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Full Length Research Paper

Micronucleus test in fish genome: A sensitive monitor for aquatic pollution

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The aquatic environment makes up the major part of our environment and resources, therefore its safety is directly related to the safety our health. In this study, three tilapia species (*Oreochromis niloticus*, *Oreochromis aureus* and *Tilapia zilli*) and *Clarias gariepinus* were employed to estimate water pollution using micronucleus (MN) test. The test has been used successfully as a mutagenic assay. It is simple, reliable, sensitive, and it does not depend on any karyotypic characteristics. Fish were collected from locations that display differential environmental stresses. Two main experiments were carried out. In the first one, blood samples were collected, fixed for 24 h and then were stained with Giemsa. In the second experiment, fish were acclimated for a week. Fish were fed and each specimen had received an IP injection of cyclophosphamide (2.6, 10, 40, mg/kg b.wt). After 24 h, blood samples were collected and MN frequencies were counted and statistically tested. Results from this study recommend the use of the micronucleus test in fish erythrocytes as a sensitive monitor for aquatic pollution. The results show also that the assay can be employed for the evaluation and the assessment of water pollution and aquatic mutagens.

Key words: Micronucleus test, tilapia, catfish, aquatic environment, water pollution.

INTRODUCTION

Fish are excellent subjects for the study of the mutagenic and/or carcinogenic potential of contaminants present in water samples since they can metabolize, concentrate and store waterborne pollutants (Al-Sabti, 1991). Since fish often respond to toxicants in a similar way to higher vertebrates, they can be used to screen for chemicals that are potentially teratogenic and carcinogenic in humans. The main application for model systems using fish is to determine the distribution and effects of chemical contaminants in the aquatic environment (Al-Sabti and Metcalfe, 1995).

Aquatic animals have often been used in bioassays to monitor water quality of effluent and surface water (Carins et al., 1975; Brugs et al., 1977). The development of biological monitoring techniques based on fish offers the possibility of checking water pollution with fast responses on low concentrations of direct acting toxicants (Poele and Strik, 1975; Koeman et al., 1977; Poele, 1977; Sloof, 1977; Badr and El-Dib, 1978).

Hayashi et al. (1998) evaluated monitoring systems that use aquatic organisms to assess the genotoxicity of water in the field and in the laboratory. In a field study, micronucleus assay was shown to be applicable to freshwater and marine fishes and that gill cells are more sensitive than hematopoietic cells to micronucleus-inducing agents.

The African walking catfish, *Clarias gariepinus*, is recognized as one of the most suitable species for aquaculture in Africa because it has high growth rate, very resistant to handling and stress, and well appreciated in a wide number of African countries. It is generally considered to be one of the most important tropical catfish species for aquaculture, has an almost Pan-African distribution, ranging from the Nile to West Africa and from Algeria to Southern Africa.

Cyclophosphamide (CP) is an alkylating agent. It causes alkylation of the purine ring, and as a result, there

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is miscoding and blockade of DNA replication. Schuler et al. (1997) evaluated the centromeric labeling to distinguish micronuclei induced by chromosomal loss and breakage *in vitro*. The *in vitro* micronucleus assay has been used to characterize the origin of the micronuclei induced by Cyclophosphamide.

Rodriguez-Cea et al. (2003) determined the sensitivity of micronucleus test in freshwater fish species for application in field surveys. Brown trout, *Salmo trutta*, European eel, *Anguilla anguilla*, and European minnow, *Phoxinus phoxinus*, three fish species inhabiting European freshwater ecosystems, were evaluated for their use as in situ pollution biomarkers using the micronucleus test in renal erythrocytes. Cyclophosphamide, colchicine, and cadmium were used as pollutants to examine their genotoxicity.

Chorvatovicová and Sandula (1995) recommended the use of Cyclophosphamide in chromosome aberration tests, sister chromatid exchanges and micronucleus (MN) formation *in vitro* and *in vivo*. This drug is mutagenic usually used as positive controls in *in vivo* tests of short duration.

Micronuclei are cytoplasmic chromatin-containing bodies formed when acentric chromosome fragments or chromosomes lag during anaphase and fail to become incorporated into daughter cell nuclei during cell division. Because genetic damage that results in chromosome breaks or spindle abnormalities leads to micronucleus formation, the incidence of micronuclei serves as an index of these types of damage. Because counting of micronuclei is much faster and less technically demanding than scoring of chromosomal aberrations, the micronucleus assay has been widely used to screen for chemicals that cause these types of damage.

For the scoring of micronuclei, the following criteria were adopted from Fenech et al. (2003). The diameter of the MN should be less than one-third of the main nucleus. MN should be separated from or marginally overlap with main nucleus as long as there is clear identification of the nuclear boundary. MN should have similar staining as the main nucleus

Ahmad et al. (2002) designed an *in vivo* study for the effects of pentachlorophenol with a pre-acclimatized fish species, *Heteropneustes fossilis*, using four sub-lethal concentrations, 0.1, 0.2, 0.3 and 0.4 ppm, and three sampling times, 48, 72 and 96 h. Cytogenetic preparations were stained by the haematoxylin-eosin technique. The incidence of micronuclei was scored by a manual and an automated method. Small-sized micronuclei appeared in the cytoplasm in addition to the main nucleus. The frequency of micronucleated erythrocytes peaked at 4 days (96 h) exposure. The percentage of single micronuclei increased with longer exposures. The micronuclei frequencies were significantly different from control ($P < 0.05$). Computer image analysis of morphological variations of erythrocytes indicated that a 1:5 ratio of micronuclei and main nucleus accompanied by a reduction in cell volume by 600 dot units.

Campana et al. (1999) detected genotoxic effects of pollutants in aquatic organisms; the genotoxicity of the pyrethroid lambda-cyhalothrin was studied using the micronucleus test in erythrocytes of *Cheirodon interruptus interruptus*. The frequency of micronuclei was examined in blood smears obtained from fish exposed *in vivo* to three different concentrations (0.05; 0. 01; 0.001 µg/L) of the compound and sacrificed at nine sampling times (24, 48, 72, 96 h and 8, 12, 15, 19 and 23 days). As a positive control fishes were exposed to 5 mg/L of cyclophosphamide. Results obtained demonstrated the genotoxic effects of the pyrethroid in the experimental model employed. The variation in the micronuclei frequencies in the different sampling times could be related to the blood cell kinetics and the erythrocyte replacement. The results could be considered as a validation of the MN test in fish for the assessment of genotoxic pollutants.

Mutagenic studies with native fish species represent an important effort in determining the potential effects of toxic agents. This study was carried out to evaluate the use of the micronucleus test (MN) for the estimation of aquatic pollution using different fish genotypes under lab and natural conditions.

MATERIALS AND METHODS

Fish species

Three tilapia species (*Oreochromis niloticus*, *Oreochromis aureus*, and *Tilapia zilli*) and the African catfish *(Clarias gariepinus)* were used in this study. Fish species, 100 - 150 g for tilapia species and 150-200 g for catfish, were collected from four natural local locations and transferred to glass aquaria.

Locations

Fish were collected from four locations that represent different levels of contaminants (Ali and El-Shehawi, 2007). Location 1 is the River Nile (Shubrakhit). This site is used as control for the main common irrigation and drinking water resource in Egypt. Location 2 is a closed drainage at Abou Homos. Location 3 is a drainage at Kafr Eldawar (Barsiwqe). Location 4 is Lake Mariout.

Cyclophosphamide (Endoxan-Asta)

Endoxan-Asta is the trade name of Cyclophosphamide. It is a white crystalline powder with molecular weight 261. It has been widely used as a positive control in genetic toxicology.

Experimental procedures

Two main experiments were carried out. In the first one, blood samples were directly collected from caught fish. In the second experiment, fish were acclimated for a week in 200 L tank with well-aerated water at 20°C. Acclimated fish were distributed in four groups. Fish were fed and each group received 2, 5, 10, or 40 mg/kg b.wt. of Cyclophosphamide by injection in the trunk muscle. The control group received the same volume of sterilized injection water.

Table 1. Averages of micronucleated erythrocytes examined in blood and kidneys of fish caught from different locations.

Species	Location							
	1		2		3		4	
	Blood	Kidneys	Blood	Kidneys	Blood	Kidneys	Blood	Kidneys
ON	0.4± 0.11	0.3± 0.1	0.8±0.1	0.4±0.12	0.61±0.12	0.42±0.12	2.2± 0.4	1.8± 0.4
OA	0.3± 0.1	0.2± 0.1	0.41±0.1	0.3±0.13	0.52±0.2	0.63±0.11	2.4± 0.41	2.3± 0.3
TZ	0.6± 0.12	0.4±0.12	1.2±0.1	0.82±0.14	1.3±0.4	0.94±0.4	3.2± 0.52	2.8± 0.4
CG	2.2± 0.22	1.01± 0.2	4.0±1.1	3.4±0.6	8.2±1.2	5.2±0.6	12.2±2.1	8.7±1.2

ON: *Oreochromis niloticus*, OA: *Oreochromis aureus*, TZ: *Tilapia zilli*, CG: *Clarias gariepinus.*

Table 2. Averages of micronucleated erythrocytes induced in blood and kidneys after treatment of fish with Cyclophosphamide.

Dose, (mg/kg.b.wt)	Species							
	ON		OA		TZ		CG	
	Blood	Kidneys	Blood	Kidneys	Blood	Kidneys	Blood	Kidneys
Control	0.4± 0.12	0.3± 0.11	0.2±0.1	0.2±0.1	0.4±0.12	0.4±0.12	1.8± 0.66	1.8± 0.66
2	2.1± 0.4	4.2± 0.46	2.3±0.14	3.2±0.6	4.1±0.8	5.3±1.1	4.8± 0.6	6.4± 1.1
5	8.4± 1.2	12.1± 1.4	4.1±0.14	6.2±0.4	6.3±0.8	10.2±1.4	8.4± 1.1	16.2± 2.3
10	10.2± 1.8	16.3± 2.2	6.2±0.8	8.4±1.1	12.2±1.4	16.6±2.2	15.1± 1.4	21.4± 2.2
40	18.3± 2.1	22.2± 2.4	12±1.4	18.2±2.1	22±3.2	30.4±3.4	28.2±2.9	42.4±3.2

ON: *Oreochromis niloticus*, OA: *Oreochromis aureus*, TZ: *Tilapia zilli*, CG: *Clarias gariepinus.*

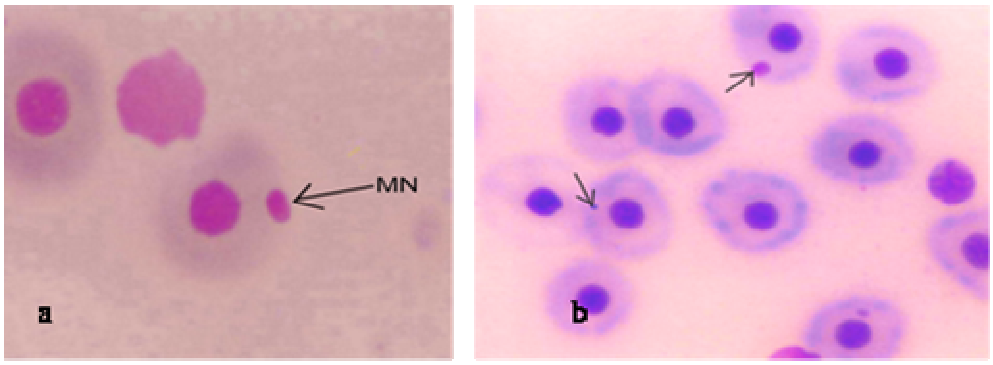

Figure 1. Photomicrograph showing micronucleated erythrocyte from CG caught from location 4 (a) and 3 (b), respectively.

Micronucleus test

The peripheral blood smears were obtained through the gills and kidneys blood by means of a medial-kidney imprint following dissection or blood smears from the gills. The slides were air-dried for 24 h, fixed in methanol for 10 min, followed by 10% Giemsa (v/v) staining. Each fish had 2000 erythrocytes examined, from both peripheral blood and the kidneys. To detect micronuclei in erythrocytes, the slides were analyzed using a 1000 X oil-immersion lens.

RESULTS

The obtained results are summarized in Tables 1 - 2 and Figures 1 - 7. Results reveal that the four fish species represent various degrees of sensitivity in monitoring genetic damage (especially clastogenic effect). This is indicated by variations in averages of the micronucleated cells among species at various locations. As previously mentioned by Ali and El-Shehawi (2007), these locations display differential environmental stress. Location number 4 was found to be highly contaminated, at the level of the estimated heavy metals. Generally, clastogenic effect represented by the formation of micronucleus was increased in the following rank: 1<2<3<4. On the other hand, peripheral blood of CG (*C. gariepinus*) was shown to be very sensitive in formation of MN depending upon the environmental stress, and might be by other factors.

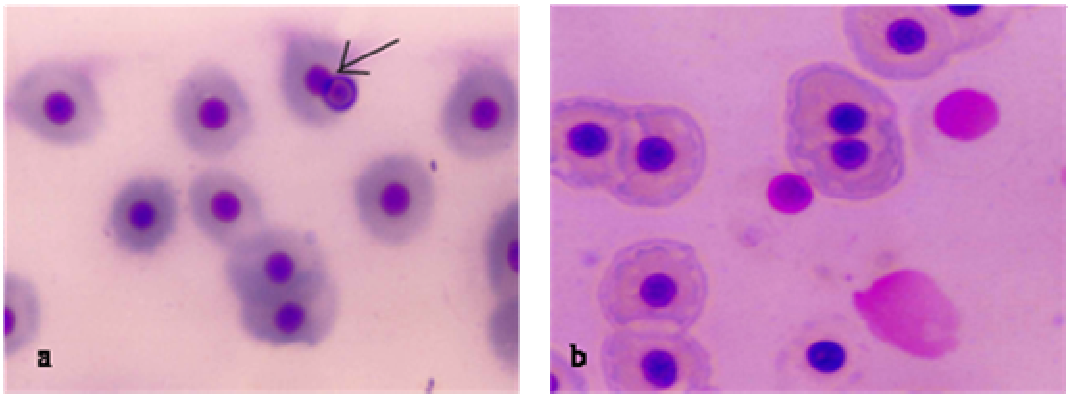

Figure 2. Photomicrograph showing binucleated erythrocyte in TZ (a) and ON (b) caught from location 4.

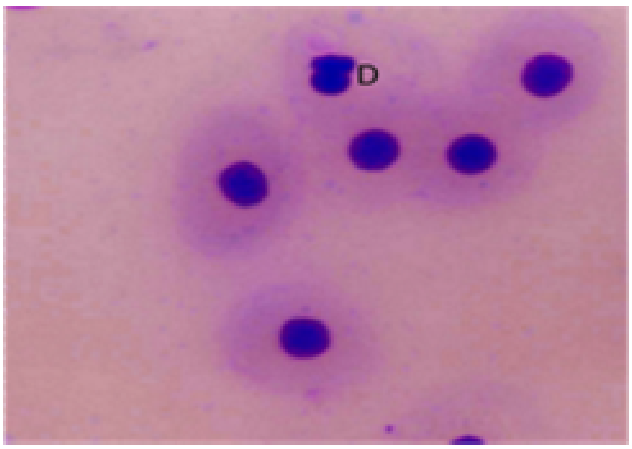

Figure 3. Photomicrograph showing Deformed Nucleus (D) in kidneys of TZ caught from location 2.

The high percentage of MN in peripheral blood of CG may represent an evidence that its genome well tolerates such type of cytogenetic damage without apoptosis. As shown in Table 1 ON (*O. niloticus*) was found to be fairly similar to OA (*O. aureus*) in formation of MN at different levels of environmental stress. In contrast, TZ (*T. zilli*) was shown to be sensitive in MN formation compared either with ON or with OA. Table 1 shows the averages of micronucleasted erythrocytes formed in kidneys of different genomes of fish at different locations. The results revealed that MN percentages were proven to be less than those obtained from peripheral erythrocytes, giving an evidence that repair system in kidneys may play a role better than that of peripheral erythrocytes. On the other hand, erythrocytes in gills may face contaminants do not reach kidney cells. In conclusion, peripheral blood as well as kidney cells were shown to be sensitive in all employed genomes for monitoring mutagenic and/or clastogenic effect induced by the aquatic environment.

Figures 1 -2 show micronuclei, binucleated cells, and deformed nucleus in different fish species caught from the four locations under study.

Cytological examination after treatment with different doses of Cycophosphamide revealed that binucleated cells, deformed nuclei in addition to the main type of aberration (micronucleus) were observed. These results are shown in Table 2. Tilapia species have close averages for micronuclei which are lower than that of catfish species. Comparing the data obtained from the pripheral blood with that of kidney cells, it is obvious that peripheral erythrocytes are sensitive for the damage induced by the aquatic contaminants (approximately 150% compared with kidneys erythrocytes). Figures 4 - 7 show micronucleated erythrocytes and binucleated one in fish caught from different locations and treated with the indicated dose of Cyclophosphamide.

DISCUSSION

Bioindicators offer several types of unique information not available from other methods: (1) early warning of environmental damage; (2) the integrated effect of a variety of environmental stresses on the health of an organism and the population, community, and ecosystem; (3) relationships between the individual responses of exposed organisms to pollution and the effects at the population level; (4) early warning of potential harm to human health based on the responses of wildlife to pollution; and (5) the effectiveness of remediation efforts in decontaminating waterways (Villela et al., 2006).

Fish serve as useful genetic models for the evaluation of pollution in aquatic ecosystems (Mitchell and Kennedy, 1992; Park et al., 1993). The erythrocyte micronucleus test has been used with different fish species to monitor aquatic pollutants displaying mutagenic features (De Flora et al., 1993; Saotome and Hayashi, 2003; Pantaleao et al., 2006) The obtained results support the fact demonstrated by Kligerman (1982) that fish inhabi-

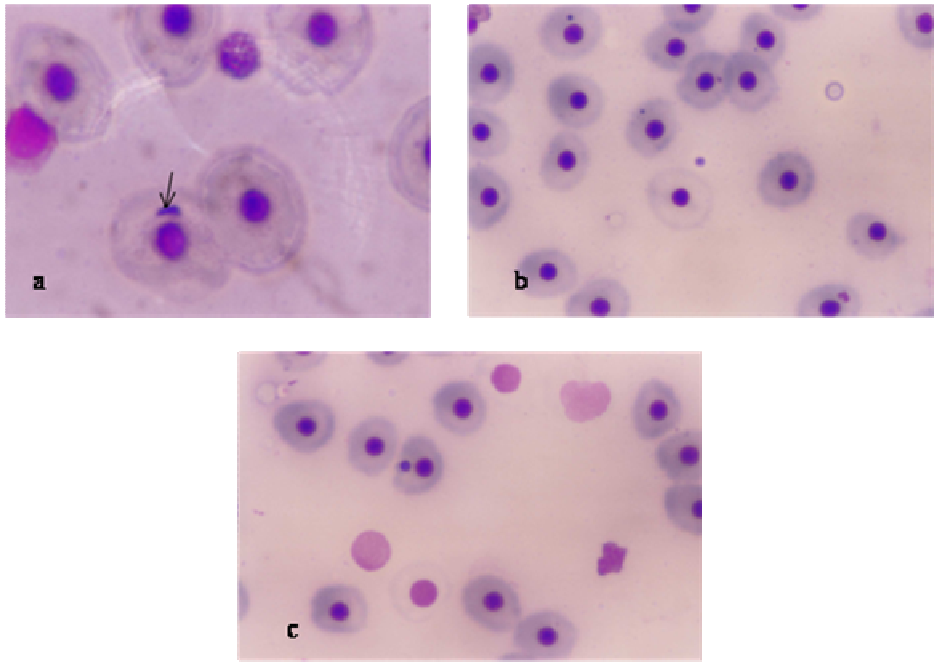

Figure 4. Photomicrograph showing micronucleated erythrocyte (MN) in ON kidneys after treatment with different doses (5, 40 and 2 mg/kg b.wt. in a, b and c, respectively) of Cyclophosphamide.

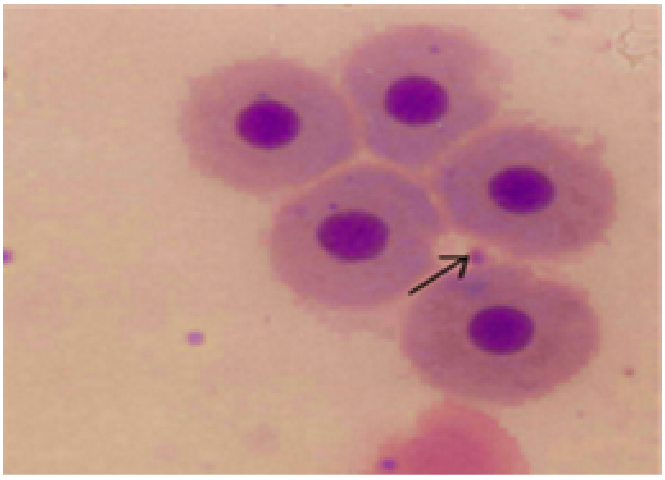

Figure 5. Photomicrograph showing Micronucleated Erythrocyte (MN) in OA after treatment with 10 mg/kg.b.wt. of Cyclophosphamide.

ting polluted waters have greater frequencies of micronuclei. The micronuclei frequencies may vary according to the season, the kind of pollution involved, and the species of fish.

In fish, the kidney is responsible for erythropoiesis as well as filtration. Upon fish exposure to toxins, defective erythrocytes undergo passage from the kidney into the peripheral blood, from where they are removed by the hemocatheresis organs (Rabello-Gay, 1991). One aim of this study was to examine if kidney erythrocytes would provide earlier and more sensitive detection of micronuclei frequencies than peripheral blood erythrocytes. Accordingly, we analyzed the frequencies of micronuclei obtained from peripheral blood and from kidneys of studied species. Results revealed that in case of fish without Cyclophosphamide treatment gills showed higher frequencies of micronuclei than kidneys, whereas, kidneys erythrocytes from fish treated with the drug showed higher frequencies of micronuclei. This goes with hypothesis of Rabello-Gay (1991). This could be attributed that the repair system in kidneys cannot recover the damage or the drug can reach the kidneys when it is more concentrated during the treatment.

The results demonstrate that different fish species can respond in completely different ways to a given genotoxic agent. Although tilapia species (ON, OA, and TZ) gave close data, their averages were obviously different from those of CG. Depending on the toxic agent and on the species, the behavior of micronuclei rates may exhibit significant variations, probably related to the pharmacokinetics of the drugs used and to the speed of the hemopoietic cycle (Kligerman, 1982). Therefore, it is suggested that micronuclei tests in fish erythrocytes be carried out at various times following treatments, thus making it possible to follow-up the changing micronuclei frequencies. Studies of the micronuclei rates of various fish species showed that they generally peaked between the first and fifth days after treatment (Al-Sabti and Met-

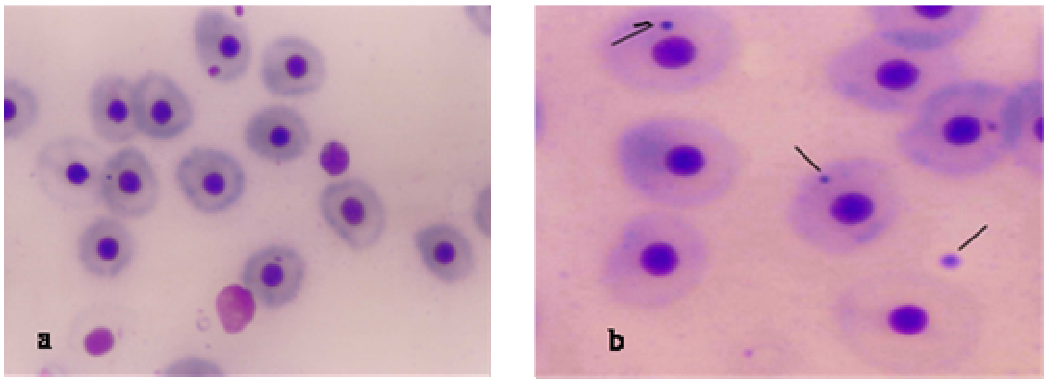

Figure 6. Photomicrograph showing micronucleated erythrocyte (MN) in CG kidneys after treatment with different doses (2 and 40 mg/kg b.wt. in a and b, respectively) of C yclophosphamide.

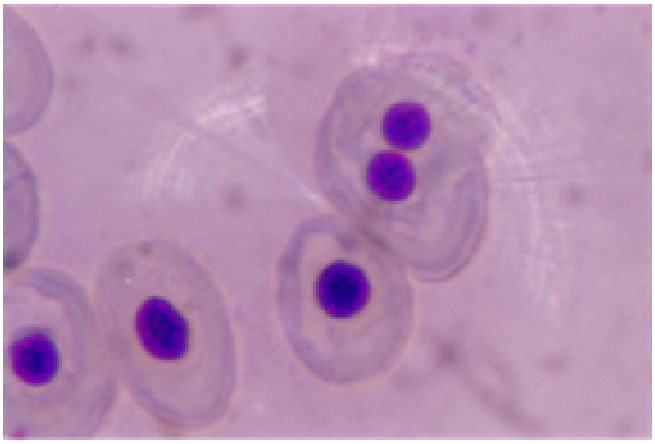

Figure 7. Photomicrograph showing micronucleated erythrocyte (MN) in TZ kidneys after treatment with 5 mg/kg.b.wt. of Cyclophosphamide.

calfe, 1995; Grisolia and Cordeiro, 2000) depending on the drug used for the induction of micronuclei.

It is concluded from this study that gills and kidneys erythrocytes can be used for estimating the genotoxic effects of waterborne pollutants. The sampling of peripheral blood is appropriate and sufficient for biomonitoring projects, as it allows collecting several samples from the same individual, without having to sacrifice it (Lyne et al., 1992)

REFERENCES

Ahmad W, Ali MN, Farah MA, Ateeq B (2002). Computerized automated morphometric assay including frequency estimation of pentachlorophenol induced nuclear anomalies (micronucleus) in catfish Heteropneustes fossilis. Chromosoma. 110(8): 570-4.

Ali FA, El-Shehawi AM (2007). Estimation of water pollution by genetic biomarkers in: Al-Sabti K (1991). Handbook of Genotoxic Effects and Fish Chromosomes. Jozef Stefan Institute, Jamova.

Al-Sabti K, Metcalfe CD (1995). Fish micronuclei for assessing genotoxicity in water. Mutat. Res. 343: 121-135.

Badr EA, El-Dib SE (1978). Effects of water pollution on the cell division cycle and chromosome behavior in *Tillapia* sp. Egypt. J. Genet. Cytol. 7: 193-200.

Brugs WA, Cormick JHM, Neiheisel TW, Spear RL, Stephan CE, Stokes G (1977). Effect of pollution on fresh water fish. J. Water Pollut. Contr. Fed. 49: 1425-1493.

Campana MA, Panzeri AM, Moreno VJ, Dulout FN (1999). Genotoxic evaluation of the pyrethroid lambda-cyhalothrin using the micronucleus test in erythrocytes of the fish *Cheirodon interruptus*.. Mutat. Res. 438(2): 155-61.

Carins J, Dickson KL, Westlake GF (1975). Biological monitoring of water and effluent quality. ASTM Publ., 607, Philadelphia.

Chorvatovicová D, Sandula J (1995). Effect of carboxymethyl-chitin-glucan on cyclophosphamide induced mutagenicity. Mutat. Res. 346: 43-48.

De Flora S, Vigario L, D'Agostini F, Camoirano A, Bagnasco M, Bennecelli C, Melodia F, Arillo A (1993). Multiple biomarkers in fish exposed in situ to polluted river water. Mutat Res. 319: 167-177.

Fenech M, Chang WP, Kirsch-Volders M, Holland N, Bonassi S, Zeiger E (2003). Human Micronnucleus Project. "HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures." Mutat. Res. 534(1-2): 65-75.

Grisolia CK, Cordeiro CMT (2000). Variability in micronucleus induction with different mutagens applied to several species of fish. Genet. Mol. Biol. 23(1): 235-239.

Hayashi M, Ueda T, Uyeno K, Wada K, Kinae N, Saotome K, Tanaka N, Takai A, Sasaki YF, Asano N, Sofuni T, Ojima Y (1998). Development of genotoxicity assay systems that use aquatic organisms. Mutat. Res. 399(2): 125-33.

Kligerman D (1982). Fishes as biological detectors of the effects of genotoxic agents. In: Mutagenicity: New Horizons in Genetic Toxicology, Heddle J (ed) Academic Press, New York. pp. 435-456.

Koeman JH, Poel CL, Slooff W (1977). Continuous biomonitoring systems for detection of toxic levels of water. In: Hutzinger O (Eds.), Aquatic Pollutants, Pergamon, Oxford. pp. 339-348.

Lyne TB, Bickham JW, Lamb T, Gibbons JW (1992). The application of bioassays in risk assesment of environmental pollution. Risk Anal.. 12(3): 361-365.

Mitchell S, Kennedy S (1992). Tissue concentrations of organochlorine compounds in common seals from the coast of Northern Ireland. Sci. Total Environ. 115: 235-240.

Pantaleao Sde M, Alcantara AV, Alves Jdo P, Spano MA (2006). The piscine micronucleus test to assest the impact of pollution on the Japaratuba river in Brazil. Environ. Mol. Mutagen. 47(3): 219-24.

Park E, Lee J, Etoh H (1993). Fish cell line (ULF-23HU) derived from the fin of the central mudminnow (*Umbra limi*): suitable characteristics for clastogenicity assay. *In Vitro* Cell Dev. Biol. 25:987-994.

Poele CL, Strik JJT (1975). Sublethal effects of toxic chemicals on aquatic animals, In: Koeman JH, Strick JJTWA (Eds), Elsevier,

Amsterdam. pp 81-91.
Poele CL (1977). Sublethal effects of Rhine water on Rainbow trout, In: Hutzinger O (Eds) Aquatic pollutants pergamon, Oxford. pp. 405-418.
Rabello-Gay MN (1991). Teste do micronúcleo em medula óssea. In: Mutagênese, Teratogênese e Carcinogênese: Métodos e Critérios de Avaliação, Sociedade Brasileira de Genética (ed). pp. 83-90.
Rodriguez-Cea A, Ayllon F, Garcia-Vazquez E (2003). Micronucleus test in freshwater fish species: an evaluation of its sensitivity for application in field surveys. Ecotoxicol. Environ. Saf. 56(3): 442-8.
Saotome K, Hayashi M (2003). Application of a sea urchin micronucleus assay to monitorin aquatic polllution: influence of sample osmolality. Mutagenesis. 18(1): 73-6.
Schuler M, Rupa DS, Estmond DA (1997). A critical evaluation of centromeric labeling to distinguish micronuclei induced by chromosomal loss and breakage *in vitro*. Mutat Res. 392(1-2): 81-95.
Sloof W (1977). Biological monitoring based on fish respiration for continuous water quality control. In: Hutzinger O (eds.), Aquatic Pollutants, Pergamon, Oxford, pp. 501-506.
Villela IV, De Oliveira IM, Da Silva J, Henrigues JA (2006). DNA damage and repair in haemolymph cells of golden mussel exposed to environmental contaminants. Mutat. Res. 605(1-2): 78-86.