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Evaluation of the genotoxic potential due to the action of an effluent contaminated with Chromium, by the Comet assay in CHO-K1 cultures

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Abstract — The comet assay technique has been considered to be more efficient in the biomonitoring of aquatic environments that the micronucleus and sister chromatid exchange techniques. The comet assay has been used to determine breaks in the DNA strands of organisms exposed to pollutants with a genotoxic potential. The comet technique was applied to CHO-K1 cells in order to evaluate the genotoxic potential of the waters of the Sapucaizinho River (Municipality of Patrocínio Paulista, State of São Paulo, Brazil), which receive tannery effluents and therefore are contaminated with chromium. The results indicated high genotoxicity of the waters collected at sites located downstream from the emission of tannery effluents, where the concentration of chromium was found to be high.

Key words: Chromium, Comet assay, genotoxicity

INTRODUCTION

Many potentially toxic chemical substances, both of natural origin or due to human actions, are released into the environment. Due to the increasing environmental exposure to these agents, there has been increasing interest in biomonitoring terrestrial and aquatic ecosystems, especially in regions compromised by chemical pollution (Silva *et al*. 2003).

Environmental pollution due to heavy metals represents a serious problem because of its high toxicity and of the bioaccumulation ability of these agents (Guecheva *et al*. 2001; Pruski and Dixon 2002). Lee and Steinert (2003) and Matsumoto (2003) reported that chromium represents a dangerous heavy metal which can directly damage DNA by promoting strand breaks or DNA-protein crosslinks, or by acting on the generation of reactive oxygen species.

The comet assay or single cell gel electrophoresis (SCGE) is a simple, sensitive and rapid technique applied to isolated cells for the detection of DNA damage (Fairbain *et al*. 1995; Klaude *et al*. 1996; Speit and Hartmann 1999), and represents a highly efficient method for application to toxicologic genetics, especially ecogenotoxicology (Pavlica *et al*. 2001). This technique has been applied by Mitchelmore and Chipman (1998) and Kassie *et al*. (2000) to studies of DNA repair, of the effect of biological radiation, and of apoptosis.

The comet assay technique has been considered to be more efficient than the micronucleus or sister chromatid exchange technique as a bioindicator of alterations of aquatic environments. Lee and Steinert (2003) have been using this technique in several *in vivo* and *in vitro* studies for the determination of strand breaks in the DNA of aquatic organisms exposed to pollutants with a genotoxic potential.

The same efficiency of this technique was reported by Pandrangi *et al*. (1995) when assessing sediments contaminated with heavy metals in the Great Lakes (Canada), where they observed in-

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duction of DNA damage caused by these contaminants. NACCI *et al.* (1992) also reported a reduction in DNA strand breaks in organisms transferred from sites with sediments contaminated with heavy metals to control sites free from these pollutants.

Using the comet assay in human lymphocytes, RAJAGURU *et al.* (2002) investigated the genotoxic potential of waters polluted with textile industry effluents in India.

Biomonitoring studies were conducted by AVISHAI *et al.* (2002) on the Kishon River (Israel) using the comet assay applied to a culture of the fish hepatoma cell line RTH-149. The authors reported a high genotoxicity of the water samples analyzed, showing the efficiency of this technique for the assessment of the genotoxic potential of an aquatic environment.

The efficiency of the comet assay was also demonstrated for CHO-K1 cell cultures (MATSUMOTO *et al.* 2003) exposed to water from the Córrego dos Bagres Stream (Franca/SP-Brazil) that receives tannery effluents. The authors observed that chromium had a genotoxic effect at concentrations of 0.01 mg/L, below the limit permitted by International Legislation (0.05 mg/L), demonstrating that this heavy metal has a high polluting action on water resources and a high genotoxic potential.

The municipality of Patrocínio Paulista-SP is a region with intense industrial activity related to leather processing (tanning). Several toxic substances, chromium among them, are used for leather tanning and are considered to be harmful to the environment. In the present study, using the comet assay technique applied to CHO-K1, we assessed the genotoxic potential of waters from the Sapucaizinho River, municipality of Patrocínio Paulista – State of São Paulo – Brazil, which receive tannery effluents and are therefore contaminated with chromium.

MATERIALS AND METHODS

Sample collect - Water collects were performed during the four seasons of the year 2002 at three different stations along the Sapucaizinho river, Municipal district of Patrocínio Paulista/SP. The collect sites selected were: before the site of tannery effluent dumping (1000 m upstream) – PA, dumping site – PB, and after the dumping site (1000 m downstream) – PC (Figure 1). At each collect, 2 liters of water were obtained at a depth of 30 cm according to routine criteria for chemical analyses. The water samples were placed in plastic bottles and carried in an insulated box with ice to

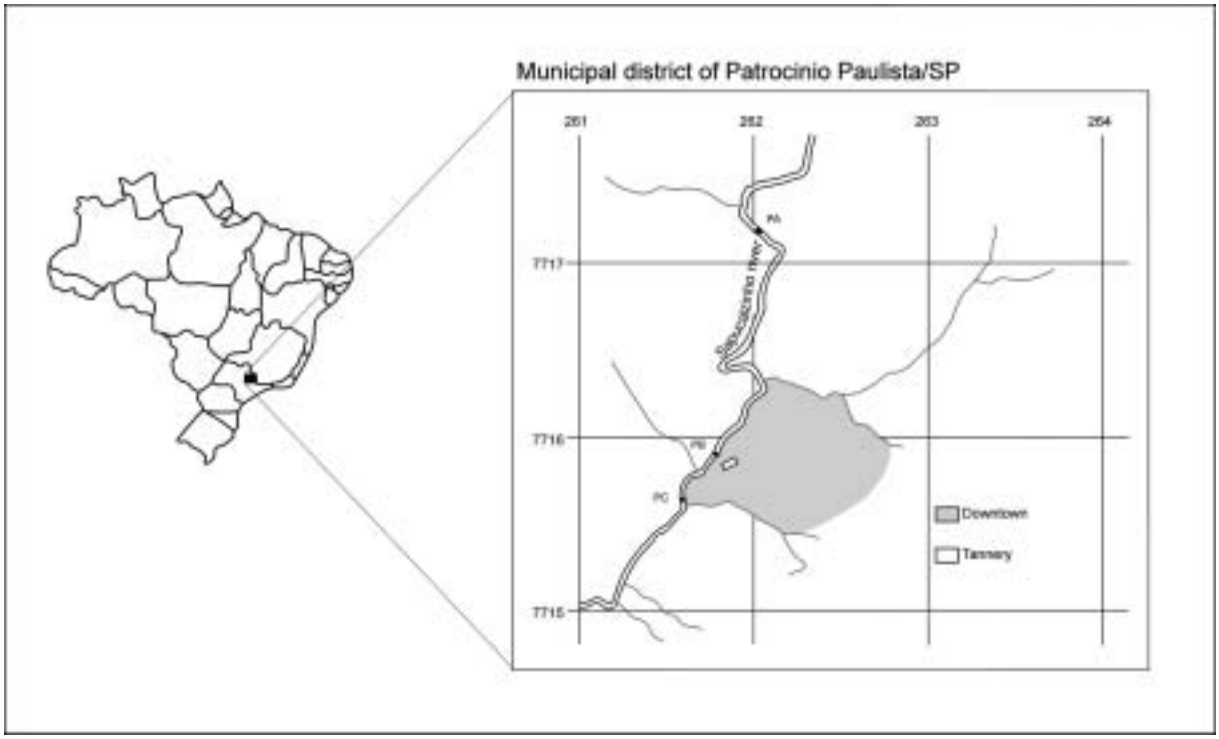

Fig. 1 — Geographical localization of the collected site along the Sapucaizinho river.

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Physicochemical analysis of the water - The analyses were based on the Standard Methods for the Examination of Water and Wastewater (FRANSON 1995).

For cation determination, the samples were first acidified with HNO_3, pH 1, and the cations were analysed sequencialy by inductively coupled

Table 1 — Analysis of the changes observed in CHO-K1 cells treated with waters collected at distinct points along the river which receives tannery effluents in the region of Patrocínio Paulista/SP.

Period	Treatment	A	B	C	D	E	F	G	H
rainy									
	Continuous	-							
	NC	-	-	-	-	-	-	-	0.0 a
	PC	0.3	1.2	0.7	0.2	0.4	2.8	-	10.95b
	PA	1	0.9	0.9	-	-	-	0.8	1.75 a
	PB-A	1.1	0.9	1	-	-	0.3	2.2	0.82 a
	PB-T	1	1	1	-	-	0.3	1.3	1.29 a
	PB-L	1.5	0.9	0.5	-	-	-	2.1	1.25 a
	PC	1	1.1	0.6	-	0.1	-	1.8	1.45 a
	20 hours								
	NC	-	-	-	-	-	-	-	0.0 a
	PC	0.3	1.2	0.7	0.2	0.6	2.8	-	10.95 b
	PA	0.6	0.4	0.4	-	0.1	0.7	0.2	2.72 a
	PB-A	2.1	0.25	0.1	-	-	0.1	0.3	2.20 a
	PB-T	0.7	0.2	0.1	0.1	0.1	-	1	1.96 a
	PB-L	1.7	0.2	0.2	0.1	0.1	0.2	0.8	1.35 a
	PC	1	0.5	0.4	-	0.2	-	1.1	2.22 a
	72 hours								
	NC	-	-	-	-	-	-	-	0.0 a
	PC	0.3	1.2	0.7	0.2	0.6	2.8	-	10.95b
	PA	0.3	0.3	0.2	-	-	-	0.5	0.55 a
	PB-A	0.4	0.4	0.2	-	-	0.1	0.7	0.71 a
	PB-T	1	0.2	0.1	-	-	-	0.3	0.50 a
	PB-L	0.9	0.3	0.2	0.4	-	0.1	1	1.60 a
	PC	0.2	0.1	-	-	-	-	0.1	0.47 a
dry									
	Continuous								
	NC	0.6	01	-	-	0.7	-	1.2	0.52 c
	PC	1.2	1.8	2.2	0.1	3	7.9	-	18.07 a
	PA	0.7	0.2	0.3		0.4	-	0.9	1.61 bc
	PB-A	0.7	0.4	0.3	0.4	0.3	0.1	1.5	3.16 b
	PB-T	0.6	0.2	0.4	1.1	0	0.2	2.3	6.06 b
	PB-L	3.1	0.7	0.6	1.8	0.3	1	4.8	3.85 b
	PC	0.9	0.5	0.31	0.5	0.7	-	2.3	3.03 b
	20 hours								
	NC	0.6	0.1	-	-	0.7	-	1.2	0.52 c
	PC	1.2	1.8	2.2	0.1	3	7.9	-	18.07 a
	PA	1.5	0.2	0.3	0.7	0.6	0.1	1.9	2.06 bc
	PB-A	1.4	0.2	0.1	0.8	0.2	-	1.4	1.52 bc
	PB-T	7.6	0.1	4.1	-	-	1.2	5.4	11.78 bc
	PB-L	1.6	0.5	0.7	1.7	1.3	-	4.2	4.84 b
	PC	0.8	0.3	0.4	1.7	0.7	-	2.1	2.99 b
	72 hours								
	NC	0.6	0.1	-	-	0.7	-	1.2	0.52 a
	PC	1.2	1.8	2.2	0.1	3	7.9	-	18.07 b
	PA	-	-	-	-	-	-		0.0 a
	PB-A	1.6	0.2	0.1	-	0.2	-	0.5	0.70 a
	PB-T	2.3	0.4	0.6	-	-	0.1	1.1	1.93 a
	PB-L	1.2	0.2	0.32	0.1	0.4	-	1	1.56 a
	PC	1.5	0.1	0.3	0.7	0.6	0.1	1.8	2.58 a

NC. negative control; PC. positive control; PA. uspstream from the site of effluent discharge; PB. site of effluent discharge; PC. downstream from the site of effluent discharge.

plasma atomic emission spectrometry (ICP-AES) with ultrasonic nebulization. The following elements were determined: calcium, magnesium, strontium, silicon, iron, manganese, aluminium, zinc, chromium, cobalt, nickel, lead, cadmium, phosphorus, copper, and barium.

The standard solutions used to construct the calibration curves for the elements analyzed in 0.1% HNO_3 medium were obtained by appropriate dilutions of the standard solutions of 1000 ppm Titrisol (Merck).

Culture conditions and treatments - CHO-K1 cells were cultured in D-Mem + HAMF10 (1:1) medium supplemented with 10% Bovine Serum Albumin at 37°C. For the treatments a concentrated culture medium (stock) was prepared, to be diluted 3:1 with the water samples collected. The water samples collected at each station were added to the concentrated culture medium at the treatment time.

After 24 hours of culture in normal medium, CHO-K1 cells were kept for 2 hours (37°C) in the mixture of medium and water collected from the stream, whose pH was adjusted to 7.2 before treatment. The positive control was treated with methylmethanesulfonate (MMS) at the concentration of 10^{-4}M and the negative control was only kept in culture medium. Three independent experiments were performed.

Comet assay - The slides for microscope observation were mounted with 20 μl of the above cellular suspension and 120 μl of low melting point agarose at 37°C. The slides were bathed in a lysis solution (1 ml of Triton X-100, 10 ml of DMSO and 89 ml of lysis stock solution [2.5 M NaCl, 100 mM EDTA, 10mM Tris, pH 10, ~8 g solid NaOH, and 10 g lauryl sarcosinate, pH 10]), in the dark, in a refrigerator at about 8°C for 1 hour.

After lysis, the slides were placed in buffer containing 0.3 N NaOH and 1 mM EDTA, pH>13, for 20 minutes for DNA unwinding. Electrophoresis was performed for 20 minutes at 25 V, 300 mA (~1.0 V/cm) and the slides were them neutralized for 15 minutes in 0.4 M Tris, fixed in absolute ethanol for 10 minutes, and stained with ethidium bromide (0.02 μg/ml).

Comet assay analysis - The comet observations were performed at 400X using a Nikon epifluo-

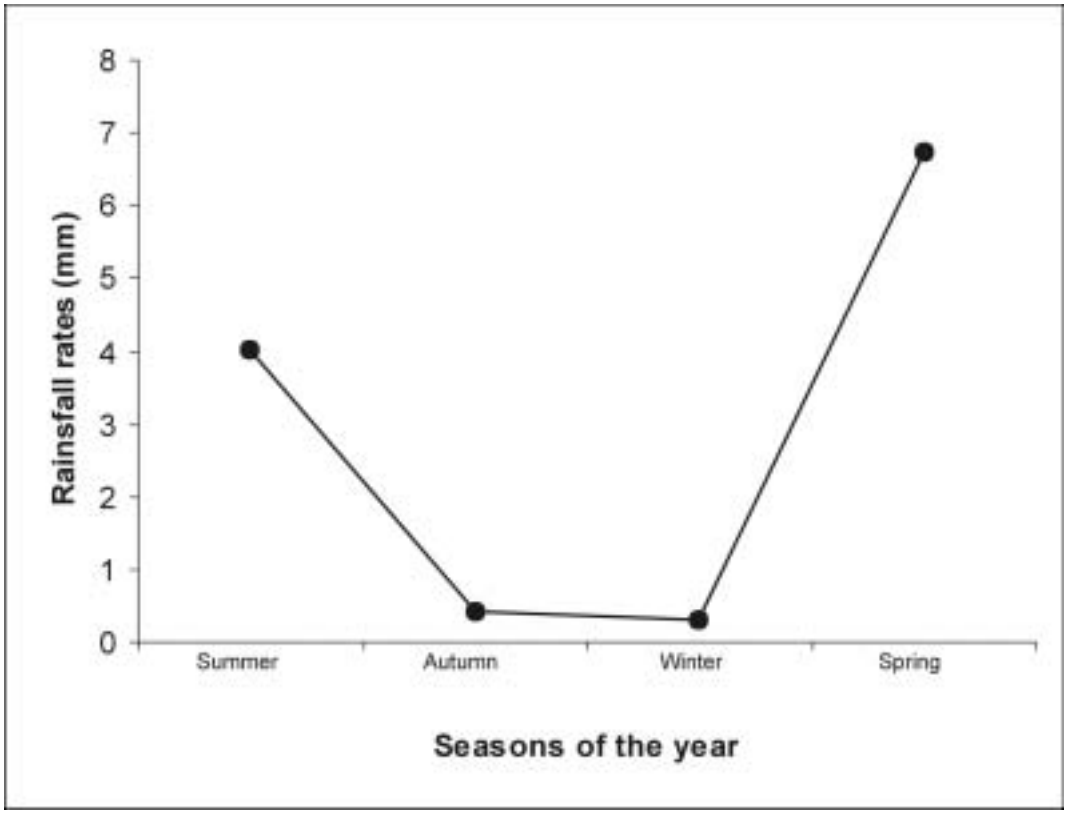

Fig. 2 — Mean rainfall indices for the seasons of the year when water samples were collected from the Sapucalizinho river.

rescence microscope (filters B-3[A]; excitation: λ = 420-490 nM; barrier: λ = 520 nM). One-hundred nuclei per sample were analyzed (treated and controls) and classified according to migration of fragments in classes 0, 1, 2 and 3 (KOBAYASHI, 1995): Class 0, cells without tail (not damaged) dont damaged that dont present tail; class 1, cells with tail smaller than the nucleus diameter; Class2, cells with size of tail among 1-2 times the nucleus diameter; class 3, cells with tail bigger than 2 times the nucleusdiameter.

Statistical analysis - Data were analyzed statistically by the χ^2 test (PEREIRA 1991) for comparison of the total number of comets of each treatment with the values observed in the negative and positive controls.

RESULTS AND DISCUSSION

CHO-K1 cultures treated with water samples collected from predetermined sites along the Sapucaizinho River and submitted to the comet assay presented DNA damage during all seasons of the year.

The frequency of damage observed for all the collection sites during all seasons was lower than that observed in the positive control and higher than that observed in the negative control (except for the summer). These data agree with those described by AVISHAI *et al.* (2002) and MATSUMOTO *et al.* (2003), who reported that the comet assay technique applied to cell cultures is efficient for application to the biomonitoring of water resources.

During the summer, the genotoxic effect observed at the three collection sites was not significant compared to control (Table 1). These results may be related to the high rainfall observed during this period of collection (Figure 2). During the other seasons, damage to the cell nuclei was observed, with the highest frequency always being recorded for the PB site. This high frequency of DNA damage at the PB site can be explained by the fact that this site corresponded to the exact place where the tannery effluent was discharged. Coincidentally, it was also this site that presented the highest concentration of total chromium, as shown in Table 2.

The highest harmful effect on the Sapucaizinho River was observed during the winter, when the rainfall rate was the lowest among all seasons of the year investigated (Table 1 and Figure 2). In winter, the highest genotoxic effect was observed for the PB site (Table 1 and Figures 3 and 4). No class 3 nuclear damage was observed in any of the samples analyzed.

The PA site (upstream to the effluent) presented a lower frequency of nuclear damage compared to the PB site. This can be explained by the location of the PA site itself, since at this site there was no emission of tannery effluents and therefore no influence of the chromium residue was present (Table 1, Figure 3).

Chromium is considered to be a carcinogen because it induces lesions in the DNA molecule (DEBETTO *et al.* 1982; MATSUMOTO 2003). The

Table 2 — Determination of elements (ppm) by ICP-AES in water samples collected at three different points in the four seasons of the year.

		Chemical elements										
		Pb	**Fe**	**Cd**	**Cr**	**P**	**Al**	**Zn**	**Cu**	**Ba**	**Co**	**Ni**
Summer												
	PA	<0.025	1.1	<0.005	<0.01	<0.1	0.12	<0.01	<0.01	0.02	<0.01	<0.01
	PB	<0.025	1.2	<0.005	0.01	<0.1	0.2	<0.01	<0.01	0.03	<0.01	<0.01
	PC	<0.025	1.1	<0.005	<0.01	<0.1	0.1	<0.01	<0.01	0.02	<0.01	<0.01
Autumn												
	PA	<0.012	0.96	<0.003	<0.005	<0.1	0.05	<0.005	<0.005	0.02	<0.005	<0.005
	PB	<0.012	0.81	<0.003	0.005	<0.1	0.1	<0.005	<0.005	0.03	<0.005	<0.005
	PC	<0.012	0.85	<0.003	<0.005	<0.1	0.07	<0.005	<0.005	0.02	<0.005	<0.005
Winter												
	PA	<0.02	0.57	<0.003	<0.005	<0.1	0.1	<0.005	<0.005	0.02	<0.005	<0.005
	PB	<0.02	0.13	<0.003	0.17	0.48	0.35	0.01	<0.005	0.01	<0.005	<0.005
	PC	<0.02	0.43	<0.003	<0,005	<0.1	0.08	<0.005	<0,005	0.02	<0.005	<0.005
Spring												
	PA	<0.02	2.6	<0.003	<0.005	<0.1	4.17	0.01	0.02	0.02	<0.005	<0.005
	PB	<0.02	3.15	<0.003	<0.005	0.11	5.27	<0.005	0.02	0.02	<0.005	<0.005
	PC	<0.02	3.24	<0.003	<0.005	0.11	5.23	<0.005	0.01	0.01	<0.005	<0.005

PA - upstream of the river; PB - disposal site; PC - downstream of the river

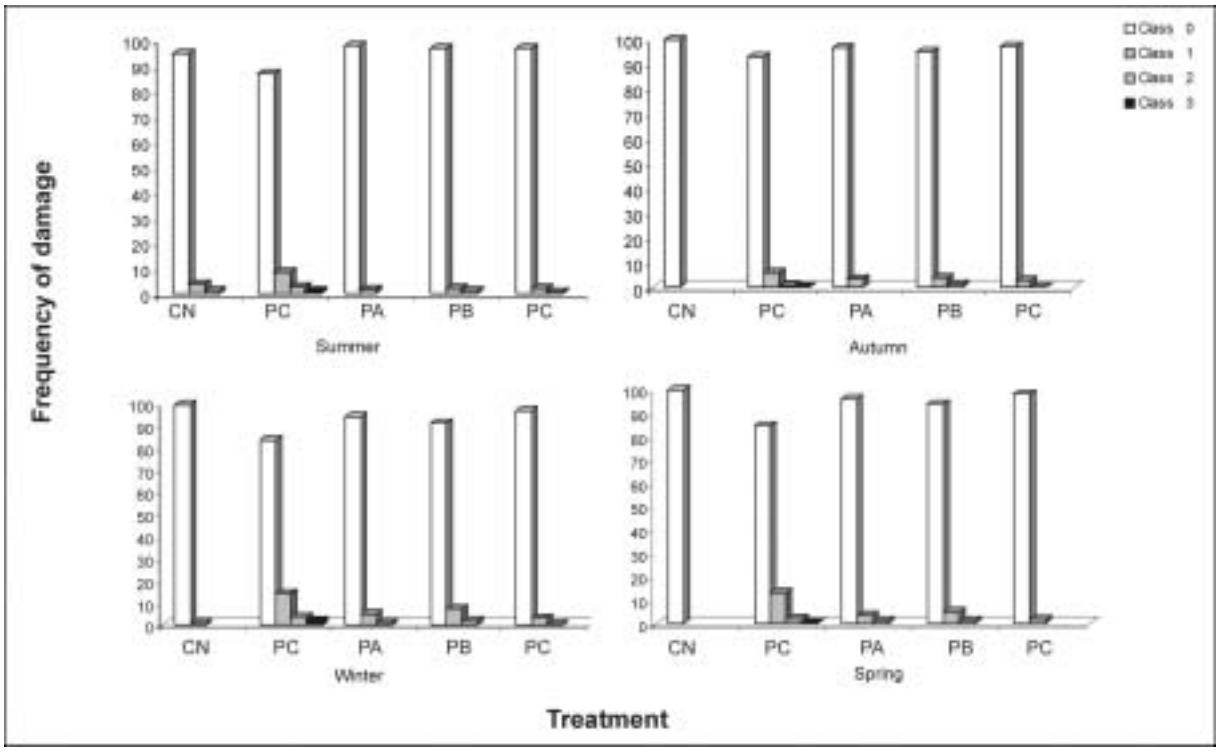

Fig. 3 — Frequency of damage observed in CHO-K1 cells submitted to a culture medium containing water samples collected from the Sapucaizinho river, for the four seasons of the year analysed.

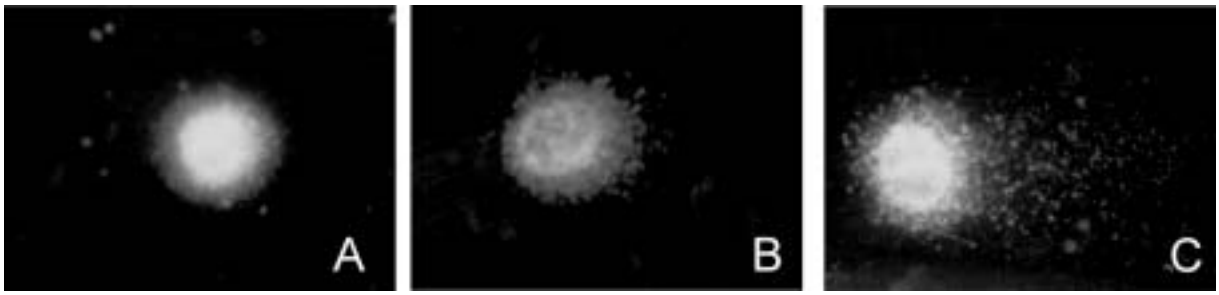

Fig. 4 — Cell without DNA damage comet class 0 (A) and cell with DNA damage: comet class 1 (B) and comet class 2 (C).

present data indicate a directly proportional relationship between the frequency of DNA damage and the concentration of chromium residues, thus confirming that chromium is a heavy metal with the potential to induce cell transformations, as also reported by Von Burg and Liu (1993) and in a previous paper by our group (Matsumoto 2003).

According to the Legislation of the State of São Paulo (1995) - Brazil, the chromium residue concentration permitted in industrial effluents discharged into Class II and III rivers is up to 0.05 mg/L. The present data indicate the presence of a genotoxic effect on CHO-K1 cell cultures as determined by the comet assay in water samples with chromium residue concentrations of less than 0.005 mg/L, confirming results obtained in a previous study (Matsumoto *et al.* 2003). These reports indicate that the genotoxic effects of chromium are already perceptible at concentrations of less than 0.05 mg/L (Table 2). Thus, leather tanneries that discharge effluents within the limits of current Brazilian Legislation may compromise the water quality of the rivers into which they dump such effluents, consequently endangering the organisms, and even man, who use these water for drinking purposes. All of our results indicate a genotoxic potential of the waters collected from the

Sapucaizinho River after they receive the tannery effluent during different seasons of the year.

The comet test proved to be efficient for the monitoring of waters that receive tannery effluents and that are contaminated with chromium. The same efficiency of the technique for environmental monitoring was also demonstrated by RAJAGURU *et al.* (2002) when they investigated the potential for genotoxicity induction of textile industry effluents, by NACCI *et al.* (1992) and PANDRANGI *et al.* (1995) who assessed the contamination of sediments with heavy metals, and by MATSUMOTO *et al.* (2003) who investigated the genotoxicity of the heavy metal chromium present in tannery effluents.

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REFERENCES

AVISHAI N., RABINOWITZ C., MOISEEVA E. and RINKEVICH B., 2002 — *Genotoxicity of the Kishon river, Israel: the apllication of an in vitro cellular assay*. Mutation research, 518: 21-37.

DEBETTO P., TOSO R.D., VAROTTO R., BIANCHI V. and LUCIANI S., 1982 — *Effects of potassium dichromate on atp content of mammalian-cells cultured in vitro*. Chemico-biological interactions, 41: 15-24.

FARBAIN D. W., OLIVE P. L. and O'NEIL K. L., 1995 — *The comet assay: a comprehensive review*. Mutation Research, 339: 37-59.

FRANSON M. A. H., 1995 — *Standard Methods for the Examination of Water and Wastewater*. 19th Ed. Washington.

GUECHEVA T., HENRIQUES J. A. P and ERDTMANN B., 2001 — *Genotoxic effects of copper sulphate in freshqater planarian in vivo, studied with single-cell gel test (comet assay)*. Mutation Research, 497: 19-27.

KASSIE F., PARZEFALL W. and KNASMÜLLER S., 2000 — *Single cell gel electrophoresis assay: a new techinique for human biomonitoring studies*. Mutation research, 463: 13-31.

KLAUDE M., ERIKSSON S., NYGREN J. and AHNSTRÖM G., 1996 — *The comet assay: mechanisms and technical considerations*. Mutation research, 363: 89-96.

KOBAYASHI H., SUGIYAMA C., MORIKAWA Y., HAYASHI M. and SOFUNI T., 1995 — *A comparison between manual microscopic analysis and computerized image analysis in the single cell gel electrophoresis*. MMS Commun, 3: 103-115.

LEE R. F. and STEINERT S., 2003 — *Use of the single cell gel eletrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals*. Mutation Research, 544: 43-64.

MATSUMOTO S.T., 2003 — *Efeitos tóxicos e genotóxicos de metais pesados, especificamente do cromo trivalente e hexavalente*. Monografia (Doutorado) Universidade Estadual Paulista. São José do Rio Preto-SP.

MATSUMOTO S. T., MANTOVANI M. S., MALLAGUTI M. I. and MARIN-MORALES M.A., 2003 — *Investigation of the genotoxic potential of the waters of a river receiving tannery effluents by means of the in vitro comet assay*. Cytologia, 68:395-401.

MITCHELMORE C. L. and CHIPMAN J. K., 1998 — *DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring*. Mutation Research, 399: 135-147.

NACCI D., NELSON S., NELSON W. and JACKIM E., 1992 — *Application of the DNA alkaline unwinding assay to detect DNA strand breaks in marine bivalves*. Marine Environmental Research, 33: 83-100.

PANDRANGI R., PETRAS M. RALPH S. and VRZOC M,. 1995 — *Alkaline single cell gel (Comet) assay and genotoxicity monitoring using bullheads and carp*. Environmental Molecular Mutagenic, 26: 345-356.

PAVLICA M., KLOBUCAR G. I. V., MOJAS N., ERBEN R. and PAPES D., 2001 —*Detection of DNA damage in haemocytes of zebra mussel using comet assay*. Mutation Research, 490: 209-214.

PRUSKI A. M. and DIXON D. R., 2002 — *Effects of cadmium on nuclear integrity and DNA repair efficiency in the gill cells of Mytilus edulis L.* Aquatic Toxicology, 57: 127-137.

PEREIRA A. A. B., 1991 — *Testes estatísticos para comparar proporções em problemas de citogenética.* In: Rabello-Gay, M.N., Rogrigues, M. A. L. R e Monteleone-Neto, R. (eds). Mutagênese, Carcinogênese e Teratogênese: *Métodos e Critérios de Avaliação*, Revista Brasileira de Genética, 113-121.

RAJAGURU P., VIDYA L., BASKARASETHUPATHI B., KUMAR P.A., PALANIVEL M. and KALAISELVI K., 2002 — *Genotoxicity evaluation of polluted ground water in human peripheral blood lymphocytes using the comet assay.* Mutation research, 517:29-37.

SILVA J., HEUSER V. and ANDRADE V., 2003 — *Biomonitoramento Ambiental. In SILVA, J.; ERDTMANN, B.; HENRIQUES, J. A. P.* Genética Toxicológica. Porto Alegre: Alcance, 8: 166-180.

SPEIT G. and HARTMANN A., 1999 — *The comet assay (Single-cell gel test)- A sensitive genotoxicity test for the detection of DNA damage and repair. In HENERSON, D. S. (ed.).* Methods in molecular biology. V.113, DNA Repair Protocols: Eukaryotic Sistems, Humana Press, Totowa, NJ, 203-212.

VON BURG R. and LIU D., 1993 — *Chromium and hexavalent chromium*. Journal of applied toxicology, 13: 225-230.