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RESEARCH ARTICLE **ARAŞTIRMA MAKALESİ**

Evaluation of DNA damage by Comet Assay in populations of endemic Beyşehir frog *Pelophylax caralitanus* (Arıkan,1988)

Endemik Beyşehir kurbağası *Pelophylax caralitanus* (Arıkan,1988) populasyonlarında Comet Analizi ile DNA hasarın değerlendirilmesi

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Abstract: In study this, the level of DNA damage in three populations of endemic Beyşehir frog (*Pelophylax caralitanus*) inhabiting on Lakes of Karamık, Eber and Beyşehir with different anthropogenic pollution was assessed by using the DNA Comet Assay technique. Frog erythrocytes cells were used for the analysis. Seven adult individuals were collected from each biotope containing minimum 100 cells were analysed. While the results showing a significant increase in the DNA comet index in the contaminated zone [(Eber Lake= Idc=0.22±0.015), ($p<0.05$)], the majority of the cells did not contain any DNA damage in the clean zone (Karamık Lake, Idc=0.08±0.001). The obtained data demonstrated t that the endemic Beyşehir frog could serve as a useful flag species in an indicator of anthropogenic impact on ecosystems.

Keywords: *Pelophylax caralitanus, endemic Beyşehir frog, DNA damage, comet assay, anthropogenic impact*

Öz: Bu çalışmada, DNA Comet Assay tekniği kullanılarak, farklı antropojenik kirliliğe sahip Karamık, Eber and Beyşehir *Göllerinde yaşayan endemik* Beyşehir kurbağası (*Pelophylax caralitanus*) üç populasyonunda DNA hasar düzeyleri değerlendirilmiştir. Analizler için kurbağa eritrosit hücreleri kullanıldı ve uygun biyotoplardan toplanılan 7 ergin bireyin, her birinden minimum 100 hücre ile analiz edilmiştir. Sonuçlar DNA comet indeksinde kontamine olmuş zonda [(Eber Gölü = Idc=0.22±0.015), ($p<0.05$)] artış olduğu gösterirken, temiz zonda, hücrelerin çoğunluğunun hasarsız DNA içerdiğini (Idc = 0.08 ± 0.001) göstermiştir. Elde edilen veriler, endemik Beyşehir kurbağasının ekosistemler üzerindeki antropojenik etkinin bayrak türü göstergesi olarak kullanılma olasılığını göstermektedir.

Anahtar Kelimeler: *Pelophylax caralitanus, endemik Beyşehir kurbağası, DNA hasarı, comet assay, antropojenik etki*

INTRODUCTION

In last few decades, intensive industrial developments have led to a dramatic increase in the concentration of numerous chemicals in the aquatic and terrestrial ecosystems that pose a potential danger to the habitat of all living beings including human beings. Anthropogenic activities such as modern farming methods, urbanization, industrialization that involve the usage of different chemical pollutants and toxicants including heavy metals, biocides, pesticides, industrial effluents etc. which ultimately reach into aquatic environments and become responsible for the degradation of aquatic ecosystem (Dautremepuits et al., 2004; Marquis et al, 2009). These pollutants contaminate and accumulate in areas such as small bays, lakes, and seas by transportation via various water connections and flows at under/over ground level. Many of these pollutants contribute to aquatic environmental degradation and in turn results biomagnifications in aquatic organisms and as well as in the consumer of aquatic products like humans. Thus, they are harmful for the health of both human and other animals (e.g. Kalay et al., 1999; Ashraf, 2005).

Amphibians are good bioindicators of environmental pollution due to their susceptibility to chemicals during their freshwater cycles. This may

be due to their sensitivity to changes of their habitat, and to the fact that their larvae inhabit aquatic environments. In addition, the presence of gills and a thin skin are both attributes of larval and adult that serve amphibians well as model organisms for study (Pollet and Bendell-Young, 2000; Huang et al., 2007; Erismis et al., 2013).

DNA damage by environmental xenobiotics is frequently assessed by single cell gel electrophoresis (SCGE) or the Comet assay (Singh et al., 1988), which detects DNA strand breakage and alkali-labile sites by measuring the migration of DNA from immobilized individual cell nuclei. In this assay, the cells are embedded in agarose gel on microscopic slides, lysed and then electrophoresed under alkaline condition. Cells with damaged DNA show increased migration of DNA fragments from the nucleus and the length of the migration indicates the amount of DNA strand breakage; the latter can be estimated by manual and computerized image scoring procedures (Kumar et al., 2013). The technique is highly sensitive for detecting DNA damage in any eukaryotic cell type and requires only a few cells. The minimum technical requirements for doing this assay in vitro and in vivo are well established (Cotelle and Férard, 1999; Hartmann et al., 2003). The Comet assay is sufficiently sensitive for detecting DNA damage in frogs (Dhawan et al., 2009). Endemic Beyşehir frogs (*Pelophylax caralitanus*) is a recently described species (Arıkan, 1988) in the Lake District of south-western Anatolia. Historically, it has been a common inhabitant of lakes and ponds at elevations of 950-1200 m (Arıkan, 1988; Budak et al., 2000; Jdeidi et al., 2001), ranging from the Konya plain to Denizli. Ongoing threats to the survival of this species include habitat loss and over-harvesting by commercial collectors, principally for the western European frogleg market. While it is remain locally abundant at some sites, overall the Beyşehir frog population is in rapid decline and is now considered an endangered species [(IUCN International Union for Conservation of Nature)-(the IUCN Red List of threatened Species - 2016)]. Although a number of studies have investigated the distribution, demographic studies, morphometry, serology, The fungal pathogen (*Batrachochytrium dendrobatidis*) and demographic data of *P. caralitanus* (e.g., Arıkan, 1988; Budak et al., 2000; Kaya et al., 2002; Erismis and Chinsamy, 2010; Erismis et al., 2013; Erismis, 2018), evaluation of DNA damage detected by micronuclei and comet assay of Turkey anurans are few (Gürkan et al., 2012; Erismis et al., 2013). The aim of the present study was to develop the first information concerning the possible biological effects of pollution from clastogenic chemicals in three lakes on endemic Beyşehir Frog (*P. caralitanus*) in Lake District of south-western Anatolia, Turkey.

MATERIALS AND METHODS

Twenty-one adults (11 males, 9 females) were collected by hand, mainly from the middle of July through the end of August 2018, which corresponds to the period of annual activity of the species studied. Blood samples from each adult frog were taken using a heparinized syringe and needle via cardiac puncture. After performing the blood sampling, the

frogs were freed to their natural habitats. Twenty-one adult endemic Beyşehir frogs were selected from three lakes of Lake District of south-western Anatolia (Figure 1) for the analysis which differed in the level of anthropogenic load.

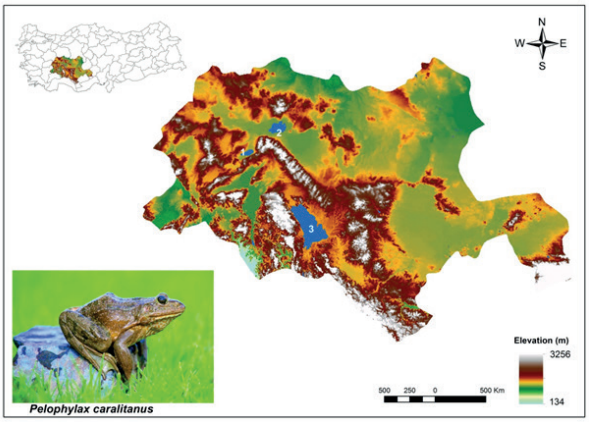

Figure 1. The map of the sampling localities; 1. Karamık Lake, 2. Eber Lake, 3. Beyşehir Lake

Characteristics of the three lakes studied are; 1. Karamık Lake (38°26'50"N 30°53'17'E; 1020 m asl) is located in the southeast of the city of Afyonkarahisar, in the west of central Anatolia region of Turkey. The wastewater of Afyonkarahisar - Çay SEKA paper factory was emptied to the lake in the past. After the year 2004, this factory was closed, and the waste water was prevented. 2. Eber Lake (38°37'53"N, 31°6'37"E; 967 asl), an "A" class wetland of south-western Anatolia, is very important for ornithology, fisheries and the native crayfish. But the structure of Eber Lake has been affected by industrial pollution from the both alkaloid and enamel factories located near it. 3. Beyşehir Lake (37°42'37"N, 31°27'10'E; 1125 m asl) is ecologically vulnerable and taken under protection as a "national park" however, domestic and industrial sewage collection and treatment is insufficient contributing to contamination of surface and ground water resource.

Single-cell electrophoresis (Comet assay) procedure and determination of DNA damage

Blood samples from each adult frog were taken using a heparinized syringe and needle via cardiac

puncture. After performing the blood sampling, the frogs were freed to their natural habitats. The samples of 5-10 µL venous blood from the frogs were collected and examined to determine if any DNA damage had occurred. Single-cell suspensions were prepared by diluting whole blood with phosphate buffered saline (PBS) (1:200, v/v) and were utilized immediately during the analyses. Then, the whole blood samples of 0.5 µL were mixed with 100 mL of 0.5% low-melting agarose in PBS at 37 °C. Subsequently, 80 mL of this mixture was layered onto a slide pre-coated with a thin layer of 1 % normal melting point agarose, covered immediately with a coverslip and stored for 5 min at 4 °C to allow the agarose to solidify. After removing the coverslips, the slides were immersed in freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100 mM EDTA-Na_2; 1 % Na-laurylsarcosine, 10 mM Tris–HCl, pH 10-10.5; 1 % Triton X-100 with 10 % DMSO being added just before use) for at least 1 h. The slides were then electrophoresed (25 V/300 mA, 25 min) after they were immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/L NaOH and 1 mmol/L EDTA-Na_2, pH 13) at 4 °C for unwinding (40 min). Experimental treatments were carried out under minimal illumination. After the electrophoresis application, the slides were neutralized (0.4 M Tris-HCl, pH 7.5) for 5 min. Each slide was stained with ethidium bromide (20 lg/mL) and covered with a coverslip. Slides were stored at 4 °C in humidified sealed containers until analysis. The nuclei were ranked by the four stages of DNA destruction. At least 100 nuclei were counted on each specimen (Fig. 2). Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells, cells near or in a trapped air bubble and superimposed comets. Results are reported as the mean ± SE. The Student-t test for paired samples was used for statistical analysis. When more than two means were compared, one-way ANOVA was performed, followed by the Duncan post-hoc test. The level of significance was set at $P<0.05$, sometimes expressed as DNA comet index (I_{dc}), using the formula: Idc = (0n0 + 1n1 + 2n2 + 3n3 + 4n4) / Σ, where n0-n4 is the number of "DNA comets" of each type, Σ is the sum of the counted "DNA comets".

RESULTS AND DISCUSSION

Blood cells with either no damage or varying degrees of damage are shown in Fig. 2. Four types of damaged DNA and non- damaged DNA in the blood cells of Beyşehir frog living in three study lakes (Fig. 2a-e) were observed. Apoptotic cells were observed (Figure 2f) but not evaluated, since they represented dead cells (Olive et al., 1993).

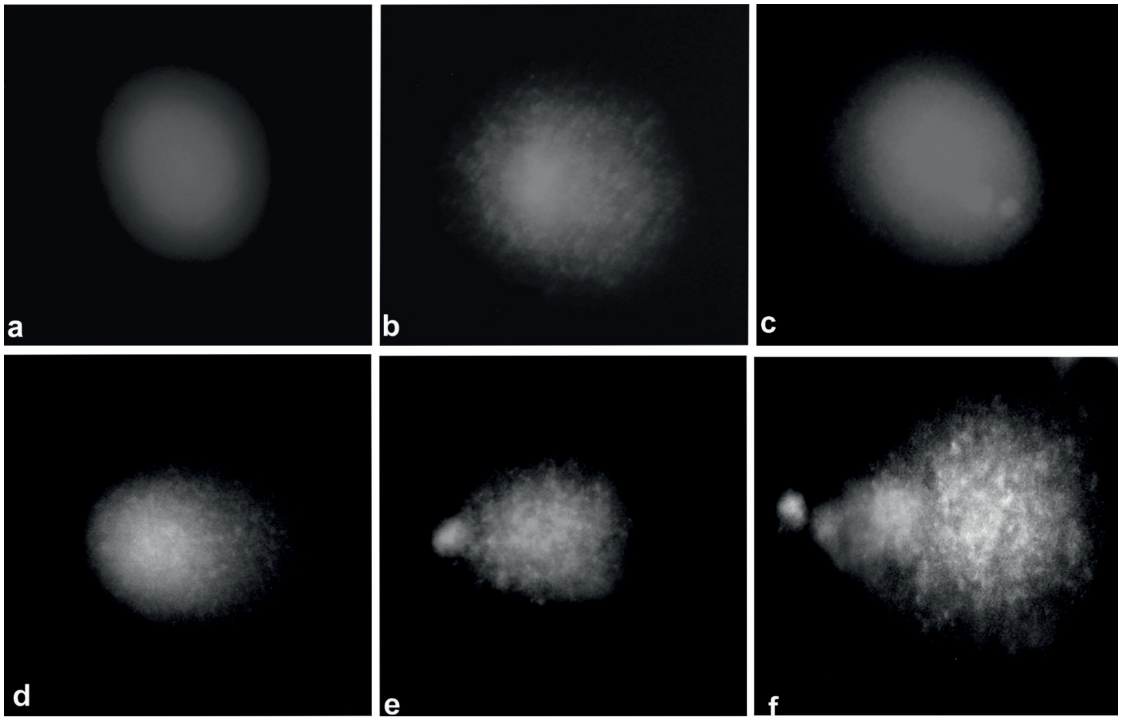

Figure 2. The classification of *Pelophylax caralitanus* blood cells by the comet assay. A: undamaged DNA; B-D: DNA damage; E: Maximum DNA damage; F: Apoptotic cell. Bar = 10µm.

In the comet assay, DNA fragments move from the nucleoid core towards the anode, resulting in 'comet' formation. The results were summarized in Table 1.

Cells sampled from Karamık Lake, in most cases, had intact nuclei and the nuclei with the third and fourth stages of DNA destruction were rarely found (Table 1). According to the information obtained in this study, due to less industrial and agricultural pollution of this lake, the level of DNA damage in the studied blood of Beyşehir frogs was considered negligible, because the average I_{dc} values reached the first stage of damage. The average value of the DNA comet index for Karamık Lake was I_{dc} = 0.089 ±0.005. In this regard, the present study indicated that the value of DNA damage was the smallest among the studied lakes, despite dwelling in an obviously polluted habitat by SEKA paper factory before 2004. This finding may indicate the absence of severe damaging factors disturbing the cytogenetic stability at the collection sites. However, it may also suggest that there are some kinds of repair mechanisms in homeostatic processes which can neutralize the detrimental effects of environmental components in the animals. Nevertheless, according to our data, certain tendencies occur towards an increase in the amount of damaged DNA. Thus, one factor ANOVA, performed using the aggregate of all studied blood of Beyşehir frog in three lakes revealed significant differences between the studied groups in the DNA comet index (ANOVA = 53.22; df = 2;18, $P_{0.00}$<0.05).

During the experiment, an increase was observed in the degree of DNA damage under the influence of industrial pollution from the alkaloid, enamel factories and weapon factories located in the vicinity of Eber Lake and Beyşehir Lake respectively. I_{dc} values of DNA damage in the collected Beyşehir frogs were analysed statistically. The blood of Beyşehir frogs in Eber Lake, which was the one of the considered areas in this study, provided the highest Idc value of DNA damage (Idc = 0.227 ±0.015). Results showed that I_{dc} values of DNA damage were statistically significant between Eber Lake and Beyşehir Lake (Student's t test = 3.83; $P_{0.002}$< 0.05). These results could be occurred due to both the alkaloid factory pollution and the enamel factory pollution. Despite the protected status of Eber Lake, fertilizers and pesticides can be drained into the surrounding lands because Eber Lake is adjacent to the agricultural fields in the Akarçay basins. A similar pattern of increasing proportion of damaged DNA was also observed in the Beyşehir Lake, probably due to the same cause.

As an environmental biomonitoring tool, the comet assay has been used increasingly by various scientists globally to assess and establish the genotoxic effects of xenobiotics on aquatic organisms (Rajaguru, 2001; Huang et al., 2007; Ismail et al., 2014). The comet technique used here, also known as single-cell gel electrophoresis (the SCGE technique), is a simple, fast, sensitive, and widespread protocol that has been used in recent years to measure and evaluate DNA damage in different cell types even with many organisms (e.g Devaux et al., 1998; Wang and Jia, 2009; Snegin, 2011a). One of the most important advantages of the technique is that it can be used with virtually all types of cells, as well as being applicable to amphibian species. Many studies carried out on amphibian species in fresh waters as well as in terrestrial can be found in the literature (e.g. Ralph and Petras, 1998; Ferreira et al., 2004; El-Zein et al., 2006; Vershinin, 2008; Wang and Jia, 2009; Dhawan et al., 2009; Zhelev et al., 2014). In this study, we found that cells from the adults of endemic Beyşehir frogs from Karamık Lake which was cleaner than the other two lakes had compressed DNA and maintained the circular form of their normal nucleus, with little or no evidence of comet formation (Fig. 2a). In contrast, blood cells from frogs living in three polluted water bodies displayed an altered appearance (Fig. 2b-e). To our knowledge, this is the first study carried out on the DNA damage in populations of Turkish endemic frog (*P. caralitanus*) in different lakes of the Lake District of south-western Anatolia. Many studies revealed that, the contaminants were the major concern in amphibians because many populations were declining dramatically and the death of entire populations increasingly occurred frequently, in some cases, due to anthropogenic changes in the environment (Pollet and Bendell-Young, 2000; Marquis et al., 2009; Erismis et al., 2013; Zhelev et al., 2014). The findings of this study are in parallel with the literature report that the amphibians are very sensitive to pollution, because they live at the interface of two environments land and water and can easily absorb pollutants through the skin. Therefore, the present study confirms that the endemic Beyşehir frogs in Lake District of south-western Anatolia, is good bio-indicators of to assess the genotoxicity and they may also serve as an early warning of natural responses to environmental contaminants

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Table 1. DNA damage indicators in the studied groups of *Pelophylax caralitanus; N:* Number of cells analysed, Na: Number of apoptotic cells

Lake	Indicators	Number of animals						
		1	2	3	4	5	6	7
Karamık Lake	N	103	106	108	114	123	102	112
	Na	0	0	0	0	0	0	0
	0 stage	97	98	103	106	118	97	108
	1 stage	3	6	3	6	2	3	2
	2 stage	1	1	-	-	2	2	1
	3 stage	2	-	2	2	-	-	-
	4 stage	-	1	-	-	1	-	1
	I_{dc}	0.106	0.113	0.083	0.105	0.081	0.068	0.071
Eber Lake	N	136	109	113	127	113	113	111
	Na	0	1	0	0	1	0	1
	0 stage	120	96	98	115	104	96	97
	1 stage	9	8	12	6	6	9	8
	2 stage	3	2	-	2	2	3	1
	3 stage	2	-	1	1	4	3	3
	4 stage	2	3	2	3	1	2	2
	I_{dc}	0.213	0.220	0.203	0.196	0.230	0.283	0.243
Beyşehir Lake	N	118	120	108	111	114	104	110
	Na	0	1	1	0	0	0	0
	0 stage	109	108	98	96	105	96	103
	1 stage	3	8	5	10	4	5	3
	2 stage	2	-	2	4	1	1	1
	3 stage	3	3	-	1	-	2	1
	4 stage	1	1	3	-	4	-	2
	I_{dc}	0.169	0.175	0.194	0.189	0.192	0.125	0.145

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