Genetic Risk Assessment of Acid Waste Water Containing Heavy Metals

E. MIADOKOVÁ[1], V. DÚHOVÁ[1], V. VLČKOVÁ[1], L. SLÁDKOVÁ[1], V. ŠUCHA[2] AND D. VLČEK[1]

1 *Department of Genetics, Faculty of Sciences, Comenius University, 842 15 Bratislava, Slovakia*
2 *Department of Mineral Deposit Geology, Faculty of Sciences, Comenius University, 842 15 Bratislava, Slovakia*

Abstract. The mutagenic/cancerogenic potential of acid-mine water from the Slovak mining area Rudňany containing a high load of toxic metals was evaluated after its application to three model test organisms (bacteria *Salmonella typhimuriumn,* yeast *Saccharomyces cerevisiae* and plant *Vicia sativa* L.). The results obtained from the modified preincubation Ames assay proved that 1000-fold diluted waste water exhibited mutagenic effect in three (TA97, TA98, TA102) of four bacterial strains. In the test on yeast the toxicity and genotoxicity increased as a function of the concentration. At the highest concentration used (0.06 %) the frequency of revertants increased 6 times and convertants increased 4.5 times above the control level. In the simultaneous phytotoxicity and clastogenicity assay, concentration dependent toxicity and statistically significant clastogenicity was proved. We can conclude that heavy metals might be responsible for the genotoxic/cancerogenic potential of the test water. However, we do not entirely exclude the possibility that its genotoxicity might be promoted by its high acidity.

Key words: Mutagenic/cancerogenic potential — Heavy metals — *Salmonella typhimurium* preincubation assay — *Saccharomyces cerevisiae* assay — Simultaneous phytotoxicity and clastogenicity assay

Introduction

Our environment is polluted due to a variety of anthropogenic activities. Environmental agents implicate potential genetic risk for man because they can be genotoxic, with a potential to induce mutations either in germinal or somatic tissues. The detection of mutagens and determination of the types of mutation induced are of importance to the understanding of the etiology of cancer and other degenerative

Corespondence to: Eva Miadoková, Department of Genetics, Faculty of Sciences, Comenius University, Mlynská dolina B-1, 842 15 Bratislava, Slovakia.
E-mail: Miadokova@fns.uniba.sk

diseases that involve mutations (Gee et al. 1994). Heavy metals belong to important environmental pollutants. They are introduced into the environment from various sources (fuel combustion, mining and industry, agricultural chemicals, liquid and solid communal waste etc.). Biological activities (e.g., physiological, biochemical, genetical etc.) of metal ions have prompted a huge number of studies on the mechanism and range of their effects (Kráľová et al. 1994; Šeršeň et al. 1997; Fargašová 1997). Cancer epidemiology has identified several metal compounds as human carcinogens. Recent evidence suggests that carcinogenic metals induce genotoxicity in many diferent ways, either themselves or by enhancing the effect of other agents (Snow 1992). According to Hartwig (1995) two modes of action seem to be predominant: enhanced formation of reactive oxygen and other radical species leading to oxidative DNA damage, and interference with DNA repair and/or DNA replication processes.

The goal of our research work was to assess if the acid water (containing high amounts of toxic metals) from the devastated Slovak mining area of Rudňany was mutagenic /cancerogenic upon its application to three model test organisms (bacteria, yeast and plant).

Materials and Methods

Toxic metals determination

A sample of the acid-mine effluent was collected at the original source of acidification (a dump site in the mining area of Rudňany, Slovakia) Toxic elements listed in Table 1 were determined by atomic absorption spectroscopy (AAS) SO_4^{2-} ions were analyzed by gravimetric analysis The arsenic content was assessed by using AAS with the hydride generation technique Aliquots of the stock sample (100 %) were diluted with sterile distilled water

Table 1. Concentrations of toxic metals in the acid water from Rudňany with the limits given by the Slovak Standards for Drinking Water (STN 75 7111), and the respective pH values

Element	Concentration (mg/l) Limit	Rudňany	Exceeding of limits
As	0 05	0 00087	
Cd	0 01	0 328	33 ×
Cu	0 1	1490	14900 ×
Fe III	2 0	17 5	9 ×
Mn	0 3	28 5	95 ×
Pb	0 05	2 7	54 ×
Zn	0 1	45 9	459 ×
SO_4^-	250	3652	15 ×
pH	6 to 8	2 5 to 3 5	

Salmonella typhimurium assay (the Ames test)

Salmonella typhimurium tester strains TA97, TA98, TA100 and TA102 were kindly provided by Dr B N Ames (Berkeley, CA, USA) The modified preincubation assay was conducted in test tubes by adding 0 1 ml of the overnight bacterial culture (approximately 1×10^8 cells/ml, cultivated in 50 ml of LB medium) (Maron and Ames 1983) to the test (1000-fold diluted) effluent in the appropriate concentrations (from 100 to 500 μl/plate), and in the case of metabolic activation 0 5 ml of S9 mixture was supplemented As positive controls the following mutagens/carcinogens were used 9-aminoacridine (9-AA, 100 μg/plate), 4-nitroquinoline-N-oxide (4NQO, 0 2 μg/plate), natrium azide (NaN_3 5 μg/plate), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 10 μg/plate) and 2-aminofluorene (2-AF, 100 μg/plate) Tubes were incubated for 24 h at 37 °C without shaking 2 5 ml of top agar were added to each tube, the content was mixed, and plated on minimal bottom agar plates His^+ revertants were counted after 72 h of incubation at 37 °C on Biotran III Colony Counter (New Brunswick Scientific Co) For each bacterial strain and activation conditions (−S9, +S9) there were three plates for the negative control, three plates for the appropriate positive control and three plates for each of the five concentrations (from 100 to 500 μl/plate) of the test sample Three consecutive experiments were performed

Saccharomyces cerevisiae assay

As a testing procedure the assay according to Zimmermann et al (1975) was used Prior to each experiment the D7 strain (*MATa/MATα, ade*2-40/*ade*2-119, *trp*5-12/*trp*5-27, *ilv*1-92/*ilv*1-92) was tested for the frequency of spontaneous convertants at the tryptophan (*trp*) locus and revertants at the isoleucine (*ilv*) locus Exponentially growing cells were treated with the test acid water at the concentration range from 0 03–0 06 % for 24 h After the treatment, washed cell suspensions were plated on appropriate media according to Zimmermann (1984), i e , $1–2 \times 10^6$ cells/plate to detect revertants on a selective medium without ilv, $1–2 \times 10^5$ cells/plate to detect convertants on a selective medium without trp, and 2×10^2 to 2×10^3 (based on survival) on synthetic medium on which mitotic crossing-over and other changes at the adenine locus and survival were evaluated The plates were incubated at 28 °C for 5–12 days Five consecutive experiments were performed

Vicia sativa assay

After 24 h of soaking at 25 °C in the test acid water (0 75–6 0 %), the seeds of *V sativa* were allowed to germinate on Petri dishes (diameter = 18 5 cm) with filter paper soaked with the same concentrations of test waste water as those used for soaking Phytotoxicity was assayed after 72 h of the dark cultivation in the thermostat The seedlings roots of *V sativa* were measured, and the growth inhibition percentages were assessed The seedlings in which the root growth was inhibited at least by 25%, 50% and 75% were fixed and used for chromosome and genome mutability evaluation The roots were fixed and permanent slides were prepared by the Feulgen method Chromosome aberrations were determined at least in 500 ana-telophases As a positive control 11 21 μg/ml of maleic hydrazide (MH) was used Kanaya et al (1994)

Results and Discussion

The results of the chemical analyses of toxic metals in the acid water from Rudňany are presented in Table 1. The metal concentrations highly exceeded those set by

Table 2. Modified preincubation *Salmonella typhimurium* assay of 1000-fold diluted waste water from Rudňany

Test agent	Concentration (μl/plate; μg/plate)	Frequency of His^+ revertants / plate							
		TA97		TA98		TA100		TA102	
		−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Control	100	87±7.9	88±6.3	30±1.6	30±3.0	159±10.2	158±14.2	246±19.3	247±18.1
Waste	500	69±4.8*	45±2.5*	28±1.8	33±1.5	255±19.2*	442±49.5*	235±14.2	335±25.4*
Acid	250	231±14.4*	43±2.6*	29±2.6	25±1.4	199±13.1*	432±43.3*	226±25.9	321±14.5*
Water	200	221±12.9*	48±2.1*	40±3.0	28±2.0	187±15.0*	380±25.7*	235±10.3	302±20.0*
from	150	172±14.6*	50±2.8*	33±1.5	31±1.7	180±12.6*	300±19.7*	240±17.3	290±19.3*
Rudňany	100	160±11.5*	35±3.9*	33±2.0	27±2.9	157±11.5	270±23.4*	210±18.8	285±14.9*
9-AA	100	221±17.3*	–	–	–	–	–	–	–
4-NQO	0.2	–	–	546±28.0*	–	–	–	–	–
NaN_3	50	–	–	–	–	536±27.4*	–	–	–
MNNG	10	–	–	–	–	–	–	978±32.1*	–
2-AF	100	–	376±22.7*	–	316±30.7*	–	415±36.3	–	479±31.4*

* significant difference in comparison with control ($p < 0.05$) ; control = sterile distilled water

Table 3. Survival and genetic changes in the cells of yeast *Saccharomyces cerevisiae* D7 after the application of the acid water from Rudňany

Waste water	Survival (%)	[1]Mitotic crossing-over	[2]Total aberrants (%)	Number of convertants per 10^5 survival cells	Number of revertants per 10^6 survival cells
Control	100 00 (8200)	– (0)	0 23 (19)	1 99 (239)	4 40 (253)
0 03	51 86 (14298)	– (0)	0 48 (69)	3 53 (144)	9 96 (230)*
0 04	33 75 (18886)	– (0)	0 54 (102)	5 03 (139)*	14 82 (248)*
0 05	23 64 (48266)	– (0)	0 57 (275)	9 20 (255)**	20 61 (248)**
0 06	20 17 (59629)	– (0)	0 67 (402)	8 96 (225)**	25 91 (276)*

* significant difference in comparison with control ($p < 0\ 05$), ** significant difference in comparison with control ($p < 0\ 01$), control = sterile distilled water, [1] Mitotic crossing-over between centromere and *ade2* locus is manifested as twin spotted (pink/red) colonies among white colonies, [2] Total aberrants are further changes observed in *ade2* locus (pink, red, white/pink, white/red, white/pink/red colonies) which reflect cumulative mitotic segregation due to mitotic gene conversion, chromosome loss or smaller structural deletions and point mutations Numbers in the parentheses indicate the actual numbers of colonies

Slovak Standards (STN 758 7111) for drinking water. The difficulty to detect genotoxicity of metals in bacteria has been attributed to the lack of their bioavailability to the cells (Hartwig 1995). As toxic metals can be detected as mutagens under conditions that enhance their bioavailability to the bacterial cell (Pagano and Zeiger 1992) a modified preincubation assay had to be used (Table 2) Due to the extremely high toxicity it was not possible to assess mutagenicity/cancerogenicity of the non-diluted water (100 %) Data in Table 2 show that 1000-fold diluted water (0.1 %) exhibited mutagenic effect in three of the four tester *S. typhimurium* strains It was mutagenic in the strain TA97 in the absence of S9 mix, but in its presence, the potential mutagenic effect was overlaped with the increased toxicity In the strain TA100 the test water was mutagenic both in the absence and in the presence of S9 mix, and in the strain TA102 only after the rat liver S9 activation. We can conclude that the test effluent induced both frameshift mutations and base pairs substitutions, and behaved as a directly-acting mutagen in the strain TA100. Pagano and Zeiger (1992) employed a similar approach and could prove the mutagenic effect of divalent heavy metals in the strain *S typhimurium* TA97 (detecting frameshift mutagens).

Data listed in Table 3 indicate that the test water was toxic and genotoxic in yeast. The toxicity and genotoxicity increased as a function of the concentration. At 0.06 % concentration the frequency of revertants at the *ilv* locus was increased 6 times, the frequency of convertants at *trp* locus was increased 4.5 times, and the frequency of total aberrants at the *ade* locus was increased 3 times above the control level.

The test water was found to exhibit a considerably strong phytotoxic effect

Table 4. Phytotoxicity evaluation of the waste acid water from Rudňany on *V sativa*

Concentration	pH	Number of germinated seeds	Root lenght	Growth inhibition
%			(mm)	%
Control	6 50	30	48 1±19 0	0 00
0 75	3 90	25	37 7±18 9	21 63
2 0	3 35	30	19 1±10 4	60 30
4 0	3 18	30	13 6±7 4	71 73
6 0	3 05	28	9 8±6 7	79 63
MH 11 21 μg/ml	3 55	30	31 9±12 5	33 68

control = sterile distilled water, MH = maleic hydrazide

Table 5. Clastogenicity evaluation of the waste acid water from Rudňany on *V sativa*

Concentration (%)	Total number of cells evaluated	Fragments	Bridges	Others	Total number of aberrations	Aberrations (%)
Control	1003	1	2	–	3	0 30±0 17
0 75	733	2	1	–	3	0 54±0 27 –
2 0	925	5	5	1	11	1 19±0 35 *
4 0	516	2	6	1	9	1 74±0 57 **
6 0	166	–	4	–	4	2 46±1 20 **
MH 11 21 μg/ml	500	18	9	24	51	10 20±1 35 **

* significant difference in comparison with control ($p < 0\ 5$), ** significant difference in comparison with control ($p < 0\ 01$), control = sterile distilled water

on *V sativa* (Table 4) The increase of its toxicity was concentration dependent The data summarized in Table 5 suggest its clastogenic effect on *V sativa* root-tip meristems

Some heavy metals (e g., Pb, Cd, Hg) are known contaminants and they are of toxicological significance (Schuhmacher et al. 1993), whereas others such as Cr, Co, Cu, Mn, Zn, Fe are essential microelements (Sommers 1974). These essential metals can be mutagenic, comutagenic or cancerogenic when their concentrations exceed acceptable limits (Babich et al. 1985; Hartwig 1995). On the basis of chemical analysis of the test water (Table 1) we can conclude that metals (Cu, Mn, Pb, Zn, Fe, Cd) the concentrations of which many times exceed limits for drinking water might be mainly responsible for the genotoxicity of the waste water tested. However, we do not exclude that the mediation of mutagenicity and clastogenicity

effects of heavy metals by some physicochemical factors (Babich et al 1985), e.g., acidic environment may play a role in, similarly as observed upon the potential risk assessment of the acid-mine drainage containing multiple heavy metals from the former mining area Banská Štiavnica-Šobov, Slovakia (Miadoková et al 1998)

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