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Short Communication

IN VITRO GENOTOXICITY OF SETTAT TOWN LANDFILL LEACHATE, MOROCCO

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With the increasing use of landfill sites, leachates produced by uncontrolled waste disposal have became a serious threat for the aquatic environment. The aim of this study was to evaluate the genotoxicity of leachate and of well water sampled close to the town of Settat in Morocco using the micronucleus test and proliferation kinetics of human peripheral blood lymphocytes *in vitro*. We also analysed a number of physical and chemical parameters, including pH, % O_2, chemical oxygen demand (COD), HCO_3^-, Ca^{2+}, Mg^{2+}, Cl^-, and conductivity.

The analysis showed much higher levels of nearly all parameters than the Moroccan standard. Increased micronucleus frequencies were also found for both leachate and well water. Preliminary results indicate that both types of water are genotoxic and pose environmental and human health risk.

KEY WORDS: *dose-effect, lymphocyte, micronucleus, physico-chemical properties, proliferation index, toxicity*

Solid waste management is a crucial environmental problem today, with its amount continuously increasing (1). In Morocco, the most usual way to dispose of waste is still the dump. As waste changes physically, chemically, and biologically, leachates are formed that enter and interact with the environment (2). This is how leachates containing high amounts of pollutants (3, 4) can contaminate ground and surface waters and affect biodiversity of aquatic ecosystems, contaminate food chains, and pose a risk to human health. Several *in vitro* and *in vivo* studies of the ecotoxicological impact of environmental chemicals have confirmed that many chemicals identified at city dumps and in leachates have a genotoxic and carcinogenic potential (5-12). Determination of the chemical composition and the genotoxic potential of wastewaters, surface, and ground waters is considered crucial for environmental protection and public health (13). It is also important to assess the toxicity of complex mixtures such as leachates or of effluents, as current chemical checks can not reliably identify the risk for human health and wildlife. Instead, biomonitoring studies can give comprehensive information about bioeffects which may not be easily identified with specific chemical analyses (14). This *in vitro* study investigated the potential genotoxicity of a leachate from a city dump by exposing human white blood cells to increasing amounts of leachate and ground water sampled near the landfill site.

STUDY DESIGN AND METHODS

The landfill site is situated 7 km away from the outskirts of the Moroccan town of Settat and 2 km to the west from the road connecting Settat to Oulad Saïd at 438 m above sea level. With a surface of some 20 ha it has been in uncontrolled use since 1984. It receives approximately 170 t of untreated domestic, industrial, and hospital waste per day. Leachate is formed and

drained under the heaps of waste threatening to contaminate local ground waters. In order to evaluate the level of contamination of local ground waters and to estimate their genotoxic potential, we conducted a physicochemical and cytogenetic analyses of leachate and well water sampled close to the landfill site.

Sampling

We sampled 1500 mL of leachate and well water from a well located 10 m away from the landfill site. Samples were kept in polyethylene bottles and transported to the laboratory at 4 °C.

Physicochemical analysis

Physicochemical analyses of leachate, well water, and control samples were performed according to standard protocols (15, 16).

Cytogenetic analysis

Venous blood (10 mL) was collected in a sterile heparinised tube from a healthy female donor (age 27 years, non-smoker) who gave informed consent for participation in the study. Cells were then cultured according to a slightly modified standard protocol for the micronucleus (MN) test (17). Briefly, 0.5 mL of whole blood was cultured under sterile conditions in a culture tube containing 5 mL of RPMI 1640 medium supplemented with 15 % foetal calf serum (Sigma-Aldrich Chemie GmbH), 1 % streptomycin-penicillin (Sigma-Aldrich Chemie GmbH), and 1 % phytohaemagglutinin (CAS # 14930-96-2, Sigma-Aldrich Chemie GmbH). Leachate or well water were added in increasing volumes (from 100 µL to 1500 µL) to the culture tubes. Volumes were increased essentially to determine those suitable for future experiments, but also to avoid confounding cytotoxic effects caused by other factors such as changes in osmolarity. We used tap water as negative control, since it is the most consumed type of drinking water.

Cell cultures were incubated in humified atmosphere with 5.0 % CO_2 at 37 °C. Cytochalasin B (CAS # 9008-97-3, Sigma-Aldrich Chemie GmbH) was added to the cultures at the final concentration of 4 µg mL^{-1}, at 44 h. Cultures were harvested after a total cultivation time of 72 h following a hypotonic shock (7.5 mmol L^{-1} KCl) and fixation with acetic acid : methanol (1:3). Slides were stained for light microscopy (x400) with 5 % Giemsa. Each was examined for the presence of micronuclei using the criteria of Fenech et al. (18). Our goal was to investigate 1000 binucleated cells per sample, but in some instances this amount was not reached (see Table 2).

The proliferation index (PI) was calculated according to the following formula (19):

$$PI = \frac{(1\times N_1)+(2\times N_2)+(3\times N_3)+(4\times N_4)}{1000 \text{ cells analysed}}$$

where N_1-N_4 is the number of cells with 1-4 nuclei, respectively.

Statistical analysis

The statistically significant difference between the control and treated samples was determined using the ANOVA.

RESULTS AND DISCUSSION

In addition to residues from bacterial activity, dump leachates contain a mixture of organic and inorganic substances that can react with each other. Sooner or later they will enter ground water (20). This was confirmed by our physicochemical analysis, especially for calcium, magnesium, and chloride concentrations. COD, conductivity, and alkalinity were also very high both in the leachate and in the well water (Table 1). Our study also confirmed that due to migration, leachate constitutes a very important source of pollution, especially for the aquatic environment (ground and surface waters) (21).

MN frequency and lymphocyte proliferation index gave us an insight into the genotoxic potential of the leachate *in vitro*. The MN test is a well-known and validated endpoint that can predict increased cancer risk in a human population (22). This test has an advantage over tests with bacteria such as the Ames assay, which are not sensitive enough for water and soil leachate genotoxicity evaluations, especially when heavy metal contamination is anticipated (12, 23). Nowadays the MN test is sometimes accompanied by counting apoptotic and necrotic cells, nuclear buds, and nucleoplasmic bridges (the cytome assay) (24).

Our study is a preliminary investigation limited to the "classical" MN test alone. We investigated a dose-effect relationship based on the MN frequency and changes in the proliferation index of lymphocytes treated with increasing volumes of leachate, well water, and tap water.

Table 2 shows that MN frequency increased with leachate volume. At an intermediate volume of 800 µL we found up to 48 MNs per 1000 binucleated cells. However, in samples treated with higher leachate volumes, almost no binucleated cells were found, indicating cytotoxicity. Taking into account the results obtained on negative control samples, we assume that cyto/genotoxicity was mediated through leachate components rather than through changes in osmolarity. Indeed, drinking water added to cell cultures in volumes up to 1000 µL did not induce a marked decrease in binucleated cells. We cannot make any firm conclusions about MN frequency in samples with only a few binucleated cells.

High micronucleus frequency was also found in lymphocytes treated with 100 µL of well water (23 MNs per 1000 binucleated cells). Similar to leachate, higher volumes of well water (>1200 µL) led to a marked decrease in binucleated cells, most probably due to toxicity. This is in accordance with an earlier study on *Vicia faba* (11) in which apoptosis was induced by genotoxic agents in water of wells located in the vicinity of dumps.

PI showed more or less the same behaviour as MN frequency. It somewhat increased in samples treated with 400 µL and 800 µL of leachate and decreased at volumes ≥1000 µL. This may again indicate toxicity and inhibition of cell division at higher volumes.

Table 1 *Results of the physicochemical characterisation of leachate, well water, and tap water*

Samples	pH	O_2/ %	COD / mg L^{-1}	HCO_3^- / mg L^{-1}	Ca^{2+} / mg L^{-1}	Mg^{2+} / mg L^{-1}	Cl^- / mg L^{-1}	Conductivity / µS cm^{-1}
Leachate	8.07	8.8	5866.67	4514	352.704	203.674	7668	15170
Well 1	7.34	38	2623.33	128.1	184.368	114.982	1136	4630
Drinking water	7.08	76	-	42.7	8.02	7.29	78.8	-
Moroccan Standard	6.5 to 8.5	70	25	-	-	50	750	2700

COD - chemical oxygen demand

Table 2 *Results of the cytogenetic tests*

Samples	Volume added to the cultures / µL	No. of binucleated cells analysed	Total No. of MN	MN frequency	No. of cells with MN	Cells with 1 MN	Cells with 2 MNs	Cells with 3 MNs	Cells with 4 MNs	Cells with 5 MNs	PI
Control	0	1007	4	0.004	4	4	0	0	0	0	1.062
Leachate	100	1000	3	0.003	3	0	0	0	0	0	1.063
	200	163	15	0.055	8	4	2	1	0	1	1.009
	400	1072	47	0.044	36	25	11	0	0	0	1.113
	800	1000	48	0.048	41	34	7	0	0	0	1.036
	1000	13	9	0.69	4	1	1	2	0	0	1.001
	1200	9	2	0.44	2	2	0	0	0	0	1.003
	1500	22	4	0.09	3	1	0	1	0	0	1.004
Water from the well	100	1117	24	0.02	23	22	1	0	0	0	1.082
	200	1000	11	0.011	11	11	0	0	0	0	1.025
	400	1048	4	0.004	4	4	0	0	0	0	1.043
	800	1010	10	0.001	10	10	0	0	0	0	1.038
	1000	108	7	0.065	6	5	1	0	0	0	1.007
	1200	180	7	0.039	4	1	0	0	1	0	1.006
	1500	163	1	0.006	1	1	0	0	0	0	1.011
Drinking Water (negative control)	100	1000	5	0.006	4	3	1	0	0	0	1.104
	400	661	5	0.007	5	5	0	0	0	0	1.018
	1000	1010	14	0.014	13	12	1	0	0	0	1.052
	1500	609	9	0.015	9	9	0	0	0	0	1.013

Table 3 *Regression analysis of the proliferation indices and MN frequency*

	Factors tested	R	F	P
Proliferation Index	Leachate/ Well Water	0.71	6.1370	<0.05
	Leachate/Tap Water	0.25	0.3837	NS
	Well Water/Tap Water	0.47	1.7040	NS
Frequency of micronuclei	Leachate/Well Water	0.88	20.943	<0.01
	Leachate/Tap Water	0.43	1.3866	NS
	Well Water/Tap Water	0.42	1.2749	NS

NS - non significant

In general, PI fluctuated a lot, and the differences between treatments were not clear (Table 2). Regression analyses showed that lymphocyte response was significantly different only between leachate and well water (Table 3). There was no significant correlation between MN frequency and PI.

It appears that the addition of volumes up to 1000 µL is suitable for (geno)toxicity assessment of water. Our findings for dump leachate and ground water are in accordance with several other studies (6, 9, 12, 20, 26, 27). Sang et al. (26) found cytogenetic damage in root tips of *Hordeum vulgare* exposed to municipal landfill leachate. Leachate decreased the mitotic index and significantly increased MN frequencies in a dose- and time-dependent manner. Sang and Li (20) had similar findings for *Vicia* faba root tips. Lah et al. (12) recently applied an integrated physico-chemical-biological approach to evaluate genotoxicity of soil *in vitro*. They performed the alkaline comet assay in Caco-2 and HepG2 cells exposed to water soil leachates. Samples were evaluated for genotoxicity with parallel Ames and *Tradescantia* micronucleus tests. Genotoxicity of all water soil leachates was demonstrated with the comet assay, but the Ames test yielded no positive results. The *Tradescantia* micronucleus assay showed increased MN frequency in half the samples. The authors concluded that the comet assay was the most sensitive assay, followed by the micronucleus test.

In conclusion, our *in vitro* study on human white blood cells confirms the genotoxic risk of pollutants present in leachates and well water collected near the Settat dump. It also demonstrates the usefulness of combining physicochemical analysis with cytogenetic methods in order to better understand the toxicity of chemical pollutants and their influence on health. Physicochemical analysis is necessary to determine the nature of the pollutants and to propose a suitable method of water treatment whereas cytogenetic analysis is useful to detect genotoxic effects of the complex mixture that constitutes the leachate. Indeed, the effect of chemical interactions and the influence of complex matrices on toxicity cannot be determined from chemical tests alone.

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Sažetak

IN VITRO ISPITIVANJE GENOTOKSIČNOSTI PROCJEDNIH VODA IZ ODLAGALIŠTA OTPADA GRADA SETTATA U MAROKU

Sve veća uporaba i stvaranje procjednih voda iz nekontroliranih odlagališta krutoga otpada postali su ozbiljna prijetnja vodenom okolišu. Cilj je ovog istraživanja bio procijeniti genotoksičnost takvih procjednih voda te podzemnih voda uzorkovanih iz bunara u blizini odlagališta otpada grada Settata u Maroku. U tu svrhu rabili smo mikronukleusni test *in vitro* i usporedno istražili kinetiku proliferacije limfocita periferne krvi zdrave dobrovoljne ispitanice. Osim toga, analizirano je više fizikalno-kemijskih parametara (nitrati, ortofosfati, nitriti, pH, otopljeni kisik, kemijska potrošnja kisika, temperatura, zamućenost vode). Te su analize procjednih voda i vode iz bunara pokazale brojna odstupanja od propisanih marokanskih standarda. Usto je u limfocitima izlaganim ovim vodama utvrđena i povišena učestalost mikronukleusa. Preliminarni nalazi pokazuju da su obje vrste voda genotoksične i da su mogući izvor rizika za okoliš i ljudsko zdravlje.

KLJUČNE RIJEČI*: fizikalno-kemijska svojstva, indeks proliferacije, limfociti, mikronukleusni test, toksičnost, učinak ovisan o dozi*

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