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RAPD Assessment of In Vivo Induced Genotoxicity of Raw and Treated Wastewater to Albino Rat

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Abstract Randomly amplified polymorphic DNA (RAPD) was applied to assess the potential genotoxicity of wastewater to albino rats. Cluster analysis using the Euclidean distance resulted in two clusters; one includes the control rats and the treated wastewater-injected rats (join at a distance of 0.57). The other one includes the rats injected with the raw wastewater (joins the first cluster at a distance of 0.6). Results confirm the ability of both raw and treated wastewater to in vivo induce genotoxic effects to rats. This demonstrates that the treatment process does not remove all mutagens found in raw wastewater completely. Consequently, the reuse of treated wastewater for irrigation poses health and environmental hazard. Therefore, we recommend genotoxicity testing be used to monitor the quality of wastewater effluents, in addition to the traditional tests used. Besides, hazardous chemicals from laboratories should be separated and treated differently. Finally, RAPD test is a reliable one that can be applied to evaluate in vivo genotoxic effects of chemicals.

Keywords Genotoxicity · Wastewater · RAPD · Rat

Industrial wastewater can contain a wide range of toxic substances that are recalcitrant to wastewater treatment process. These substances reduce the efficiency of wastewater treatment by inhibiting biological activities and limiting the possibilities of wastewater reuse. Genotoxins are substances that cause alterations in DNA structure such as deletions, insertions, inversions, rearrangements and recombination (Noel and Rath 2006). These alterations affect DNA expression and increase the incidence of tumors (Singh and Roy 2001).

Randomly amplified polymorphic DNA technique (RAPD) is a simple, sensitive and reliable method that utilizes molecular biology knowledge to assess genotoxicity, phylogeny, taxonomy, genotoxicity, and epidemiology (Marillia and Scoles 1996; Zhiyi and Haowen 2004). Conte et al (1998) used RAPD to monitor the genotoxic effects of heavy metals in *Arabidopsis* plant. Zhiyi and Haowen (2004) implemented RAPD to detect the genotoxicity of some chemicals to zebrafish. Castaño and Becerril (2004) used RAPD to assess DNA damage caused by Benzo(a)pyrene to a cell line. The method was applied to evaluate genotoxicity of ethyl methanesulfonate in Swiss mice (Noel and Rath 2006). Swaileh et al (2008) used RAPD to evaluate in vivo genotoxicity of wastewater to oat plants. Doganlar (2012) assessed phytotoxicity and genotoxicity of quizalofop-*p*-ethyl to *Lemna*. The present study aims at applying RAPD analysis to evaluate the potential of raw and treated wastewater to in vivo induce genotoxicity to albino rats.

Materials and Methods

Samples of treated and raw wastewaters were collected in clean, sterile plastic bottles from Birzeit University Treatment Plant on a weekly basis during the experiment. In the laboratory, samples were first filtered using gauze swabs then Minisart® syringe filters of 0.2 μm pore size (Sartorius AG, Germany). Filtered samples were stored in the refrigerator at 4°C for later use. Samples of tap water (control) were treated the same way. 12 female adult Sprague-Dawley albino rats of similar age were obtained

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from the animal unit of the Department of Biology and Biochemistry in Birzeit University. Animals were divided into two categories (duplicate) with 6 rats in each experiment. Thereafter, rats in each experiment were divided randomly into 3 groups (2 rats/cage) and marked on their tails. Rats were weighed and given *ad libitum* access to food and water throughout the experiment. Then, rats of each group were injected intraperitoneally (IP) with 1 m tap water (Control), treated wastewater or raw wastewater. The injection took place on three non-consecutive days/week for a total of 4 weeks. With each injection, animals' weights were recorded. At the beginning and the end of the experiment, blood samples for DNA extraction were obtained from each rat by retro orbital bleeding.

Isolation of DNA from whole blood was performed using AccuPrep® Genomic DNA Extraction Kit (Bioneer Coorporation, Korea). The isolation procedure was according to the accompanied manufacturer's instructions. DNA yield was determined spectrophotometrically (Spectronic 601) and the index of DNA purity (OD 260/280) was calculated. Additionally, the quality of the obtained DNA was checked by staining 10 µL of DNA sample and resolving it in an electrophoresis system and observing the DNA bands under UV light. A total of 21 decamer primers were screened. The amplification reaction volume was 25 µL and contained 4 mM $MgCl_2$, 0.5 mM dNTPs, 1 µM of the primer, 0.25 U Taq DNA polymerase, and 13.3 µL double distilled water and 5 µL of template DNA. The amplification mixture was subjected to the following PCR cycling program: initial denaturation for 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 40 s annealing at 36°C and 1 min for extension at 72°C, and last 5 min at 72°C for final extension in a Gene Cycler (Bio-Rad Laboratories, UK). Amplification products were resolved on 1.5 % agarose, stained with ethidium bromide (Sigma, St. Louis, USA) and detected using UV light. Finally, the RAPD profiles were documented using a Fire Reader XS D-56-26.MX gel documentation system (UVITECH, UK). Changes observed in RAPD profiles were scored by identifying the appearance and disappearance of bands compared to the control. RAPD profiles were analyzed by making a binary matrix, where the presence of a band was scored as 1 and absence of a band was scored as 0 (Padmesh et al. 1999). Thereafter, a numerical analysis based on banding patterns obtained from the treatments (raw and treated) was compared with the control via hierarchical cluster analysis; resulting in a dendrogram which was created by the group linkage method using squared Euclidean distance measurement (Enan 2006). The analysis was made and graphs were plotted using the computer statistical program "SYSTAT 12 For Windows® version 12.02" (SYSTAT Software Inc., USA). DNA fingerprint similarities between treatments and the control were calculated according to Nei and Li (1979).

Results and Discussion

During the 4-week experiment, all rats remain healthy and did not show any abnormal health condition or weight loss (Fig. 1). Therefore, the intraperitonial injection of rats did not affect the general health of the rats as far as weight is considered. The purity index of the isolated DNA was determined spectrophotometrically as 1.8. This indicates a high degree of DNA purity. The quality of the DNA isolated from the 12 rats was checked and found to be high (Fig. 2), which indicates good DNA integrity. The consistency of the RAPD technique was checked (Fig. 3) and the results indicated that the DNA isolated gives always the same banding pattern with the same primer. This also assures that the integrity of the DNA isolated was good. Out of 21 primers screened, 12 gave good polymorphic bands (Table 1). The total number of bands generated was 101 and the number of bands generated by each primer ranged between 4 and 16 with an average of 8.4 bands/primer. The overall number of polymorphic bands obtained was 59 (Table 1). Thus, polymorphic bands constitute 58.4 % of the total bands generated. These results are

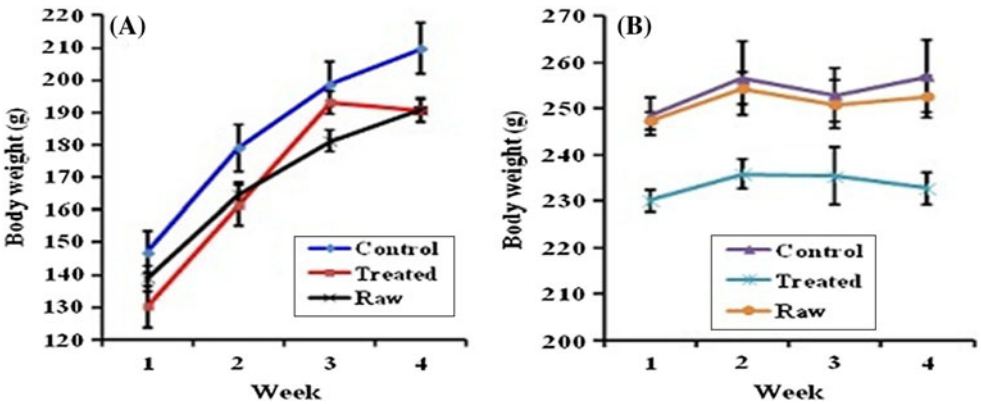

Fig. 1 Rat weights throughout the 4 weeks of the experiment. Each value represents mean ± SE of 6 weight measurements taken for 2 rats each week. Experiment was done in duplicates A and B

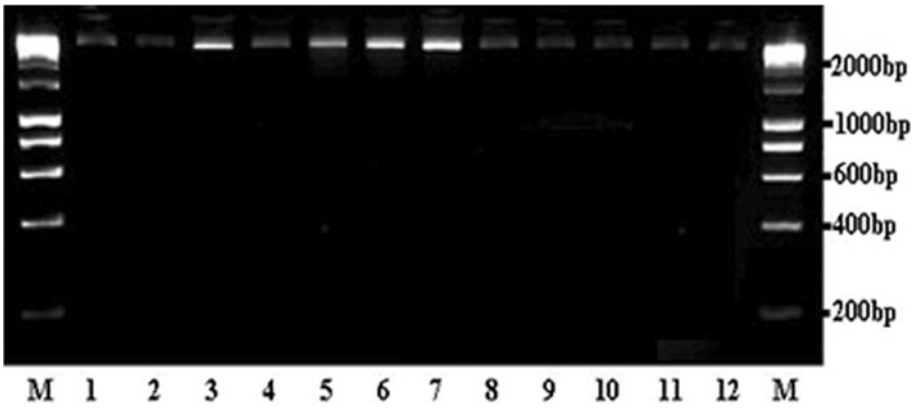

Fig. 2 The quality of DNA isolated from blood samples of the rats used in the experiment. M: marker (Bioline HyperLadder^{TM} 1). 1–12: DNA isolated from the 12 rats

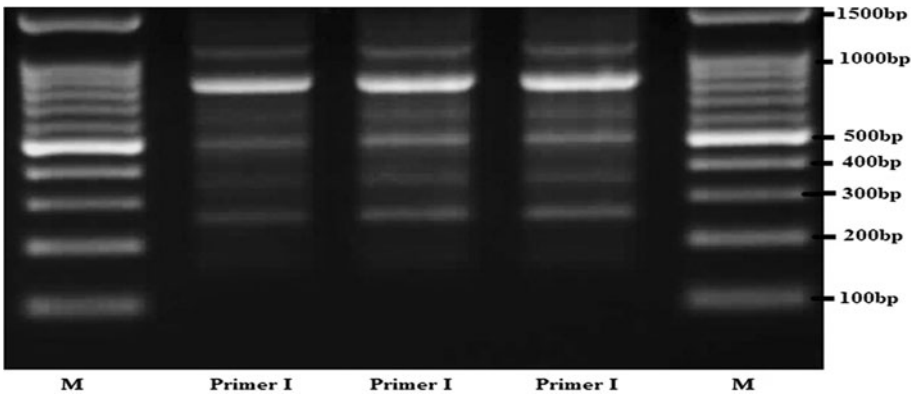

Fig. 3 Reproducibility of RAPD profiles generated from rat DNA. M: marker (GeneDirex® 100 bp DNA Ladder RTU)

Table 1 Primers used in the present study, total number of bands and polymorphic bands generated by each primer

Primer	Sequence 5′→3′	G–C content (%)	Total bands	Polymorphic bands
1	AGTCAGCCAC	60	5	2
2	CTCACCGTCC	70	5	4
3	GACGGATCAG	60	8	6
4	GTCCCGACGA	70	14	13
5	CCAGCCGAAC	70	16	11
6	GGT TGTACCC	60	7	1
7	TCGGCGATAG	60	9	7
8	GTTGCGATCC	60	4	2
9	TGCGCCCTTC	70	7	4
10	TGTCATCCCC	60	4	1
11	GCCTCATACC	60	8	1
12	GTGTCTCAGG	60	14	7
Total			101	59

consistent with those reported by other authors (Yoon and Kim 2001; Enan 2006; Swaileh et al. 2008). Examples of RAPD profiles generated using 12 decamer primers are shown in Fig. 4. the profiles shown represent the RAPD profiles for the rats at the start of the experiment (C_0) and those after 4 weeks of injection with raw wastewater (primer 4, R_4), treated wastewater (primer primer 5, T_4) and tap water (primer 3, C_4). The differences in the banding patterns of those rats injected with wastewater are clearly exhibited, while the banding profiles for the control group remained unchanged. DNA finger prints for rats injected with raw wastewater showed that a total of 44 bands that existed before the start of the experiment disappeared. In addition, 3 new bands appeared in the profiles after injection with raw wastewater. In rats injected with treated wastewater, a total of 23 bands disappeared and 6 new bands appeared. This indicates the genotoxic effect of both treated and raw wastewater from Birzeit University Treatment Plant as genotoxic substances are known to change the DNA sequence, thus, alter the primer binding sites and the banding profile. However, as can be expected, the results show that raw wastewater is more genotoxic than treated one. This is reflected by the higher number of polymorphic bands (appeared/disappeared) generated by raw wastewater (47 bands) compared to those generated by treated wastewater (29 bands). Bands disappear when the primer fails to bind to a certain site on the DNA that was altered by the genotoxic substance, while new bands appear when some sites on the DNA become accessible to the primer after structural change by the genotoxic agent (Pietrasanata et al. 2000; Enan 2006).

Cluster analysis method is considered one of the most effective methods in numerical computation regarding

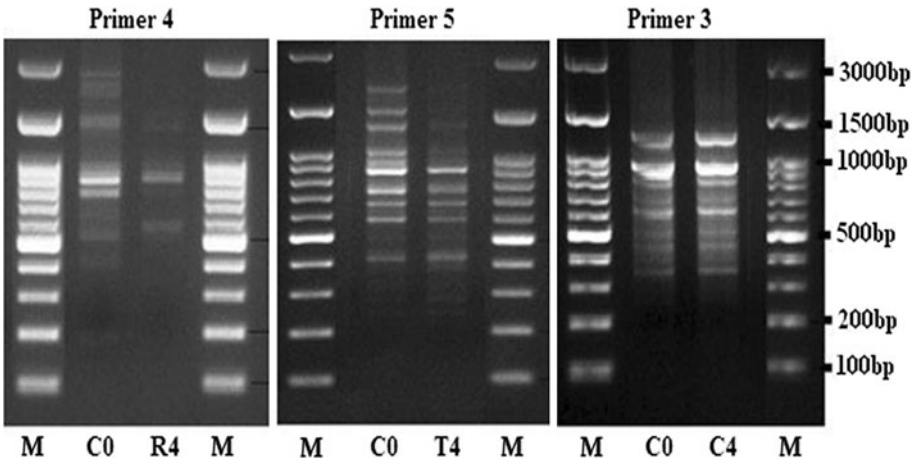

Fig. 4 Examples of RAPD profiles generated from DNA samples of rats at the beginning of the experiment (C_0) and after being injected with raw wastewater (R_4), treated wastewater (T_4) and tap water (C_4). M: marker (GeneDirex® DNA Ladder H3 RTU)

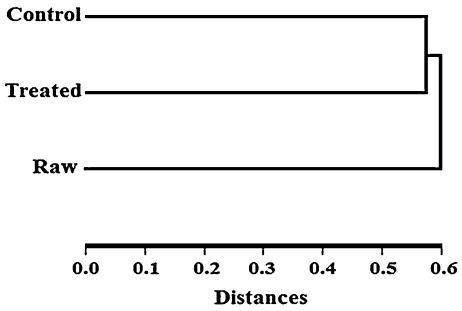

Fig. 5 Euclidean distances dendrogram generated from DNA profiles of rats injected with tap water (control), treated wastewater and raw wastewater

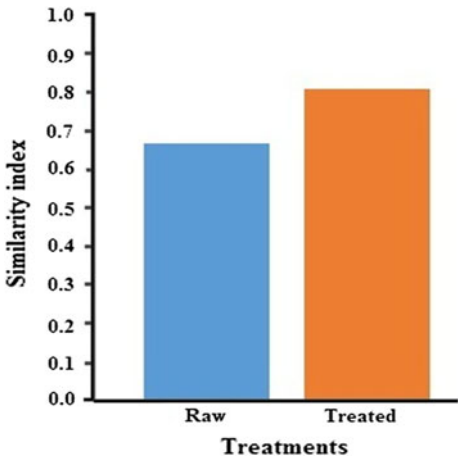

Fig. 6 Genetic similarity indices calculated for rats injected with wastewater (raw and treated) to those injected with the tap water (control)

band scoring in the analysis of RAPD fingerprints. The cluster analysis results for RAPD fingerprints from rats injected with tap water and wastewater are shown in Fig. 5. The constructed dendrogram showed the presence of two clusters; one containing the control rats and the rats injected with treated wastewater, while the other cluster contains rats injected with raw wastewater. The dendrogram shows that the rats injected with treated wastewater join the control ones at a Euclidean distance of 0.57, while the rats injected with raw wastewater join the first cluster at a Euclidean distance of 0.6. These results confirm that both treated and wastewater can cause in vivo genotoxicity to rats and that the raw wastewater is more genotoxic than the treated one. Results of the present study confirm previous results regarding the genotoxicity of the influents and effluents of Birzeit University Treatment Plant to oat plants (Swaileh et al. 2008). Furthermore, many studies confirmed the genotoxic effects of wastewaters of different origins using other molecular assays (Chromosomal aberration, DNA strand break, DNA laddering), microbial tests (the Ames test and SOS test), zooplankton bioassays (*Daphnia*) and plant bioassays (*Allium*) (Codina et al. 1994; Grisolia et al. 2005; Movahedian et al. 2005; Krishnamurthi et al. 2008; Radić et al. 2010). Results of these studies, along with those of the present one, confirm the need to include the mutagenicity tests, along with the conventional chemical tests, of wastewater before considering its reuse.

The DNA-fingerprint similarity, which is defined as the fraction of shared bands in the amplification profiles of two organisms or groups of organisms, was calculated. The similarity indices for rats injected with wastewater to those injected with tap water (control) were found to be 0.805 and 0.667 for treated wastewater-injected and raw wastewater-injected rats, respectively (Fig. 6). In addition, rats injected with both types of wastewater had a similarity index of 0.714 to each other. These findings confirm results of the cluster analysis that both raw and treated wastewaters cause genotoxicity; however, treated wastewater contains less genotoxic substances. The 80 % similarity between the control rats and those who received treated wastewater explains their clustering in one group in the dendrogram, whereas the less similar (66.7 %) rats who received raw wastewater were clustered in a separate group.

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