EVALUATION OF DNA DAMAGE BY THE WASTEWATER OF MARRAKESH

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The present study was aimed to integrate genotoxicity test with routine toxicity test in 28 days repeated dose oral toxicity of Marrakesh's wastewater in mice. Wastewater was administered at the dose of 25%, 50% and 75% per oral repeatedly for 28 days in mice. The endpoints of evaluation for routine toxicity testing included body weight (*BW*), organ weight (*OW*), food intake (*FI*), water intake (*WI*), hematology (*HE*) and histology (*HI*), while for the genotoxicity testing micronucleus (*MN*) and chromosomal aberration (*CA*) assay were used. The body weight (*BW*) significantly decreased at the highest dose of wastewater treatment as compared to the control group. Histological data (HI) revealed morphological alterations in the liver and lung cells at the highest dose of wastewater treatment. Micronucleus (*MN*) assay results indicated that the highest dose of wastewater led to a significant increase as compared to the control group. Chromosomal aberration (*CA*) assay results indicate significant DNA damage in different organs induced by wastewater as compared to the control group. Integration of a genotoxicity test with routine toxicity test would reduce the cost of additional animals and test items, while also providing further information at an early stage of product development.

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INTRODUCTION

Many studies on genotoxicity of industrial wastewater, sludge/effluents, and soil in short-term bioassays and gene mutations in Salmonella strains have already been reviewed.[1] Bioluminescence inhibition assays have demonstrated the cytotoxic impact on *Vibrio fischeri* by wastewaters and *Photobacterium phosphoerum* by small and large industrial discharges (Microtox and Tox–Alert tests),[2] and notable genotoxic alterations were reported through in vivo tests on aquatic amphibian larvae *Xenopus laevis* exposed to aqueous samples of industrial waste.[3] Plant bioassays provide meaningful parameters to assess the toxicity of complex mixtures like industrial wastes even without knowledge of its chemical composition. Previously many workers have successfully employed plant bioassays viz. The Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) legislation emphasizes that the new technologies require a shift on the way in which safety assessment is conducted for different chemicals. Further, it has been realized that the Integrated Testing Strategies (ITSs) are already in use for toxicological evaluation, but the concept of their integration and application in a regulatory setting have yet to be fully implemented.[4] The Joint Research Centre of the European Commission estimated that the highest number ofinvivo genotoxicity tests are essential to evaluate the chemicals under REACH program.[5] In a recent workshop hosted by the European Centre for the Validation of Alternative Methods (ECVAM) held from 24 to 25 June 2008, it was emphasized that integration of genotoxicity endpoints into the repeated-dose toxicity study in a scientifically justified manner can reduce the number of animals used in toxicity testing.[6]

MATERIALS AND METHODS

Animals: Experiments were performed on male mice (weight range in between 22 and 25 g) kept at controlled environmental conditions with room temperature (22±2 °C), humidity (50±10%) and automatic controlled dark and light cycles (0600–1800 h). Six animals were used in each group. Standard laboratory animal feed and water were provided from Morocco. Animals were acclimatized to the experimental conditions for a period of 1 week before the initiation of the experiment.

Study site: Industrial wastewater was taken from Draa Lasfar mine located about 12 km south-West (Fig. 1) of the city of Marrakech, Morocco and are part of a systematic wastewater quality monitoring program performed on a monthly basis.

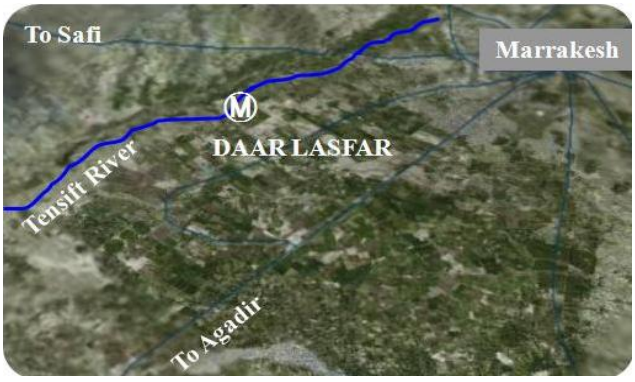

Figure 1. Geographic location of the sampling sites.

Each wastewater sample was collected monthly over a three month period (from April to June). It was transported in 1 L bottles and refrigerated at 4 °C until required for analysis and exposure experiments. The sample was mixed in varying proportion with distilled water to obtain the different percent of wastewater in mixtures (0, 5, 10, 25, 50, 75, and 100 %) for the toxicity test.

Dose selection and animal treatment: The present doses of wastewater were selected based on our own experiences. The wastewater was given at the doses of 5, 10, 25, 50, 75, and 100% per orally. Animals were divided in group I to IV (n=6) and they received vehicle 5, 10, 25, 50, 75, and 100% of wastewater, respectively. The volume administered was 10 ml per day for 28 days.

Animal observations: All animals were observed twice daily for any toxicity, morbidity and mortality. Body weights were immediately recorded pretest, weekly and on the day prior to sacrifice. On day 28, animals were fasted overnight and on day 29, animals were sacrificed.

Histopathology: The liver and lung were isolated for histopathology. Tissues were fixed in 10 % formalin solution. Paraffin blocks were prepared after completing the tissue processing in different grades of alcohol and xylene. Sections (5 micro m) were prepared from paraffin blocks using microtome, stained with hematoxylin and eosin. Images were taken using an OLYMPUS camera connected to the microscope.

Estimation of oxidative stress markers malondialdehyde (MDA) and glutathione (GSH) level in liver and brain: MDA level in the liver and brain was measured according to the method described by.[7] Further the liver and brain tissue homogenate were used for the estimation of glutathione (GSH) content as described by.[8] Protein content in tissue homogenate supernatant was determined as described by.[9]

Peripheral blood micronucleus chromosomal aberration assay: Blood samples were collected by cardiac puncture at the time of sacrifice for hematological analysis. A peripheral blood smear was prepared as described by Holden et al. with some modifications.[10] Blood was collected from the tail tip before the sacrifice of the animal and the blood smear was prepared on pre-cleaned slides. The smear was allowed to dry at room temperature and fixed in absolute methanol for 5 min. After fixation slides were stained with acridine orange (AO) and washed twice with phosphate buffer (pH 6.8).[11] Slides were observed under oil immersion objective (10) using an Olympus fluorescent microscope. In total, 2000 cells were scored for the incidence of MN and chromosomal aberration in the erythrocytes, which includes both immature reticulocytes (RETs) as well as mature normocromatic erythrocytes (NCEs).

Statistical analysis: Results were shown as means ± SEM for each group. Statistical analysis was performed using the statistical programme SigmaStat 3.0 (SPSS Inc., 233 South Wacker Drive, 11th Floor, Chicago, IL 60606-6307) software package. For multiple comparisons, one-way analysis of variance (ANOVA) was used. In this case, ANOVA showed significant differences. Post hoc analysis was performed with Tukey's test. $P < 0.05$ was considered statistically significant. Differences between corresponding controls and exposure treatments were considered statistically significant at 0.05.

RESULTS

General observation, body weight, organ weight, feed intake and water intake: No mortality was observed in the 28 days repeated dose oral toxicity testing in mice using wastewater. There were no statistically significant differences observed in the food and water intake (data not shown). Body weight was taken once in a week and significant differences in the body weight ($P < 0.01$) was observed at the highest dose of wastewater as compared to the control (Fig. 2). After sacrificing the animals, organ weight was measured (liver, heart, brain, lung and kidney) and no significant difference was found in the organ weight (Table 1).

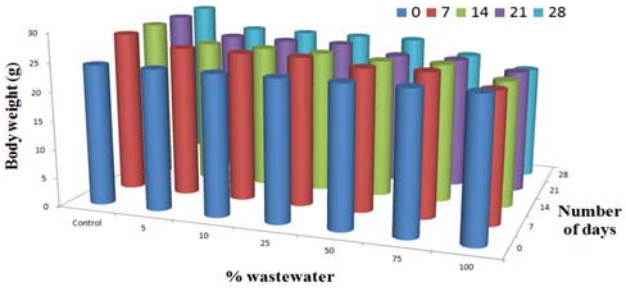

Figure 2. Effect of wastewater treatment on the body weight of mice in 28 days repeated oral toxicity study.

Histological examination: Toxicity at the cellular level was evaluated by histological examination of liver and lung tissue. Treatment with wastewater led to extensive vacuolization in the liver cells. There was considerable irregularity in nuclear size in wastewater treated mice, in which hepatic nuclei were significantly larger than those of the control animals. Further, histology of the lung revealed that treatment with wastewater led to diffused alveolar damage that included consolidation and honey combing as well as infiltration that included reticular shadows and ground glass shadows (Figs. 3 and 4).

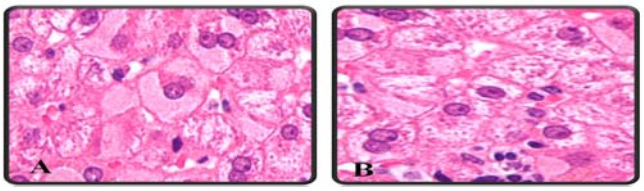

Figure 3. Photomicrographs showing the liver sections of mice stained with hematoxylin and eosin (H&E) at magnification 100°. Control animal receiving only vehicle (A) and animal receiving the highest dose of wastewater 100% (B). Wastewater treatment led to extensive vacuolization of nuclei (shown in the encircled area) in 28 days repeated oral toxicity study.

Table 1: Effect of wastewater treatment on different organs weight in 28 days repeated oral toxicity study in mice. All values are expressed as means ± SEM (n = 6).

Treatment	Liver (g)	Heart (g)	Brain (g)	Lung (g)	Kidney (g)
Control	1.20±0.01	0.18±0.01	0.40±0.01	0.26±0.02	0.39±0.02
5%	1.11±0.05	0.18±0.01	0.40±0.01	0.26±0.01	0.34±0.01
10%	1.09±0.01	0.18±0.01	0.38±0.04	0.25±0.02	0.34±0.03
25%	1.08±0.01	0.18±0.03	0.39±0.07	0.20±0.03	0.33±0.06
50%	1.08±0.03	1.06±0.03	1.37±0.03	0.20±0.03	0.18±0.03
75%	1.04±0.02	1.06±0.03	1.37±0.03	0.19±0.03	0.18±0.01
100%	1.00±0.03	0.16±0.03	0.37±0.09	0.19±0.03	0.18±0.06

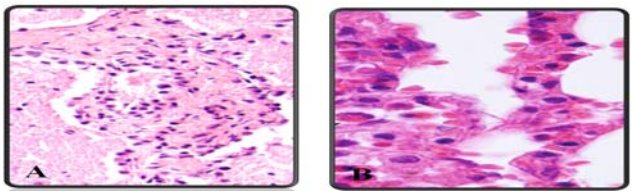

Figure 4. Photomicrographs showing the alveoli density in lung sections of mice stained with H&E at a magnification 100° Control animal receiving only vehicle (A) and animal receiving the highest dose of Wastewater (B). Wastewater treatment led to the formation of honeycomb like structure in the lung alveoli in 28 days repeated oral toxicity study.

Measurement of malondialdehyde (MDA) and glutathione (GSH) level in liver and brain tissue: The levels of liver and brain MDA were increased after treatment with Wastewater and found to be significantly higher than those in the control animals. Further, wastewater treatment decreased the liver and brain GSH levels significantly as compared to those in the control animals (Figs. 5 and 6).

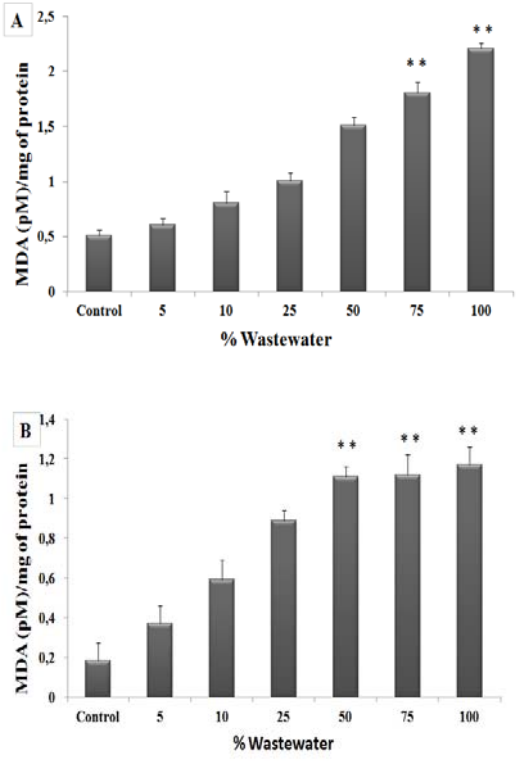

Figure 5. Effect of wastewater treatment on the MDA level in 28 days repeated oral toxicity study in liver (A) and brain (B) of mice. All values are expressed as means ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001.

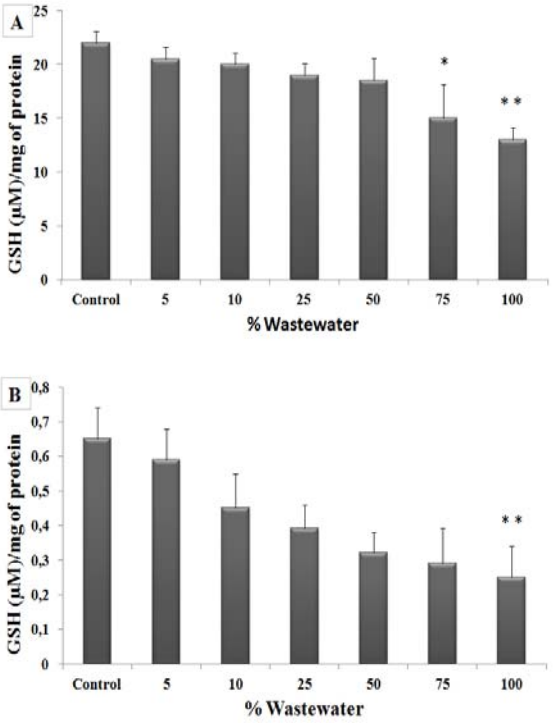

Figure 6. Effect of wastewater treatment on the GSH level in 28 days repeated oral toxicity study in liver (A) and brain (B) of mice. All values are expressed as means ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001.

Peripheral blood micronucleus chromosomal aberration assay: Treatment with wastewater at the highest dose led to significant increase in MNERTs/2000ERTs ($P < 0.001$) as compared to the control group (Fig. 6). Further, percentage of chromosomal aberration (% CA) was significantly decreased at the highest dose of wastewater as compared to those in the control group (Table 2).

Table 3. Frequency of MNERTs/2000ERTs, % of CA in peripheral blood of control and wastewater treated groups (n = 6). All value s are expressed as means ± SEM (n = 6).

Treatment	MNERTs/1000ERTs	% Chromosomal aberration
Control	1.9± 0.11	1.01±0.10
5%	3.1±0.15	1.4±0.60
10%	5.8±0.29	2.30±0.11
25%	10.5±0.1**	3.12±0.25
50%	10.9± 0.1**	3.21± 0.22
75%	11.12± 0.25**	4.77± 0.29
100%	11.55± 0.2	3.20± 0.12

DISCUSSION

In the present investigation, 28 days repeated oral doses of wastewater treatment (5, 10, 25, 50, 75, and 100%) induced significant changes in the routine toxicity parameters as well as DNA damage as observed by MN and chromosomal aberration. The highest dose of wastewater induced significant decreases in the body weight of the animals as compared to the control group at the end of 28 days. Hematology parameters such as count showed statistically significant reduction with the highest dose of wastewater as compared to the control group. Earlier studies haves already proven the hematologic and myleosuppressive effects of wastewater.[12] The liver histology of the highest dose group revealed extensive vacuolization of nuclei as compared to the control. Further, in the lung the density of alveoli declined at the highest dose of wastewater as compared to the control group. The hepatic and pulmonary toxicity of wastewater has been well established in clinical situations.[13,14] Furthermore, wastewater induced oxidative stress, as evident from significant increases in the MDA and decreases in the GSH level in both the liver and brain of mice. Our findings well corroborated with the literature that wastewater induced an increase in the lipid peroxidation and a decrease in glutathione content in the liver and brain of rodents.[15,16]

Evaluation of genotoxicity using in vivo MN assay is one of the primary test in a standard battery of genotoxicity tests recommended by the regulatory agencies. Presently, the MN assay is widely used to detect genotoxicity and well established guidelines are recommended for the conduct of the study for regulatory acceptance.[17] Attempts have already been made to integrate genotoxicity evaluation with systemic toxicity studies. Krishna et al. suggested the integration of the MN assay with routine toxicology studies.[18] Several lines of evidence support that the MN assay can be used as suitable endpoints for genotoxic risk assessment in regulatory set-ups.[19-21]

The advantages of the chromosomal aberration assay over the MN assay include sensitivity for detecting low levels of DNA damage, requirement of a small number of cells per sample and flexibility, versatility and an ease of application using automated software.[22-24] A recent study conducted by Vasquez emphasized that combining both MN and comet assay with differences in sensitivity, endpoints measured and type of data generated can significantly improve the current standard for the detection of genotoxicity without requiring additional animal studies.[25] It is clear that no single test is capable of detecting all relevant genotoxic agents. The usual approach is to conduct a battery of in vitro and invivo tests for genotoxicity.[26]

CONCLUSION

In conclusion, a chromosomal aberration assay can be successfully integrated and should gain practical implementation along with the MN assay in repeated-dose toxicity studies in the early stages of product development. The rationale behind the integration of a chromosomal aberration into the repeated-dose toxicity testing is to investigate the DNA damage in the additional tissues and to broaden the spectrum of DNA damage. In addition to this, integration of genotoxicity test involving both comet and MN assays with routine toxicity tests would reduce the cost of additional animals as well as provide information on target organ specific DNA damage. Further, an integration approach would reduce the use of additional test items, which is often limited in supply at the early discovery stage. After the 4th International Workgroup on Genotoxicity Testing (IWGT), the chromosomal aberration assay is gaining maximum acceptability by international regulatory agencies. The efforts will gain its justification, if the scientific community can orient their approach and integrate the assays into the routine toxicity studies at an early stage of product safety evaluation on an international level for regulatory acceptance. Perhaps this is one step in the right direction as it has been truly emphasized in the recently completed 5th IWGT workshop for the integration of both MN and the chromosomal aberration assays into the repeated-dose toxicity studies.[27] The results of the present study clearly indicate that the genotoxicity assay parameters (micronuclei and chromosomal aberration assays) can be successfully integrated into the routine toxicity test for regulatory compliance. However, further studies are required using different direct acting genotoxic chemicals to validate both the assays and to integrate the chromosomal aberration assay into routine toxicity testing.

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