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Monitoring of nuclear abnormalities in peripheral erythrocytes of three fish species from the Goksu Delta (Turkey): genotoxic damage in relation to water pollution

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Abstract Goksu Delta is a specially protected area in the Mediterranean region of Turkey. The delta is classified as a Wetland of International Importance according to the RAMSAR Convention on Wetlands of International Importance. Increases in population have recently taken place in this region due to heavy agricultural activities and discharges of anthropogenic wastes. In the present study, frequencies of erythrocytic nuclear abnormalities such as, micronuclei (MN) and nuclear buds (NB) were investigated in peripheral blood of three fish species; *Clarias gariepinus* (Catfish), *Alburnus orontis* (Bleak), and *Mugil cephalus* (Mullet) from Akgol (AG) and Paradeniz (PD) lagoons of Goksu Delta. Concentrations of heavy metals (Cu, Cd, Ni, Pb) were also measured in the water, sediment samples. MN and NA frequencies were elevated in fish from AG and PD lagoons in comparison with those from upstream regions. The results of this study indicate that the lagoons of Goksu Delta contaminated with genotoxic pollutants and that the genotoxicity is related to the agricultural activities and to the discharge of anthropogenic waste waters.

Keywords Goksu Delta · Lagoons · Nuclear abnormalities · Genotoxicity · Heavy metals

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Introduction

Over the last decades aquatic ecosystems have been contaminated by persistent pollutants, of agricultural and industrial origin (Sanchez-Galan et al. 2001; Cavaş and Ergene Gözükara 2005a, b). Goksu Delta is an internationally important wetland due to its location along a bird migration route, providing refuge to a large number of waterfowl and constituting a breeding range for rare species such as sea turtles (*Caretta caretta*, *Chelonia mydas*) and blue crabs (*Callinectes sapidus*). The Delta is formed by Goksu River near the Southern part of Silifke town in the Mediterranean region, Turkey.

In Goksu Delta fishery is typical production for Akgol and Paradeniz lagoons, and coastal zones of the Mediterranean which are not far away from the beaches. Because the most widespread land use pattern is agriculture in the delta, agricultural inputs caused high levels of contamination within the lagoons of Goksu Delta. Previous studies indicated the presence of heavy metal and pesticide pollution in the area (Ayas and Kolonkaya 1996; Ayas et al. 1997; Ergene and Saraymen 1999). However, there is study on the genotoxic effects of contamination on fish species inhabiting Goksu Delta.

The micronucleus (MN) test, considered to be one of the most useful methods for evaluating genotoxicity in aquatic systems, has been extensively applied on fish species (Al-Sabti and Metcalfe 1995; Çavaş and Ergene Gözükara 2005a, b). Micronuclei are chromosome fragments or whole chromosomes that lag at cell division due to the lack of centromere, damage, or a defect in cytokinesis. Micronuclei are formed by both clastogenic and aneugenic compounds. (Heddle et al. 1991). The simultaneous expression of morphological erythrocytic nuclear abnormalities (NAs) such as nuclear buds (NB) together with micronuclei has received considerable attention. Although the mechanisms underlying the formation of 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 (Serrano-Garcia and Montero-Montoya 2001; Fenech and Crott 2002; Çavaş and Ergene-Gözükara 2005a, b).

The objectives of this study were to determine heavy metals (Cu, Cd, Pb and Ni) in water and sediment samples from Akgol and Paradeniz lagoons of Goksu Delta and to assess their genotoxic effects on three common fish (*Clarias gariepinus*, *Alburnus orontis* and *Mugil cephalus*) species found in the Delta by means of the evaluation of micronuclei and other nuclear abnormalities such as nuclear buds and binuclei formations in peripheral erythrocytes.

Materials and methods

Study area

Goksu Delta is an important wetland (13,000 ha) where the Goksu River reaches the sea in the eastern of town Tasucu-Mersin (Fig. 1). There are two aquatic ecosystems in the Delta. Paradeniz (PD) Lake is a saltwater lagoon connected to the sea and located at the west point of Goksu River. The salinity level of Akgol Lake (AG) is as low as that of freshwater. The shores of lake are 50–200 m wide and covered with dense reed beds. The north of the lakes and eastern part of delta consist of farmland where rice, cotton and peanuts are grown all year. The salt steppes between this farmland and the lakes are inundated in winter forming extensive marshes.

Selected fish species

Three fish species commonly found in the Goksu Delta was selected as sentinels mainly according to their feeding habits. African catfish, *Clarias gariepinus* (Burchell, 1822) belonging to family Clariidae is a benthic species, with high commercial importance, mainly occurs in quiet waters, lakes and pools and feeds on insects, plankton, invertebrates and fish but also takes young birds, rotting flesh and plants (Ergene et al. 1999). Grey mullet, *Mugil cephalus* L., 1758 belonging to family Mugilidae is also a commercially important species. The habitat of *M. cephalus*, which is an omnivore, is benthopelagic, usually inshore, entering estuaries and lagoons (Ergene and Kuru 1999). Bleak fish, *Alburnus orontis* Sauvage, 1882 belonging to family Cyprinidae is a pelagic fish generally occurs in shoals near the surface. Feeds mainly on plankton, including crustaceans and insects (Bogutskaya 1997).

Sampling of fish

Samplings were carried out between March and April 2004. Mullet, bleak and catfish samples were collected Akgol (AG) and Paradeniz (PD) lagoons of the Goksu Delta. Bleak and Mullet samples collected from upstream regions 1 (UPS1) and 2 (UPS2), respectively, were used as control groups. Because Catfish were living only in the Delta, a group of them were transferred and kept in clean water under laboratory conditions for a period of 2 months to constitute a control group.

Analysis of micronuclei and other nuclear abnormalities

Peripheral blood samples were obtained from the caudal vein of the collected specimens and smeared onto pre-

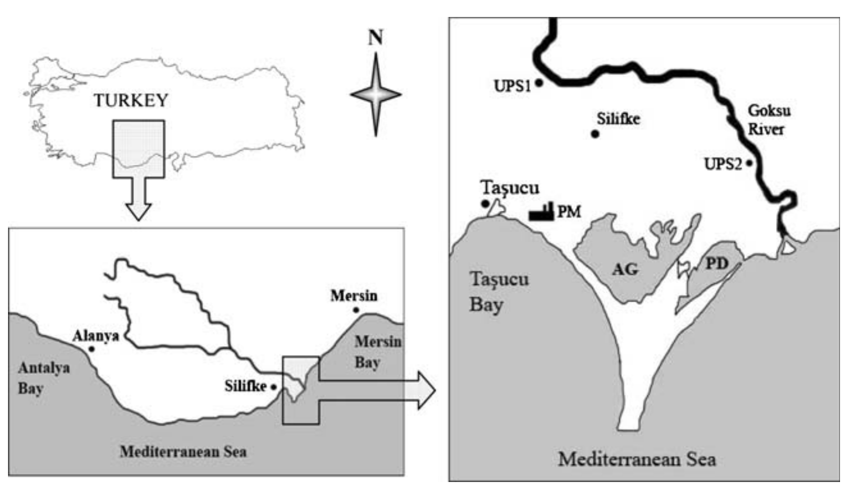

Fig. 1 Map of the study area. *AG* Akgol lagoon, *PD* Paradeniz lagoon, *UPS1* upstream region 1, *UPS2* upstream region 2, *PM* paper mill

cleaned slides. After fixation in pure ethanol for 20 min, the slides were allowed to air-dry and then the smears were stained with 5% Giemsa solution. Three slides were prepared for each fish, and 1,500 cells were scored from each slide under 100× magnification. Small, nonrefractive, circular, or ovoid chromatin bodies, displaying the same staining and focusing pattern as the main nucleus, were scored as mironuclei (Al-Sabti and Metcalfe 1995). Blebbed nuclei had a relatively small evagination of the nuclear membrane and contained euchromatin Carrasco et al. (1990). Total of 45 *Clarias gariepinus*, 50 *Mugil cephalus* and 50 *Alburnus orontis* specimens were collected and analyzed.

Chemical analysis of water and sediment samples

Temperature, pH, and dissolved oxygen of water samples were measured in situ. All the samples were brought to the laboratory in the same day. Surface water samples (about 15 cm) were filtered and analyzed directly. The extraction of metals in the water samples were performed by a 100 mL sample was placed in a volumetric flask, then 5 mL of HNO_3 and 0.5 mL HCl were added. About 4 cm of the top of sediment samples were dried at 105°C for 24 h. The dried sediments were passed through a 60 mesh stainless sieve to remove larger particles. One gram of the sediment sample was dissolved in concentrated nitric acid in a Teflon beaker and small amount of hydro fluoric acid was added. 5 mL of the concentrated HNO_3 was added very slowly and continued heating at 120°C. When the sample solution became liquid, hydrogen peroxide was added and continued heating at the same temperature for 30 min. The hydrogen peroxide was added until the sample remained clear for 2 h at 150°C. After that, the sample was diluted to 100 mL with 2% HNO_3 in a volumetric flask. The instrument was calibrated with standard solutions that were prepared from commercial materials. Deionized water was used. The metal analyses of samples (Cd, Cu, Ni and Pb) were carried out by using Perkin Elmer Atomic Absorption Spectrophotometer (AAS). Absorption wavelength and detection limits were as follows: 228.8 nm and 0.0006 mg/L for Cd; 324.8 nm and 0.003 mg/L for Cu; 232.0 nm and 0.008 mg/L for Ni; 217.0 nm and 0.02 mg/L for Pb, respectively.

Statistical analysis

Multiple comparisons were performed using one-way analysis of variance (ANOVA), followed by the least significant difference test (LSD). Student's t test was used to compare differences between two groups when necessary. The relationship between MN and NB frequencies was analyzed using the regression analysis. Linear regression analysis was used to test correlations between the total heavy metal content of water samples and frequencies micronuclei and nuclear abnormalities. The correlations were made with the sum of all metal concentrations. The method of least squares was used for comparison and Pearson correlation coefficients were calculated. All statistical analyses were performed using SPSS 9 for the PC (SPSS, Chicago, IL, USA).

Results

The age, weight and length values of fish are given in the Table 1. Some hydrological parameters as well as heavy metal concentrations in water and sediment samples from study areas are given in the Tables 2 and 3, respectively. Chemical analysis of water samples indicated that water from the downstream sampling sites Akgol and Paradeniz lagoons had higher concentrations of heavy metals (Cu, Ni, Cd, Pb) than sites from the upper river (UPS1 and UPS2) (Table 2). For comparison, Table 3 also shows the pollutant limits established by the Turkish Water Pollution Control Regulation (2001). Especially downstream sampling sites Akgol and Paradeniz lagoons had Ni, Cd, and Pb concentrations at or in excess of these limits.

The frequencies of micronuclei and nuclear buds in peripheral blood erythrocytes are summarized in Table 4. MN frequencies in *Clarias gariepinus* collected in the AG and PD lagoons were significantly higher than in catfish samples reared in clean water under laboratory conditions ($P < 0.001$). Similarly NB frequencies in catfish showed a significant decline in control group reared under laboratory conditions ($P < 0.01$). MN ($P < 0.01$) and NB ($P < 0.05$)

Table 1 The age, weight and length of fishes caught from the Goksu Delta

Fish	Length (cm)	Weight (g)	Age
Clarias gariepinus	28 (16–56)	267 (960–70)[a]	1–5
Alburnus orontis	14 (11–17)	66 (58–76)	1–2
Mugil cephalus	15 (12–18)	59 (23–109)	1–2

[a] Minimum and maximum values are given in parentheses

Table 2 Some hydrological parameters in the Goksu Delta water

Parameter	TR STD	UPS1	UPS2	Akgöl	Paradeniz
Temperature (°C)	25	18	19	20	22
PH	6.5–8.5	8.0	8.1	8.3	8.1
Dissolved oxygen (mg/L)	8	7.5	13.8	5.1	11.6
Suspended solid material (mg/L)	500	107	89	0.23	0.24

Table 3 The concentration of heavy metals in water (μg/L) and sediment (mg/kg) samples from Goksu Delta and Goksu River (UPS1 and UPS2 regions)

Region	Samples	Cu	Ni	Cd	Pb
Turkish standards	Water	20	20	3	10
UPS1	Water	<1.5	22	3	<0.015
UPS2	Water	<1.5	26	3	<0.015
Paradeniz	Water	<1.5	32	5.5	4.0
	Sediment	2.0	12	8.5	125
Akgöl	Water	<1.5	50	6.5	89
	Sediment	<1.5	15	9	257

frequencies in *Mugil cephalus* collected from the AG and PD lagoons were also significantly higher than in mullets from the UPS2 region. The same relation also observed for *Alburnus orontis* in which the MN and NB frequencies were the highest in AG and PD lagoons and the lowest in the UPS1 region ($P < 0.001$). According to least square estimation analysis, there was a significant linear relationship ($P < 0.05$) between total heavy metal concentrations and MN ($R^2 = 0.473$) as well as NB ($R^2 = 0.450$) frequencies.

Comparison of MN and NB frequencies revealed a strong correlation between the expressions of micronuclei and nuclear buds in peripheral erythrocytes ($R^2 = 0.843$, $P < 0.001$) (Fig. 2). On the other hand, comparative analysis the nuclear abnormalities in different fish species indicated that both MN and NB frequencies were significantly higher in fish from AG lagoon than in fish from PD lagoons ($P > 0.05$) with the exception of NB frequencies in catfish ($P < 0.05$). Furthermore, interspecies comparisons showed that the Catfish had the higher level of frequencies of MN and NB while the Bleak fish had the lowest frequencies in upstream and lagoon areas.

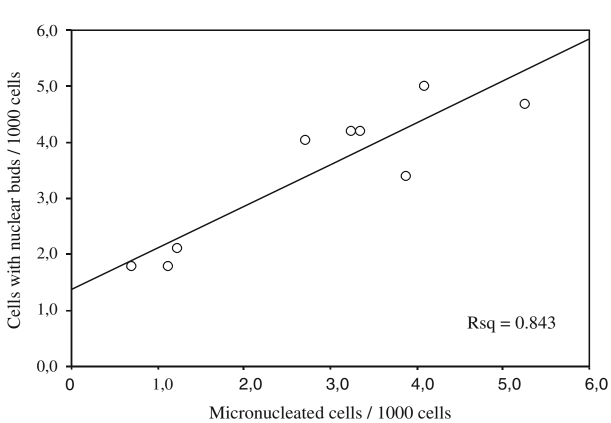

Fig. 2 Correlation between micronucleated erythrocytes/1,000 cells and cells with nuclear buds/1,000 cells. ($R^2 = 0.843$, $P = 0.001$)

Discussion

Lagoons are economically and ecologically important to the area for recreation and fishing activities. However, agricultural inputs cause high levels of contamination within the lagoons. The biogeochemical processes associated with the lagoon can alter the environmental characteristics of these contaminants, thus making them more toxic to aquatic organism (Kjerfve and Magill 1989). The Environmental Protection Department of the Ministry of Environment has declared the Goksu Delta as a Special Environmental Protection Zone to protect the area against pollution and exploitation, and to ensure that natural resources and cultural assets have a future. The delta is classified as a Wetland of International Importance according to the Ramsar Convention on Wetlands of International Importance. The Goksu Delta has also a special significance for being one of the few remaining areas in the world where sea turtles (*Caretta caretta*, *Chelonia mydas*) and blue crabs (*Callinectes sapidus*) lay their eggs (Glen et al. 1997; Ayas et al. 1997).

The concentrations of pollutants in water usually depend on enrichment and dilution phenomena caused by rainfall and drainage of water from irrigation systems. On the other hand low rainfall and low river flows can have an adverse effect through reduced dilution of pollutants, while high rainfall can also increase pollution through greater leaching of pollutants from the soil into rivers, or overflows from the sewerage system. The Goksu River flow regime is also strongly dependent on the seasonal rains and temperature. Average flow of Goksu River is 130 m^3/s where it reaches the highest value during May. Thus, we performed the samplings during March and April.

Analysis of micronucleus frequencies in peripheral erythrocytes of fish from Goksu Delta showed that mullets and bleak fish in AG and PD lagoons had higher MN frequencies than in relatively clean reference areas, UPS1 and UPS2 regions. Because it was not possible to found catfish in upper stream of Goksu River we transferred some of the catfish samples collected from channels of the Goksu Delta to our laboratory and kept them in clean water under laboratory conditions for a period of 2 months to constitute a negative control group for this species. As results significant a significant decrease was observed in fish reared under laboratory conditions indicating the baseline MN frequency would be much lower than determined in the field samples.

In the recent years, considerable attention has been paid the simultaneous expression of morphological nuclear abnormalities (NAs) such as nuclear buds and nuclear blebs and micronuclei in the piscine MN test (Cavas and Ergene-Gozukara 2003; Cavas and Ergene-Gozukara 2005a, b; Da silva Souza and Fontanetti 2006; Matsumoto et al. 2006). It

Table 4 Frequencies of erythrocyte nuclear abnormalities in fish collected from the Goksu Delta

Fish species	Number of fish (n)	Micronucleated erythrocytes (‰)			Erythrocytes with nuclear buds (‰)		
		AG	PD	Control	AG	PD	Control
Clarias gariepinus	50	5.23 ± 0.81^{c,*}	4.10 ± 0.70^{c}	1.10 ± 0.33	4.75 ± 0.085^{b}	4.90 ± 0.090^{b}	1.86 ± 0.39
Mugil cephalus	50	3.86 ± 0.95^{b,*}	3.10 ± 1.05^{a}	1.26 ± 0.40	4.20 ± 0.80^{b,*}	3.33 ± 0.92^{a}	2.10 ± 0.30
Alburnus orontis	50	3.35 ± 0.73^{c,*}	2.70 ± 0.49^{c}	0.68 ± 0.20	4.15 ± 0.50^{c,*}	3.45 ± 0.87^{b}	1.76 ± 0.25

* $P < 0.05$ comparison between fish from AG and PD lagoons

[a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$ compared to negative control group

has been suggested that problems in segregating tangled and attached chromosomes or gene amplification via the Breakage–Fusion–Bridge cycle could cause lobed nuclei or blebbed nuclei during the elimination of amplified DNA from the nucleus (Tolbert et al. 1992; Shimizu et al. 1998, 2000). In the present study we found significantly elevated NB frequencies in peripheral erythrocytes of all three fish species captured from the lagoons of Goksu Delta and observed that the expression of NB were strongly correlated with the MN induction.

Comparative analysis of MN and NB frequencies showed significant interspecies differences where catfish had the highest values and the bleakfish had the lowest values. Differences in nuclear abnormalities could, however, be attributed to the different feeding habits of the two species, as uptake of metals via the food plays an important role. For example, Kargin (1996) stated that due to variations in feeding habits, habitats and behaviour of species, the levels of metals found in tissues of the benthic fish were always higher than those found in pelagic fish. Romeo et al. (1999) pointed out that cadmium, copper, mercury and zinc concentrations in edible muscles of pelagic fish species are lower than for benthic fish species. Thus, differences between the feeding patterns of *C. gariepinus*, *M. cephalus* and *A. orontis* could be the main reason for the significant differences between these three species.

The elevated heavy metal levels of various environments and organisms (soil, water, sediment, fish and waterbird) in the Goksu Delta were reported in previous works (Sanin et al. 1992; Ayas and Kolonkaya 1996; Ergene and Saraymen 1999). As indicated by Alloway (1995) the heavy metal sources of intensive farming regions could be mineral fertilizers (Cd, Cr, Mo, Pb, U, V, Zn) and pesticides (Cu, As, Pb, Mn, Zn). Obtained data showed that about 94 ton pesticides and 431 ton mineral fertilizers were used within 1 year at Goksu Delta (Cetinkaya 1996). Although the genotoxins present in the Goksu Delta were not extensively characterized in this study, some chemical analyses were performed, such as pH, dissolved oxygen (DO), and heavy metal content (Tables 2, 3). Analysis of water samples revealed elevated levels of Pb, Cd and Ni in the lagoons of Goksu Delta in comparison with the upstream of Goksu River. Pb, Cd and Ni have been previously shown to cause genotoxic effects.

The mechanisms of Pb genotoxicity may involve indirect damage to DNA affecting the stabilization of chromatin (Johansson and Pellicciari 1988) or by the interaction of the metal with repair processes (Hartwig et al. 1990). Also, Bonacker et al. (2005) reported that Pb salts may induce micronuclei by disturbing microtubule function. It was suggested that Cd genotoxicity is mainly manifested as single strand DNA breaks generated by direct Cd-DNA interactions as well as by the action of incision nucleases and/or DNA-glycosylases during DNA repair (Privezentsev et al. 1996]. Ni compounds generate specific structural chromosomal damage as well as micronuclei (Sen et al. 1987; Arrouijal et al. 1990). In addition to chromosomal damage, DNA-protein crosslinks and oxidative DNA base damage were observed in Ni(II)-exposed cells (Patierno et al. 1985; Kasprzak 1991). Also, Ni genotoxicity may be potentiated through the generation of DNA-damaging reactive oxygen species and the inhibition of DNA repair by this metal (Kazimierz et al. 2003).

Induction of micronuclei was reported previously for fish exposed to Cd (Ayllon and Garcia Vazquez 2000; Sanchez-Galan et al. 2001; Arkhipchuk and Garanko 2005; Cavas et al. 2005), and Pb (Cestari et al. 2004; Ferraro et al. 2004). Therefore, it is plausible that elevated concentrations of heavy metals could have contributed to the elevated MN frequencies in fish from the lagoons of Goksu Delta. On the other hand, it should be noted that the increased erythrocyte micronucleus frequencies in fish from Goksu Delta could also be due to other genotoxic chemicals that may present in the water.

In conclusion, our results suggest that higher frequencies of micronuclei and nuclear buds determined in peripheral erythrocytes of *Clarias gariepinus*, *Mugil cephalus* and *Alburnus orontis* from Akgol and Paradeniz lagoons of the Goksu Delta may be indicative of damage caused by pollutants in these lagoons. The genotoxic effects were generally correlated with heavy metal content of water samples. These results highlight the genotoxicity of the pollution in this area, and suggest that chemical contaminants originated from domestic and agricultural activities in the Goksu Delta may place local populations at risk of disease. The positive and significant relationship between MN and NB induction suggests that NB formations in erythrocytes may be a useful complementary assay for genotoxicity analyses when fish are used as experimental animals. Our results further demonstrated the sensitivity and suitability of three fish species as sentinel organism in environmental genotoxicity assessments. Finally, we recommend that Goksu Delta water be analyzed chemically for additional classes of toxicants to identify other contaminant(s) that may be responsible for the genotoxic effects and prolonged genotoxicity monitoring studies on other feral organisms be performed.

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