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MICRONUCLEUS TEST IN PERIPHERAL ERYTHROCYTES OF *Rana ridibunda* AS AN INDICATOR OF ENVIRONMENTAL POLLUTION

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ABSTRACT

Clastogenic effects of industrial and agricultural contaminants of the Porsuk River flowing through Kütahya and Eskişehir cities were investigated in peripheral erythrocytes of *Rana ridibunda*. Examination of blood smears showed that the formation of micronuclei was 5-6 times more abundant in the frogs from industrial sites where nitrogen, textile and sugar factory were located than in the animals from the least polluted sites of the river. This increase in the formation of micronucleus indicates that industrial and agricultural pollution exerts clastogenic effects on organisms and may has similar effects on the human population located around the region.

Key Words: Micronucleus test, Frog peripheral erythrocytes, Environmental genotoxicity.

ÇEVRE KİRLENMESİNİN BİR İNDİKATÖRÜ OLARAK *Rana ridibunda*' NIN PERİFERAL ERİTROSİTLERİNDE MİKRONÜKLEUS TESTİ

ÖZ

Eskişehir ve Kütahya illerinden geçen Porsuk çayında sanayi ve tarımsal kirleticilerin klastojenik etkileri, *Rana ridibunda*'nın periferal eritrositlerinde araştırılmıştır. Kan yayma preparatlarının incelenmesi, mikronükleus oluşumunun, azot, tekstil ve şeker fabrikalarının bulunduğu sanayi bölgelerinden alınan kurbağalardan nehrin en az kirli kısımlarından alınanlara göre 5-6 kez daha fazla olduğunu göstermiştir. Mikronükleus oluşumundaki bu artış endüstriyel ve tarımsal kirlenmenin organizmalar üzerinde klastojenik etkiler gösterdiğini, dolayısıyla bölgede yaşayan insan topluluğu üzerinde de benzer etkilerin olabileceğini göstermektedir.

Anahtar Kelimeler: Mikronükleus testi, Kurbağa periferal eritrositleri, Çevre genotoksisitesi.

1. INTRODUCTION

The micronucleus test, an *in vivo* and *in vitro* short-term screening test, developed by Schmid (1975) and Heddle (1973) is widely used to detect genotoxic effects (He et. al., 1998; Villarini et al., 1998). It is one of the simple, reliable, least expensive and rapid screening system for both clastogenic (chromosome breakage, formation of acentric fragments) and aneugenic (chromosome lagging and effects on spindle) effects (Heddle et al., 1983; Orhon and Menevşe, 1986). Clastogenic and aneugenic agents affect the spindle apparatus, which can be differentiated on the basis of the relative induced micronucleus sizes or with the presence of kinetochores (Heddle et al., 1983; Yamamoto and Kikuchi, 1980; Heddle et. al., 1991). In anaphase, any chromosome fragment or whole chromosomes which lack a centromere may not be integrated in the nucleus, because of the lack of an indispensable element for orientation in the spindle apparatus. After telophase, the fragments or whole chromosomes give rise to one or several secondary nuclei which are smaller than the main daughter nucleus and are therefore called micronuclei (Schmid, 1975; Heddle et al., 1983).

The usefulness of the micronucleus test for mutagenicity screening has been well established in several systems i.e. ovary, bone marrow, epithelial tissue, peripheral blood, liver, exfoliated buccal cells and fetus cells of several laboratory animals or human (Orhon

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and Menevşe, 1986; Heddle, 1990; Krishna et al., 1991; Hayashi et al., 1992; Catena et al., 1994; Konopacka, 1994; Agarwal et al., 1994; Emecen and Ünlü, 1995; Ghose and Parida, 1995; Ludwikow et al., 1996; Hrelia et al., 1996). Micronuclei formation can be occured in any dividing tissue of any species (Heddle et al., 1983) as shown by the values of spontaneous micronucleated erythrocytes (MNE) of some laboratory animals and mammals (Zuniga et al., 1996). Some organisms such as plants, fish, birds and frogs were also investigated by micronucleus test to detect environmental pollution capable of producing genotoxic damage (Ma et al., 1984; Bhunya and Jena, 1993; Zhuleva and Dubinin, 1994; Chu et al., 1996).

In the present study, we have investigated the clastogenic effects of industrial and agricultural contaminants of a river on the formation of micronucleus in erythrocytes of peripheral blood of lake frog *Rana ridibunda*. Porsuk River is heavily contaminated by many factories such as nitrogen, textile, sugar, some others and agricultural facilities along Kütahya and Eskişehir cities (Yücel et al., 1995; Anon, 1991; 1992). However, the mutagenicity of pollutants of Porsuk River has not been investigated. Therefore, it was a great interest to examine the mutagenicity of water at various sites in the area where water is either used as drinking water or consumed directly by the people living around it. Simultaneously, the influence of mutagenic contaminants of the water on the human gene pool in the region was analyzed.

2. MATERIALS AND METHODS

2.1. Animals

The frogs, *Rana ridibunda*, for the present study were trapped from four different aquatic localities in Porsuk River flowing through Kütahya and Eskişehir cities. The animals were collected in June 1996. Animals from village of Gelin Kayası, 10-Km southwest of Kütahya, were used as controls, because of this region of the river is the least affected area from environmental pollutant (Yücel et al., 1995). The second station was near the location of nitrogen factory in Kütahya where the waste of factory is thrown into the river. The third station was near the Sümerbank textile factory in Eskişehir, and the fourth station was near the Eskişehir sugar factory where the waste of them is released into the water. Finally, animals were trapped from the pool at Anadolu University as a second control. Approximately ten frogs were collected from each station and weighed in the laboratory. Heavier frogs were identified as female.

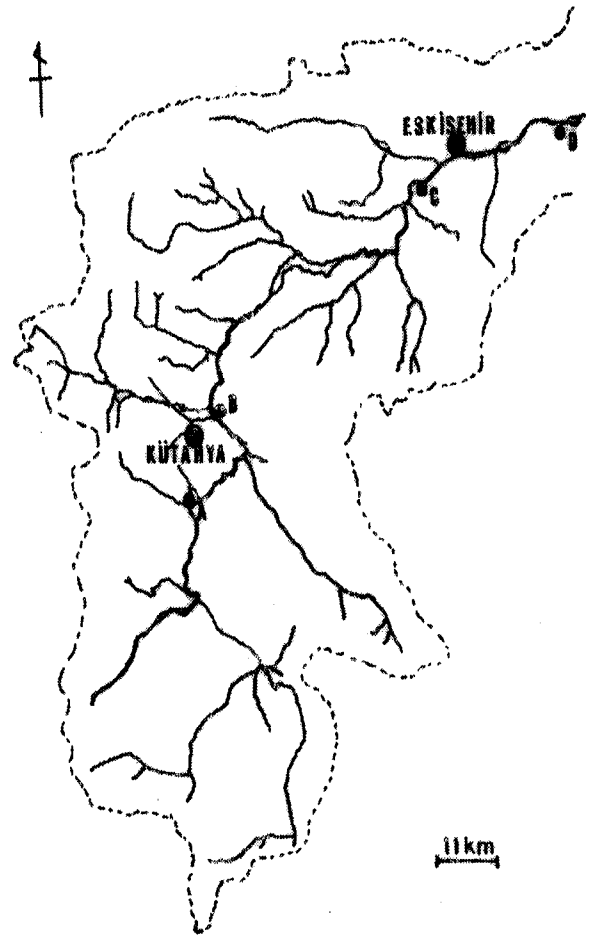

Figure 1. Sites Tested for Genotoxicity In The Porsuk River Flowing Through Kütahya and Eskişehir Cities. A, Village of Gelin Kayası; B, Nitrogen Factory; C, Textile Factory and D, Sugar Factory.

2.2. PREPARATION FOR MICRONUCLEATED ERYTHROCYTES

Animals were killed after etherizing at the same day and venous blood was collected from the each animal using syringes containing 4:1 heparine: distilled water. Blood smears were made on precleaned microscope slides, and air-dried. Slides were stained with May-Grünwald (Sigma) and Giemsa (Merck) based bloodstain according to Mohr (1981).

The frequency of MNE was manually scored through an immersion objective lens. Only well-individualized intact cells with distinc nuclear and cellular membranes were scored. The criteria for scoring and identification of MN were similar to earlier studies (Schmid, 1976; Heddle et al., 1983; Zhuleva and Dubinin, 1994; Mersch et al., 1996): (1) they are spheric cytoplasmic inclusions with a sharp contour, (2) their diameter ranges between one-tenth and one-third of the nucleus, (3) they resemble the nucleus in both texture and colors, (4) they have no contact with the nucleus. For each individual, 2000 erythrocytes were scored and

the mean was presented as its own value, then the mean values of all individual animals of each group was presented as the final value for that group. The frequencies of MNE were determined and results were analysed by using Student's t-test.

3. RESULTS AND DISCUSSION

Animals were collected at four different sites along the Porsuk River flowing Kütahya and Eskisehir as shown in Fig.1, and tested for the mutagenicity of the water using micronucleus test in peripheral blood cells.

Table 1. The Result of Micronucleus Assay In Peripheral Erythrocytes of Frogs (Rana Ridibunda) Collected From The Four Different Locations of Porsuk River.* Animals Collected From The Pool at Anadolu University. Animals Collected From Gelin Kayası on Porsuk River. A: One Micronucleus Per Cell, B: Shoot-Shaped Nuclear Material, C: 2 Micronuclei Per Cell, D: 3 Micronuclei Per Cell. MNE: Micronucleated Erythrocytes; MN: Micronucleus.**

Frog no. control	Weight (g)	MNE/ 2000 ery	Type of MN				MNE/ery %
			A	B	C	D	
1	20.0	14	10	4	-	-	0.7*
2	20.5	10	6	4	-	-	0.5*
3	22.5	16	14	2	-	-	0.8*
4	23.0	24	17	8	-	-	1.2*
5	29.7	8	6	2	-	-	0.4**
6	30.3	10	10	0	-	-	0.5**
7	31.3	14	8	6	-	-	0.7**
8	35.0	12	12	0	-	-	0.6**
9	41.8	8	6	2	-	-	0.4**
10	45.6	18	10	8	-	-	0.9**
Nitrogen fact.							
1	22.7	61	47	14	-	-	3.05
2	23.5	60	38	22	-	-	3.0
3	29.5	50	32	18	-	-	2.5
4	31.0	76	58	18	-	-	3.8
5	35.3	60	40	18	1x2	-	3.0
6	36.3	104	68	36	-	-	5.2
7	43.8	162	48	114	-	-	8.1
8	50.1	86	52	34	-	-	4.3
9	100.5	158	100	58	-	-	7.9
10	112.3	150	86	60	2x2	-	7.5
Textile fact.							
1	70.1	58	52	4	1x2	-	2.90
2	70.1	58	52	4	1x2	-	2.90
3	72.8	65	51	12	1x2	-	3.25
4	73.4	53	34	19	-	-	2.65
5	73.5	53	36	17	-	-	2.65
6	75.4	66	36	28	1x2	-	3.30
7	75.8	57	46	8	-	1x3	2.85
8	82.3	76	52	24	-	-	3.80
9	123.0	112	70	30	3x2	2x3	5.60
10	130.5	71	58	13	-	-	3.55
Sugar fact.							
1	20.6	48	40	6	-	-	2.4
2	23.0	52	36	16	-	-	2.6
3	27.2	50	34	16	-	-	2.5
4	32.0	62	44	18	-	-	3.1
5	33.0	76	32	44	-	-	3.8
6	40.3	60	36	24	-	-	3.0
7	43.2	68	44	24	-	-	3.4
8	45.8	62	42	20	-	-	3.1
9	55.7	60	26	34	-	-	3.0
10	105.3	100	64	36	-	-	5.0

The results of the micronucleus assay in peripheral blood erythrocytes of frogs are shown in Table 1. Animals collected from the affected areas had increased MNE frequencies. Results of micronucleated erythrocytes were also expressed as the mean of all animals of each group as shown in Table 2. Frequency of total micronucleus was very low in control animals from Gelin Kayası which is the least polluted region of Porsuk River and the pool at Anadolu University (0.58 % and 0.80 % respectively). The most affected animals were from near the nitrogen factory (MNE was 4% of erythrocytes, $P<0.01$), and then textile and sugar factories (3%, $P<0.05$) (Table 2).

Table 2. Summary of Experimental Results. *:P<0.01 and **:P<0.005 Compared With Control Results Using Student's t-test.

Locations	Number of animals	Total numbers of ery. counted	MNE/ ery.counted	MNE/ ery. %
Anadolu Unv.	4	8000	64	0.38
Gelin Kayası	6	12000	70	0.58
Nitrogen factory	10	20000	963	4.81*
Textile factory	10	20000	668	3.34**
Sugar factory	10	20000	638	3.19**

Micronuclei formation also showed variation in their shapes and the number per cell as shown in Fig. 2. The formation of one micronucleus per cell (A) was found in groups, two (C) and three (D) micronuclei per cell were found more abundant in the animals from the textile factory than the other affected region, not in controls. Almost in each animal, shapeless or shoot-shape nuclear material (B) were observed. Most of the affected cells contained only one micronucleus or shoot-shaped nuclear material, a few of them were found to contain 2 or 3 nuclei, which are smaller than the nuclei of other cells.

The overall results of this study demonstrate that the water in Porsuk River may contain some genotoxic agents and the increase in micronuclei may be due to contaminants in the river. The most affected animals were from around the nitrogen factory, and followed by the textile and sugar factories. The cytogenetic effects (MN yield) observed at these sites of the river were not much different from each other.

However, as seen in Table.1, various numbers of micronuclei were observed such as 2 and 3 micronuclei per cell in the animals collected from the most polluted sites. These kinds of anomalies are formed by retardment in anaphase or metaphase and by an aberration of chromosomes caused by mutagens in the environment.

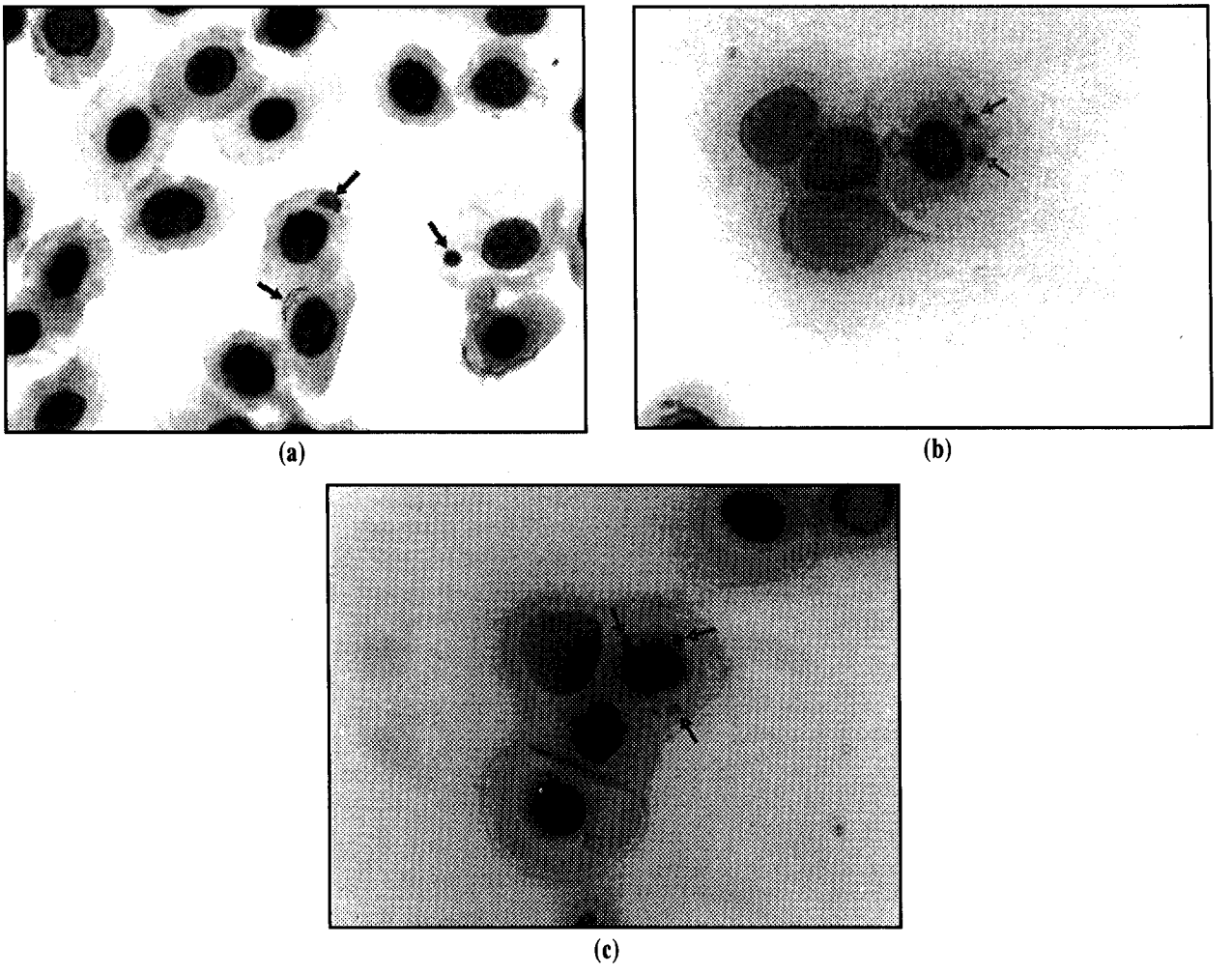

Figure 2. Micronucleated Erythrocytes of *Rana ridibunda*: (a) One Micronucleus (Right Arrow) and Shapless or Shoot-Shape Nuclear Materials (Left Arrows), (b) Two Micronuclei and (c) Three Micronuclei In a Cell. Magnification: X300.

The genotoxic agents which the effects were detected in the present study were not identified. However, as reported previously (Yücel et al., 1995; Anon, 1991, 1992), the water is contaminated with many kinds of substances especially heavy metals such as Cd, Pb, Cr and Zn and various insecticides. Especially from the sugar factory, 25 000 kg/ year of nitrogen and 900 kg /year phosphor, and from the nitrogen factory, 6454 kg/day waste containing mostly nitrate, nitrogen and its compounds have been disposed to the waste water. In order to clean the color of the Porsuk River which is caused by the textile factory, the water is treated with 1300 kg of sodium hypochloride regularly. It is well documented that all these pollutants excerts very hazardous effects on living organisms and on the human body (Vural, 1984; Aiyar, 1991; Abe and Urano, 1994; Chu et al., 1996).

Porsuk River is a major source of drinking or tap water and consumed directly by the majority of people living around the river. It is very important to identify of the genotoxic agents in the water. Therefore, genotoxic effects of pollutants can be examined by using various organisms from polluted water such as fish, plants (Ma et al., 1984; Zhuleva and Dubinin; 1994; Chu et al., 1996) or frogs as indicated by the present work. It will be very helpful for reducing the genotoxic agent levels in the river.

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