Zn) is presented in Table 1 as the percentage of DNA in the tail (% Tail DNA) and the tail moment values (TM). Both these parameters indicated a significant increase in the occurrence of chromatin integrity abnormalities in the erythrocytes of Prussian carp. Erythrocytes with different levels of DNA damage in the comet assay are shown in Fig. 1.

The highest percentage of DNA in the tail, 37.87 ± 0.76% Tail DNA, was observed in fish simultaneously exposed to Cd and Zn and was noted after 14 days of the experiment. Subsequently, the level of DNA damage to that observed in the control fish on day 28 of the experiment. In fish exposed to Zn, the percentage of chromatin breaks also significantly decreased after 21 and 28 days. In the specimens exposed to cadmium for 14 (31.78 ± 0.69) and 21 days (31.84 ± 0.59%), there was a statistically significant increase ($P < 0.05$) in DNA damage in the comet tail compared to the control group (26.30 ± 0.56% and 28.08 ± 0.65%, respectively). The level of observed damage in these cadmium-exposed fish rose by 5.48 % after 14 days of exposure, and after 21 days of exposure, the percentage of DNA damage was 3.76% higher than in the control group (Table 1).

DNA migration in the nucleus presented with an auxiliary parameter – TM – ranging from 2.80 ± 0.35 (after 28 days) to 53.58 ± 1.80 (after 21 days) in the control group. In fish exposed to heavy metals, the highest TM values were recorded after 14 days of exposure: 69.51 ± 1.91 in the group simultaneously exposed to Cd and Zn, 64.46 ± 1.80 in fish exposed to Cd, and 63.77 ± 1.85 in those exposed to Zn. The TM values for all the treatment groups were significantly higher as compared to the control group, except for the group exposed for 28 days. The TM values decreased with exposure time in all the groups. After 21 days of the experiment, the control group showed an increase in the value of the TM parameter (Table 1).

Erythrocytes micronucleus assay. The EMN assay revealed various types of cellular defects within the nucleus and cytoplasm (Fig. 2). Table 2 presents a comparison of the frequency of MN (one or more micronuclei) and nuclear and cellular abnormalities in the erythrocytes of Prussian carp after 14, 21, and 28 days of exposure to Zn, Cd, and the Cd and Zn mixture.

The average frequency of MN erythrocytes in the control group ranged from 1.29 ± 0.21 to 3.11 ± 0.34. The highest induction of micronuclei in erythrocytes was observed on day 14 in fish exposed to Cd and Zn (16.07 ± 1.67), the Cd group had approximately 20% fewer MN (12.49 ± 1.08) and the Zn group had more than 50% fewer (7.34 ± 0.80). The average number of micronucleated erythrocytes in the treatment groups exposed to heavy metals was statistically significantly higher than the number in the control group. In all the

groups, the MN frequency decreased along with the exposure time. In fish intoxicated with either Cd or Zn, the MN frequency after 28 days of exposure was similar to that found in the control group. The frequency of MN induction remained significantly higher in fish simultaneously exposed to both these metals with reference to the control and cadmium-exposed groups (Table 2).

Table 1. Comet assay results for Prussian carp exposed to different treatments of cadmium, zinc and their binary mixture

	Group			
	Control	Cd	Zn	Cd + Zn
Day	DNA in Tail (%)			
14	26.30 ± 0.56 Aa	31.78 ± 0.69 Ab	33.35 ± 0.67 Ab	37.87 ± 0.76 Ac
21	28.08 ± 0.65 Aa	31.84 ± 0.59 Ab	24.40 ± 0.53 Bc	31.89 ± 0.64 Bb
28	32.04 ± 0.61 Ba	32.87 ± 0.67 Aa	28.58 ± 0.51 Cb	31.90 ± 0.59 Ba
	Tail moment			
14	48.82 ± 1.49 Aa	64.46 ± 1.82 Abc	63.77 ± 1.85 Ab	69.51 ± 1.91 Ac
21	53.58 ± 1.80 Ba	46.11 ± 1.33 Bb	30.39 ± 1.05 Bc	38.81 ± 1.39 Bd
28	2.80 ± 0.35 Ca	2.18 ± 0.28 Ca	3.56 ± 0.33 Ca	4.14 ± 0.54 Cb

Small letters denote statistically significant differences ($P<0.05$) between the groups on different exposure days (a–d), while capital letters indicate significant differences in the groups between successive days of exposure (A–C)

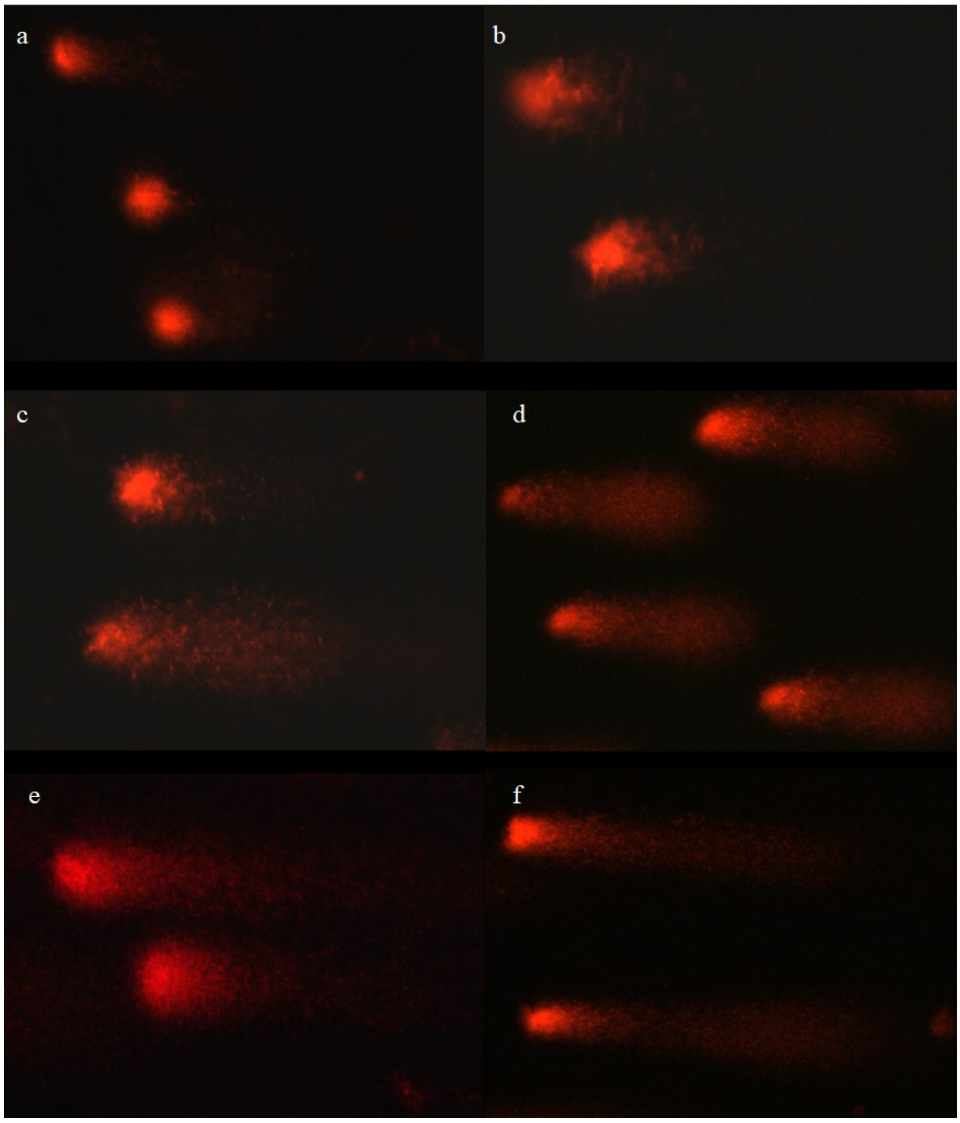

Fig. 1. Erythrocytes of Prussian carp with different levels of DNA damage in the comet assay: cells from control samples after 14 days (a), after 21 days (c), and after 28 days (e); cells after exposure to Cd for 14 days (b), for 21 days (d) and for 28 days (f). 400×

Erythrocyte nuclear and cytoplasmic abnormalities. Defects such as BUD, BNC, AN, NT and A occurred with a considerably lower frequency than MN. Significant differences in BUD incidence were found after 28 days of exposure between the cadmium-exposed group and the control group. The BNC frequency was significantly higher in the Cd + Zn group after 21 days compared to the frequency in the control and cadmium-exposed groups, and in all experimental groups after 28 days of exposure relative to the control group. In the case of erythrocytes with abnormal shape of the nucleus, a significant increase in the number of these lesions was observed with lengthening experimental time, in the control group between 14 and 21 days and 14 and 28 days, and in the Cd-exposed fish between 21 and 28 days of exposure. Significant differences in the number of ANs were observed after 21 days of the experiment between the control and Zn-exposed groups. In contrast, after 28 days, significant differences in the number of ANs were found between all experimental groups. As for NT-type abnormalities, there were significant differences in their number between all tested groups after 14 days of the experiment. The 21-day exposure resulted in a significant increase in NT in the Cd-exposed group compared to control fish. Erythrocyte damage after continuing the experiment to 28 days was characterised by similar levels of NT in the Cd and Zn groups, with significant differences noted between the Zn-exposed group and the unexposed group, while the Cd and Zn group showed a significant decrease in the number of NTs compared to the other groups examined. Apoptotic cells were not identified in all experimental groups during the exposure periods studied. The A frequency was significantly higher in the Cd group after 14 days compared to the control and combined Cd and Zn-exposed groups. Significant differences in the frequency of apoptotic cells were found in the group of fish with Cd in their water between 14 and 28 days of exposure. In addition, significant differences in the number of A were found after 28 days of exposure between the Cd and Zn-exposed groups. The frequency of AC in fish from the control group was below 1.0 in all experimental periods and fell significantly after 21 days. After 14 days of the experiment, there were significant differences in the number of AC between the Cd-exposed group and the control group, and between the Zn and combined Cd and Zn groups with respect to the Cd group and the control group. The 21-day exposure was characterised by significant differences in the number of AC between the experimental groups and the control group. The 28-day exposure of fish to Zn in their water resulted in a significant increase in the number of AC relative to the control group (Table 2).

Table 2. The frequency of erythrocyte nuclear and cellular abnormalities in Prussian carp exposed to different concentrations of cadmium, zinc and their binary mixture

	Group			
	Control	Cd	Zn	Cd + Zn
Day	MN (one or more micronuclei)			
14	2.30 ± 0.36 Aa	12.49 ± 1.08 Ab	7.35 ± 0.80 Ac	16.07 ± 1.68 Ab
21	1.29 ± 0.21 Ba	4.00 ± 0.48 Bb	4.61 ± 1.14 ABb	3.67 ± 0.43 Bb
28	3.11 ± 0.34 Aab	2.33 ± 0.298 Ca	3.69 ± 0.34 Bbc	4.53 ± 0.63 Bc
	BUD (nuclear buds)			
14	0.26 ± 0.05 Aa	0.16 ± 0.08 Aa	0.15 ± 0.05 Aa	0.34 ± 0.08 Aa
21	0.11 ± 0.05 Ba	0.19 ± 0.06 Aa	0.21 ± 0.10 Aa	0.12 ± 0.05 Ba
28	0.07 ± 0.03 Ba	0.28 ± 0.07 Ab	0.17 ± 0.06 Aab	0.18 ± 0.08 ABab
	BNC (binuclear cells)			
14	0.25 ± 0.22 Aa	0.30 ± 0.09 Aa	0.22 ± 0.06 Aa	0.12 ± 0.07 Aa
21	0.19 ± 0.07 ABa	0.23 ± 0.06 Aa	0.32 ± 0.12 ABab	0.54 ± 0.14 Bb
28	0.09 ± 0.03 Ba	0.43 ± 0.11 Bb	0.69 ± 0.17 Bb	0.51 ± 0.08 Bb
	AN (abnormal shapes of nucleus)			
14	0.01 ± 0.01 Aa	0.04 ± 0.02 Aa	Not found	0.18 ± 0.09 Aa
21	0.21 ± 0.07 Ba	0.03 ± 0.02 Aab	0.012 ± 0.011 Ab	Not found
28	0.15 ± 0.05 Ba	1.68 ± 0.43 Bb	0.009 ± 0.009 Ac	0.036 ± 0.036 Ad
	NT (different shapes of notched nuclei)			
14	2.28 ± 0.45 Aa	0.07 ± 0.03 Ab	0.91 ± 0.18 ABc	3.94 ± 0.52 Ad
21	0.67 ± 0.10 Ba	2.91 ± 0.42 Bb	0.66 ± 0.12 Aa	1.30 ± 0.26 Ba
28	0.69 ± 0.14 Ba	1.15 ± 0.24 Cab	1.57 ± 0.36 Bb	0.28 ± 0.07 Cc
	Total nuclear abnormalities (MN+BUD+BNC+AN+NT)			
14	5.50 ± 0.63 Aa	14.57 ± 1.27 Ab	8.35 ± 0.80 Ac	20.61 ± 1.84 Ad
21	2.39 ± 0.23 Ba	7.36 ± 0.69 Bb	5.80 ± 1.10 Ab	5.62 ± 0.65 Bb
28	4.08 ± 0.41 Aa	5.66 ± 0.48 Cb	6.12 ± 0.38 Ab	5.53 ± 0.64 Bab
	A (apoptotic cells)			
14	0.02 ± 0.013 a	0.44 ± 0.24 Ab	0.00 ± 0.00	0.10 ± 0.06 c
21	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.001 Aa	0.00 ± 0.00
28	0.00 ± 0.00	2.04 ± 0.56 Bb	0.011 ± 0.001 Aa	0.00 ± 0.00
	AC (abnormal shapes of cytoplasm)			
14	0.86 ± 0.16 Aa	22.11 ± 1.99 Ab	1.33 ± 0.12 Ac	2.16 ± 0.55 Ac
21	0.39 ± 0.12 Ba	1.31 ± 0.16 Bb	4.60 ± 1.13 Bb	1.21 ± 0.15 Bb
28	0.94 ± 0.16 Aa	1.31 ± 0.25 Bab	1.94 ± 0.39 Ab	1.32 ± 0.18 Bab

Small letters denote statistically significant differences ($P<0.05$) between the groups on different exposure days (a–d), while capital letters indicate significant differences in the groups between successive days of exposure (A–C)

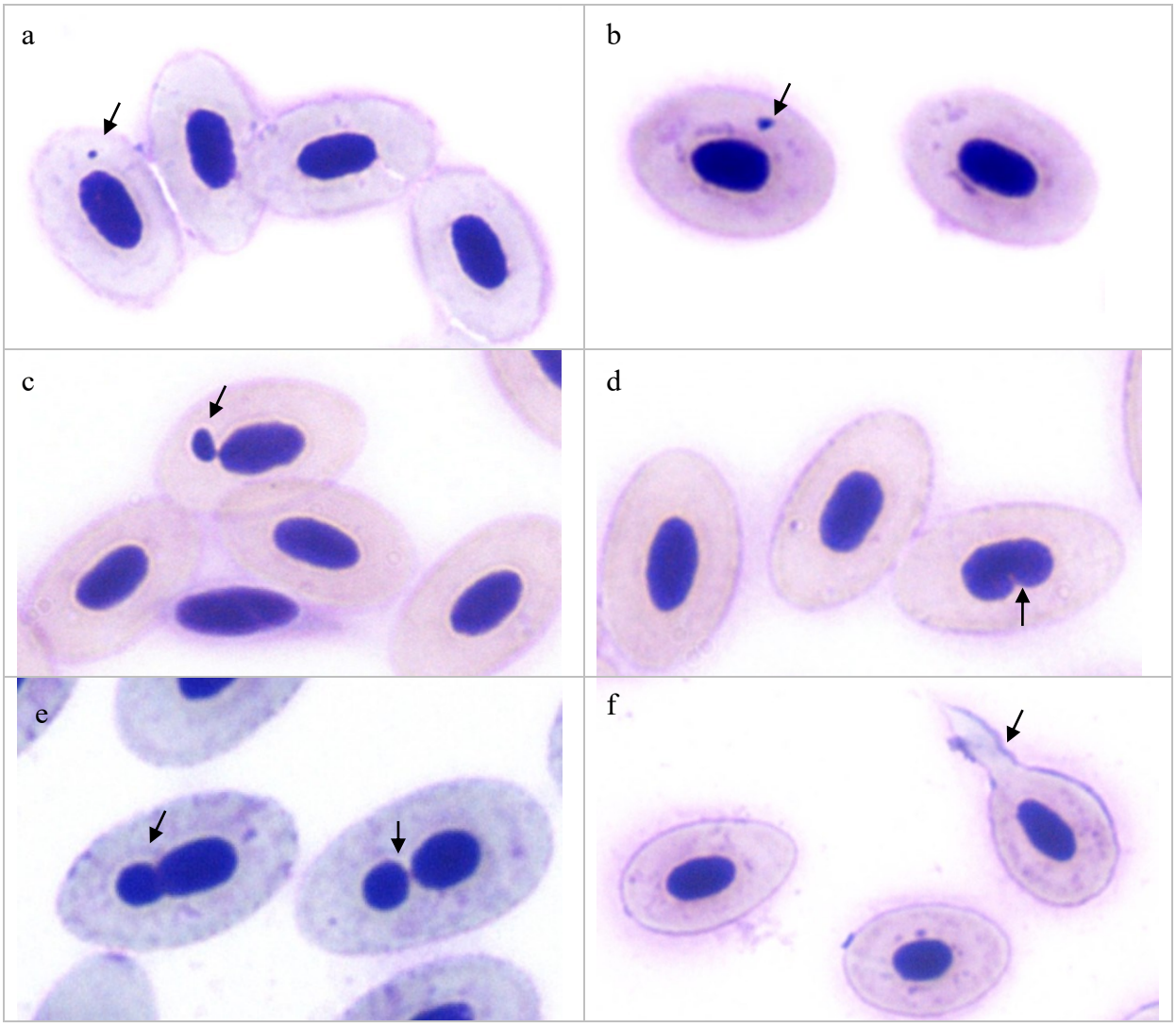

Fig. 2. Giemsa-stained peripheral blood smear of erythrocytes of Prussian carp with micronuclei (a and b), a nuclear bud (c), a notched nucleus (d), binucleated cells with unequal nuclear division (e), and a cell with abnormal cytoplasm (f). Arrows show the abnormalities. Erythrocytes are from the control group after 21 days of the experiment (c and d), exposed to Cd after 14 days (f) and exposed to Cd after 28 days (a, b and e). 1000×

Discussion

The awareness of potential risks related to the presence of heavy metals in the aquatic environment has evoked considerable interest in the use of fish for the biomonitoring of carcinogens, teratogens, and mutagens in the aquatic environment. The subject literature clearly indicates that potential genotoxic effects leading to mutations and population decline in fish exposed to such toxicants are not well understood (3, 19). This study was planned to assess the effects of such pollutants as cadmium and zinc in the peripheral blood erythrocytes of Prussian carp. Assessment of these in the wild-caught fish could allow for early detection and warning of habitat pollution serious enough to threaten the extinction of some of the fish populations. Genotoxicity assessment is performed using cytogenetic assays (as genotoxicity biomarkers), including the comet assay and the erythrocyte micronucleus assay (21, 36, 40). The results of the assays conducted in the present study confirmed the genotoxic effect of sublethal Cd and Zn concentrations on the peripheral erythrocytes of Prussian carp exposed to Cd and Zn, used individually or in combination.

The comet assay provides important information on cellular DNA integrity defects caused by teratogenic or carcinogenic agents (25). This technique is used to assess the genotoxicity caused by environmental pollution, which also affects the aquatic environment comprising fish habitats (21, 34). The most commonly used parameters that indicate DNA damage in the comet assay are the percentage of DNA in the tail and the tail moment (39). The present study demonstrated that the exposure of fish to the combination of Cd and Zn caused considerably greater DNA damage than individual exposure to each of these metals. It was also shown that the highest intensity of DNA integrity defects (% Tail DNA and TM) in each of the treatment groups was observed on day 14 of exposure, followed by a decrease in the percentage of DNA in the comet

tail in the subsequent weeks (Table 1). Such results corroborate previous reports on the effect of cadmium and zinc on other fish species and cell types (1, 19). Singh *et al.* (36) demonstrated that after 30-day exposure of spotted snakeheads (*Channa punctata*) to cadmium at 0.05 mg/L there was a significant increase in the occurrence of DNA damage as shown in a comet assay, which was 5.25 ± 0.62% Tail DNA as compared to the control group's result of 0.78 ±005%. Cadmium induces single-stranded DNA breaks as a result of direct Cd–DNA adduct formation and through the activation of incision nucleases and glycosylase (36). Hussain *et al.* (19) conducted a study the aim of which was to determine the freshwater pollution profile in the River Chenab, Pakistan and assess its toxic effect on Rohu labeo (*Labeo rohita*). Among the metals contaminating the water in this river were cadmium and zinc at concentrations of 0.01 mg/L and 5 mg/L, respectively. These authors demonstrated that for fish in the most polluted experimental sites, the percentage of DNA in the comet tail was as high as 42.21 ± 2.06% and the tail moment was 17.71 ± 1.79 (19). The DNA damage noted by these authors was more extensive than that observed in Prussian carp in the present research after 14 days of exposure to Cd + Zn. After 28 days of the experiment described here, a significant decrease in the value of TM was observed in the control and experimental groups, which confirms the presence of natural fluctuations in the level of nuclear damage, not only due to exposure to xenobiotics. This observation confirmed the desirability of maintaining the control group during the entire exposure period in long-term studies, and not relating the occurring changes only to the baseline TM value.

There are various testing methods used to detect genotoxins in the aquatic environment, including the erythrocyte micronucleus assay, which also makes identifying other types of structural defects in the cell possible. These abnormalities are related to nuclear and cytoplasmic defects within the cell (21, 34). The EMN assay performed on erythrocytes has been successfully used as an indicator of genotoxic stress in fish caused by heavy metals both in the field and in laboratory conditions (11, 18). Micronuclei are formed as a result of abnormal cell division involving the loss of a fragment or an entire chromosome. The resultant chromatin structures are separated from the daughter nuclei during anaphase, and after cell division become enclosed by a nuclear membrane, forming the so-called micronuclei. Such segregation of chromosomes or their fragments is a sign of genotoxic effects. In the control group of fish there was a fluctuation in the MN frequency at the level of 1.29–3.11%, while in the groups exposed to Zn and Cd for 14 days, it was significantly higher (7.35–16.07%). The present study showed a significant increase in the frequency of MN in the peripheral blood cells of the fish exposed to sublethal concentrations of Zn (over three times higher than the control fish), Cd (over six times higher) and combined Cd and Zn mixture (over eight times higher than the control fish). But after another week, the MN frequency had stabilised at a level close to that in the control group. Abu Bakar *et al.* (1) and Singh *et al*. (36) also demonstrated significant increases in the frequencies of MN in the blood of fish exposed to sublethal concentrations of Cd and Zn. In their study on Nile tilapia (*Oreochromis niloticus*), Abu Bakar *et al.* (1) measured maximum MN frequencies of 0.033 ± 0.057% in the control group, 2.700 ± 0.436% in the group exposed to cadmium, 2.133 ± 0.126% in the group exposed to zinc, and 1.500 ± 0.100% in the group exposed to a Cd and Zn mixture. On the other hand, in the study by Singh *et al*. (36), the frequency of MN formed as a result of exposure to cadmium was 2.75 ± 1.25%, with no micronuclei observed in the control group. Studies by Hussain *et al.* (19) and Naik *et al*. (26) also demonstrated that contamination with heavy metals causes an increase in the frequency of MN in erythrocytes. Hussain *et al*. (19) reported a frequency of 5.0 ± 0.63% for single micronucleus induction and 1.44 ± 0.25% for double micronucleus induction in Rohu labeo from the Chenab River.

Naik *et al.* (26) also observed an increase in the frequency of MN in Mozambique tilapia (*Oreochromis mossambicus*) exposed to cadmium at 7.4 mg/L, 3.7 mg/L and 1.85 mg/L. Just as in the case of DNA integrity defects, MN induction decreased with time of exposure to metals, which may suggest an attempt by the organism to restore the physiological balance despite the persisting environmental pollution. The decrease in the occurrence of the cellular abnormalities of different types with exposure time stemmed from the activation of DNA repair mechanisms in the cells. The activation of these mechanisms occurs upon accumulation of chromatin damage to a sufficiently large extent in cells exposed to a given agent (15). This type of association was observed by Abu Bakar *et al*. (1) in Nile tilapia after exposure to Cd and Zn. The authors of that study found increases in the MN frequency of up to 2.700 ± 0.436% and 2.133 ± 0.126% in the group exposed to Cd and the group exposed to Zn, respectively, followed by decreases down to 1.600 ± 0.229% (Cd) and 1.700 ± 0.229% (Zn). Özkan *et al*. (30) demonstrated a similar relationship in this fish species between this abnormality's occurrence and long-term exposure to Cd.

Assays to detect ENA and ECA have been used to detect the genotoxic potential of various pollutants not only in the field, but also in laboratory conditions (21, 22, 36). In the present study, various kinds of nuclear abnormalities, such as nuclear buds (BUD), binuclear cells (BNC), abnormal shapes of the nucleus (AN), different shapes of notched nuclei (NT), apoptotic cells (A) and abnormal shapes of the cytoplasm (AC), appeared in the erythrocytes of fish exposed to Cd, Zn and their binary mixture at all exposure times. The highest frequency of BUD, A, AN and AC was found in the erythrocytes of fish exposed mainly to cadmium.

Regarding the other two nuclear defect types, a high BNC and NT frequency was observed in the erythrocytes of fish exposed to the Cd Zn mixture. Similar results were reported by Jindal and Verma (21) and Singh *et al.* (36) in the peripheral erythrocytes of Rohu labeo and spotted snakeheads exposed to sublethal doses of Cd. Likewise, time-dependent effects were observed in the erythrocytes of Nile tilapia exposed to Cd, Zn, and combined Cd and Zn (1).

In the present study, the assay to detect ECA revealed considerable variability in the effects exerted by the metals studied, suggesting the involvement of various defence mechanisms affecting the level of their toxicity. When analysing the results obtained, it was demonstrated that the frequency of BUD and NT in the erythrocytes of fish treated with the Cd and Zn mixture was the highest on the 14th day of exposure. In the cadmium group, the peak frequency of AC and AN appeared after 14 and 28 days, respectively. The frequency of all these abnormalities decreased with the exposure time, suggesting that the cells exposed to the toxins activated a pathway of defence mechanisms reducing the number of nuclear and cellular defects. The frequency of such structural abnormalities as BUD and BNC following exposure to Cd, or BUD, A, AC, and AN following exposure to Zn did not significantly decrease by the end of the experiment. Furthermore, the A and AN frequency in the Cd group, and BNC and NT frequency in the Zn group increased with the time of exposure, which may confirm the toxic effect of these metals on fish erythrocytes. On the other hand, in the control group, a decrease in the number of NT-damaged erythrocytes was observed with the passing of the experiment's time, which could be related to the greater repair activity of the damage or possibly to the further transformation of these nuclear changes into micronuclei.

Other authors found much higher values of the studied parameters of genotoxic effects than those observed in the present experiment. In their study on Nile tilapia, Kehinde *et al*. (22) observed nuclear abnormalities in the assay to detect MN with a the frequency of 13.00 ± 1.00% for BNC and 27.00 ± 5.00% for NT as compared to 5.00 ± 1.00% and 3.50 ± 0.50%, respectively, in the control group. A study by Abu Bakar *et al*. (1) conducted on the same fish species revealed that the BNC frequency increased following exposure to metals, from a low of 0.77 ± 0.21% after exposure to combined Cd and Zn, through 0.97 ± 0.16% after treatment with Zn, to a high of 1.07 ± 0.10% after exposure to Cd, as compared to 0.05 ± 0.05% in the control group. As for the other parameter, *i.e.* NT, the authors demonstrated a frequency increase to 1.28 ± 0.27% after exposure to Cd, 1.07 ± 0.08% after treatment with the mixture of Cd and Zn, and 0.67 ± 0.08% after exposure to Zn, as compared to 0.05 ± 0.05% in the control group. Apart from BNC and NT, Omar *et al*. (28) also observed the presence of nuclear buds in the cells of tilapia at the level of 5.75 ± 0.56% BUD/cell at the reference site, and 23.0 ± 2.35% BUD/cell in fish from Lake Qaroun. The study by Singh *et al*. (36), conducted on the erythrocytes of spotted snakeheads exposed to cadmium, demonstrated NT and BUD abnormalities present at 2.00 ± 0.23% for NT (0.50 ± 0.23% in controls) and 2.00 ± 0.28% for BUD (with no such damage identified in the control group).

Cadmium has chemical affinities similar to essential metals such as iron, zinc, and calcium and can enter cells through the mechanism of "ionic and molecular mimicry", thus disturbing the metabolic processes of the organism (5, 9). The reasons for cadmium's toxicity are its ability to disrupt enzymatic systems in cells by means of essential metal ion substitution *(i.e.* Zn^{2+}, Cu^{2+} and Ca^{2+}) and its strong affinity to biological structures containing sulfhydryl groups, such as proteins, enzymes and nucleic acid (14, 33). Simultaneous exposure of the aquatic ecosystem to the mixture of both essential and toxic microelements may produce a different effect from that caused by these agents individually. An additive toxic effect may be exerted as the total impact of multiple toxicants on the organism. A mixture may be more weakly toxic (an antagonistic effect) or significantly more strongly toxic (an synergistic effect) than the sum of the toxic effects of its individual components (16, 29). The analysis of the results obtained in the present study points to different outcomes of fish exposure to the metal mixture used, suggesting the involvement of various genotoxicity mechanisms. The analysis of frequencies of MN and DNA damage in the comet assay suggested the occurrence of a synergistic effect during the simultaneous treatment of fish with Cd and Zn, while the results of the ECA assay indicated an antagonistic effect of these metals. The antagonistic effect observed during the analysis of A, AC or AN frequencies may suggest that the presence of Zn in water reduces the genotoxic effect of Cd on erythrocytes. In this case, zinc is a microelement that can protect the cell against the negative toxic effects of cadmium. Jamarkala and Rani (20) demonstrated that cadmium administered to rats significantly increased the lipid peroxidation level, and made findings of reduced activity of catalase, superoxide dismutase, glutathione peroxidase and glutathione S transferase. In contrast, treatment with Zn and/or Fe caused a significant reversal in the profile of oxidative stress enzymes. In their study on rabbits, Bulat *et al.* (7) suggested that the presence of zinc ameliorated some of the cadmium-induced alterations in tissue bioelement levels. Similar results were obtained by Brzóska *et al.* (6), who indicated that zinc has a protective effect against oxidative stress and its consequences in the brain. Interactions between cadmium and zinc may take place at different stages of the absorption, distribution, and excretion of metals. The analysis of the results obtained in the present study points to different effects of fish exposure to the metal mixture used, suggesting the

involvement of various genotoxicity mechanisms. Thus, further studies are needed to elucidate molecular mechanisms in order to better understand the interaction between the actions of cadmium and zinc.

To conclude, the results of the present study have demonstrated that peripheral erythrocytes are an adequate model for testing the genotoxicity of the elements accumulating in fish. The differences in the frequency of chromatin abnormalities at various levels of its packaging observed in the SCGE and EMN assays following exposure to selected heavy metals and their mixture point to the a genotoxic character of these microelements. Furthermore, it was found that the differences in the pace of chromatin damage formation in the cells studied might result from the individual susceptibility of organisms within a given species to the agent analysed. The chemodynamics and bioaccumulative potential of metals may vary depending on the species, organ exposed, exposure time, and dose. Therefore, an integrated and comprehensive approach, using a set of assays for genotoxicity profile determination, should be adopted during ecotoxicological studies and environmental risk assessment pertaining to these elements.

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