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DETECTION OF GENOTOXICITY INDUCED BY HEAVY METAL IONS AND GAMMA RADIATION USING MICRONUCLEUS ASSAY IN MICE: PATHOLOGICAL EVALUATION

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Abstract: Micronuclei (MN) test is used as markers of radiosensitivity or chemosensitivity. In present study, it was investigated the frequency of MN in erythrocytes and body weight gain in 80 *Mus musculus* var. *albinos* exposed to 10 Gy gamma (γ) radiation and heavy metal ions. For this aim, it was used MN assay as an indicator of genotoxicity induced by γ–radiation and heavy metal toxication. The animals were divided into four groups: control, radiation, Hg and Pb treatment groups. They were treated with three dose levels (10, 15 and 20 µg/mL) of Hg and Pb metal ions and 10 Gy γ–radiaton was applied twice during 14 days. The initial and final weights of all mice were determined by sensitive balance in order to investigate the effect of heavy metal ions and radiation on the weight gain of mice. As a result, the frequency of MN was higher in the Hg, Pb and γ–radiation treated animals than animals in control group. Besides, MN frequency was higher in mice exposed to γ–radiation than in Hg and Pb treated mice, and differences was statistically significant ($p<0.05$). Histopathologically, periaciner necroses, hydropic degenerations in the liver and villous atrophy in the intestine, gastric glandular mucosae necroses were observed in treatment groups. These results indicate that MN assay is very sensitive and a useful biomarker for the evaluation of the genotoxicity.

***Key words*:** Gamma radiation, *In-vivo* micronuclei assay, Lead and mercury toxicity, Pathology, Weight gain.

FARELERDE MİKRONUKLEUS TESTİ KULLANILARAK GAMA RADYASYONU VE AĞIR METAL İYONLARI TARAFINDAN TEŞVİK EDİLEN GENOTOKSİSİTENİN BELİRLENMESİ: PATOLOJİK DEĞERLENDIRMELI

Özet: Mikronukleus (MN) testi, radyasyona veya kimyasallara duyarlılığın belirteci olarak kullanılmaktadır. Bu çalışmada, ağır metal iyonları ve 10 Gy gama radyasyonuna maruz kalan 80 adet *Mus musculus* var. *albinos*'da, eritrositlerde MN sıklığı ve vucüt ağırlığı kazanımı araştırılmıştır. Bu amaçla, γ–radyasyonu ve ağır metal toksikasyonu tarafından uyarılan genotoksisitenin indikatörü olarak MN testi kullanılmıştır. Hayvanlar kontrol, radyasyon, Hg ve Pb uygulama grupları olarak dört gruba ayrılmıştır. Bu gruplar ayrı ayrı Hg ve Pb metal iyonlarının üç dozuna (10, 15 ve 20 µg/mL) ve 14 gün süresince iki kez 10 Gy γ–radyasyona maruz bırakılmıştır. Farelerin ağırlık kazanımları üzerine ağır metal iyonları ve radyasyonun etkilerini araştırmak için, tüm farelerin başlangıç ve son ağırlıkları hassas terazi yardımıyla belirlenmiştir. Sonuçta MN sıklığı Hg, Pb ve γ–radyasyonu ile muamele edilen hayvanlarda, kontrol grubundakilerden daha yüksek bulunmuştur. Ayrıca, MN sıklığının, γ–radyasyonuna maruz kalan farelerde, Hg ve Pb ile muamele edilen farelerden daha yüksek olduğu bulunmuştur ve farklar istatistiksel olarak önemlidir ($p<0.05$). Histopatolojik olarak ise, uygulama gruplarında karaciğer hepatositlerinde hidropik dejenerasyon, periasiner nekroz, barsakta villus atrofisi ve mide glandular mukoza nekrozları gözlenmiştir. Bu sonuçlar, MN testinin radyasyon ve ağır metal iyonlarının etkilerini değerlendirmek için çok hassas ve faydalı bir biyolojik belirteç olduğunu göstermiştir.

***Anahtar kelimeler*:** Gama radyasyonu, *In-vivo* Mikronukleus testi, Kurşun ve civa toksisite, Patoloji, Ağırlık kazanımı.

I. INTRODUCTION

As a result of rapid industrial development, about 50000 chemical substances have been released to the environment during the last decade and caused ecological system pollution [1, 2]. Among these heavy metals, Pb and Hg are considerable important environmental pollutants. They occur naturally in the earth, but are spread through the environment by human activities. For many years they were used in various industries such as medicine, dentistry, batteries, science, military applications and gasoline. People may expose to Pb and Hg with drinking, eating, breathing and touching to any contaminated material in the environment. They accumulate in the body organs such as kidney and liver, which may lead to poisoning or even death in acute cases [3–6]. There are several reports suggesting that the intake of foods contaminated with heavy metals adversely affecting the health. Heavy metals also cause an enhanced level of chromosomal aberrations in living cells [1, 2].

Likewise, the radiation has many applications in various industries as medicine, communication and domestic life in recently. Hence, the biological effects of radiation have been investigated for a long time by scientists. Some biological effects the observed of radiation are change in cell function, cell death, delayed mitosis, disruptions in cell growth, permeability changes, tissue damage and chromosome damage [7]. These effects of radiation are basically similar for different kinds and dose of radiation [8, 9].

The *MN* assay is a widely used technique for monitoring of these clastogenic effects of chemical and radiation [10]. Micronuclei originate from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during metaphase or anaphase phase of division. They occur in the event of chromosomal damage and may thus provide a marker of early-stage carcinogenesis [11–13]. The presence of *MN* in eukaryotic cells has been known for a long time [14]. The *MN* has been observed in plants and in numerous tissues of many animal species such as lymphocytes, erythrocytes, fibroblast and epithelial cells [15–17]. Nowadays, *MN* serves as an important indicator to detect the genetic damage induced by chemicals or radiation in invitro and invivo studies. When compared with other traditional methods, *MN* method is rapid, time saving, easy to learn, and obtained results from different groups can be compared [18–20].

The aim of this study was detection of genotoxicity induced by γ–radiation and heavy metal ions using as an indicator the *MN* assay in albino mice.

II. MATERIALS AND METHODS

The present study has been carried out on 80 mice. Six weeks old male mice (*Mus musculus* var. *albinos*) were used for *MN* analysis and determine of the alterations in body weight. Healthy mice were obtained from the Animal Research Center of Refik Saydam Hıfzissiha Institute (Ankara, TURKEY). Their mean body weight was 33.63±1.53 g. The mice were kept in metal cages with 5 albino mice per cage at 22 °C (±3 °C) temperature and a 12 h light/dark cycle. The mice were treated by heavy metal ions and 10 Gy γ–radiations. In order to investigate the effect of γ–radiation and heavy metal ions on the weight of mice, the initial and final weights of all mice were determined by sensitive balance. We collected the peripheral blood samples from mice at 7^{th} and 14^{th} days and were determined the effect of exposure time on *MN* frequency. In this study, the methods and techniques applied to mice carried out favorably to ethical standards of Kırıkkale University-Faculty of Veterinary Medicine and favorable to the guidelines set by the World Health Organisation (Geneva, Switzerland).

Chemicals: The Pb (II) citrate (CAS No.:512-26-5), Hg (II) chloride (CAS No.:7487-94-7) and Grünwald Giemsa (CAS No.:51811-82-6), o-Xylene (CAS No.: 95-47-6), Glutaraldehyde (CAS No.:111-30-8), Hematoxylin (CAS No.:517-28-2) were obtained from Interlab A.S., Istanbul, TURKEY.

Experimental Design and Heavy Metal Exposure: The mice were divided into the following four groups: group I: control, group II: radiation treatment group, group III: Pb treatment group, group IV: Hg treatment group. Control group mice (n: 10) were housed in cage, and fed with standard pellet diet (Samsun Food Industry, Samsun, TURKEY) and fresh water. Pb (n: 30) and Hg (n: 30) treatment group mice were fed with standart pellet diet and were treated with 0.5 mL of solutions prepared from Pb (II) citrate and Hg (II) chloride once a day for 14 days by gavage. The each solution contained 10, 15 and 20 µg/mL of Pb and Hg. Heavy metal solutions were prepared daily in distilled water.

Radiation Exposure: Mice were exposed to radiation at the Ankara University-Department of Radiation Oncology. Radiation procedure was carried out using a Cobalt 60-gama source (Cirus, Cis-Bio Int-France). Totally ten mice were

treated with 10 Gy γ–radiation twice (at seventh day and at first day of treatment) during two weeks. This dose was chosen because it induces an increase in the MN formation. The radiation was applied on a single peak whole-body to a depth of 3 cm as described literature during 15 min [21].

MN Assay: The *MN* frequency is scored in mature normachromatic erythrocytes (NCE) in the circulating blood. Blood samples were obtained from the tail vena of each mouse and 3–4 smears were prepared. Five µl blood sample spread on slide, then cells on slides were fixed with methanol and stained with Grünwald Giemsa staining. After stain process, the slides were examined in binocular light microscope (Japan, Nicon Elipse E600). One thousand erythrocytes were scored in each slide for determining the *MN* frequency.

Pathologic Examinations: After necropsy procedure, systematic organ samples of the mice in each group were collected and fixated in 2.5 % glutar aldehyde for 48 h. After 15 times washing in cacodylate buffer, second fixation was done in osmic acid for 2 h. Then routine histological tissue procedure was applied in alcohol, xylene series and tissue samples were embedded in paraffin and sectioned at a thickness of 4–5 µm. Hematoxyline eosine stained sections were evaluted in binocular light microscope and photomicrographs were taken.

Statistical analysis: For the statistical analysis, differences between the groups were tested by analysis of variance (ANOVA) and Duncan test using SPSS for Windows version 10.0. The data were displayed as means ± SD, and P values less than 0.05 were considered significant.

III. RESULTS

Micronuclei Frequency

There was only several the *MN* formation in erytrocytes of mice belong to control group. But *MN* formation fairly increased in all mice exposed to radiation, Hg and Pb ions (Fig. 1, 2). The results related with the *MN* frequency were given in Table 1 and 2. The differences of *MN* frequency between control and treatment groups were found statistically significant ($P<0.05$). The highest *MN* frequency was observed at 10 Gy γ–radiation dose at the end of fourteenth day. The Hg treated group showed a higher frequency of *MN* than Pb treated group. The frequency of *MN* increased with the applied doses in Hg and Pb treated

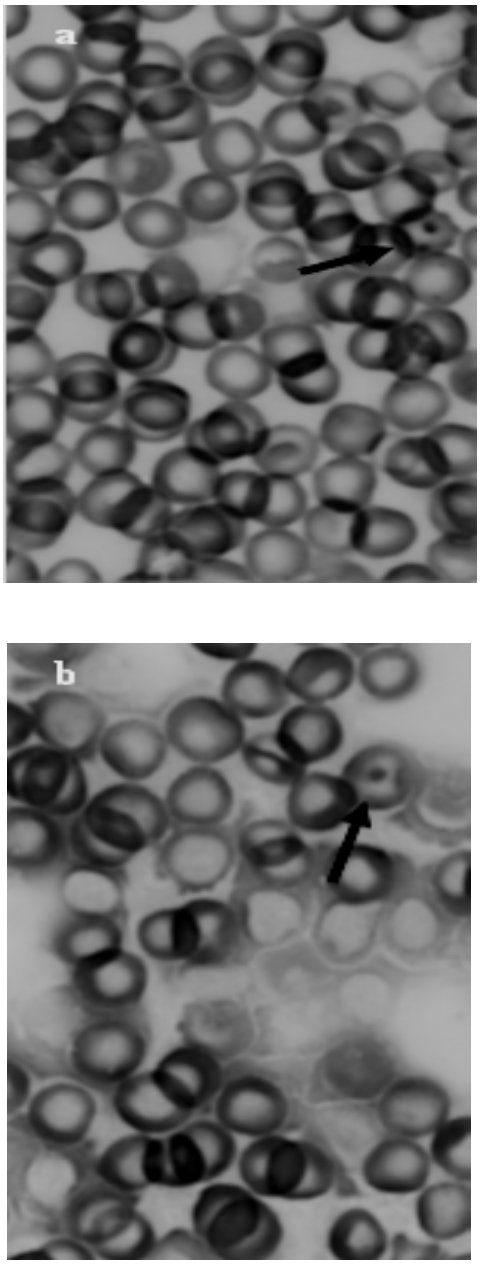

Figure 1. The appearance of micronucleated erythrocytes in mice. a: Pb treated group, b: Hg treated group. Giemsa staining (X350)

groups and reached a peak level at 20 µg/mL dose at the end of fourteenth day. In Hg and Pb treated mice, the least frequency of *MN* was observed at 10 µg/mL doses at the end

of seventh day. Besides, the morphologic damage in blood cells was compared between treatment group mice and control group mice. In erythrocytes of heavy metal ions treated mice and control group mice morphologic damage was not found, but in erythrocytes of mice exposed to γ–radiation a great deal morphologic damage was observed (Fig. 3).

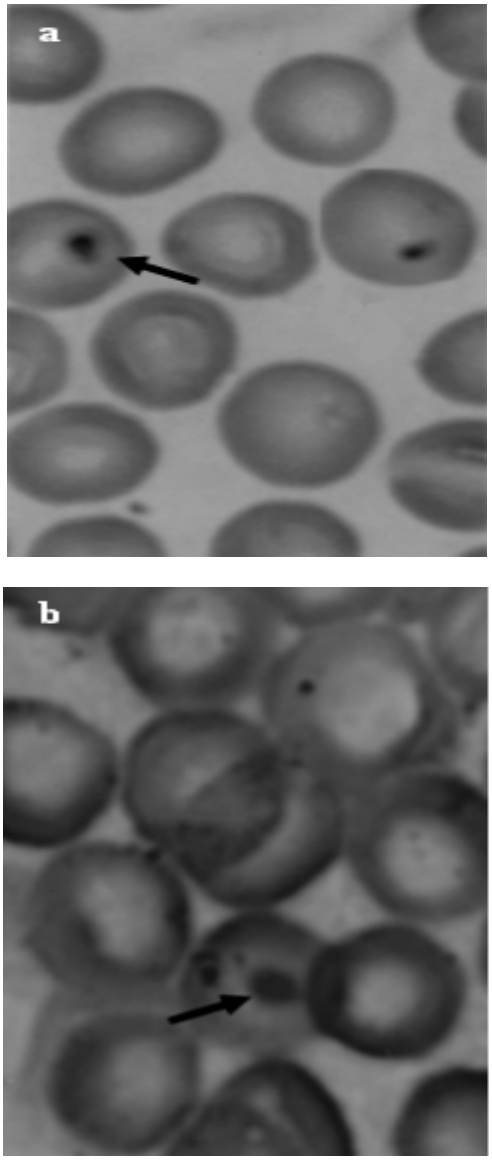

Figure 2. The appearance of micronucleated erythrocytes in mice exposed to γ–radiation. Giemsa staining (a,b X500)

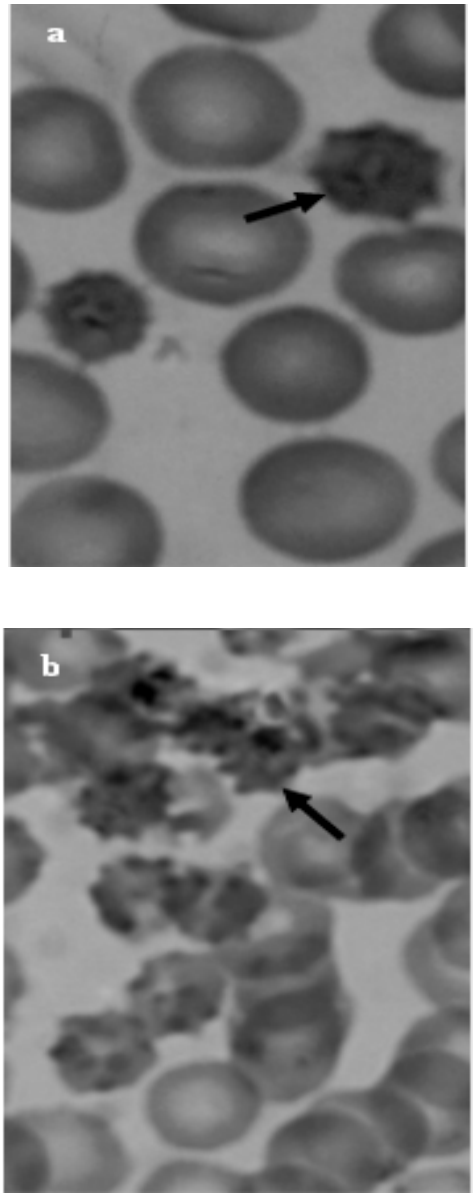

Figure 3. The appearance of erythrocyte damage induced by γ–radiation. Giemsa staining (a,b X500)

Table 1. The mean of *MN* frequency in treatment group mice at the end of 7th ; number of scored cell was 1000

Groups µg/mL	Min.	Max.	Average ±SD
Control	1	4	2.50±0.85[a]
Radiation	33	48	39.40±4.38[b]
10 Hg	13	19	16.30±2.06[c]
15 Hg	20	28	23.00±2.62[d]
20 Hg	24	33	27.50±2.68[e]
10 Pb	7	13	10.20±2.15[f]
15 Pb	13	18	15.40±1.51[c]
20 Pb	18	25	21.30±2.11[d]

* One-hundred cells were analyzed per animal (10 animals/group, for a total of 1000 cells/treatment). All values are the mean±SD (n=10). Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Duncan as a post ANOVA test (P<0.05). Means with the same letter within the same parameter are not significantly different.

Table 2. The mean of *MN* frequency in treatment group mice at the end of 14th

Groups (µg/mL)	Min.	Max.	Average ±SD
Control	2	4	2.80±0.63[a]
Radiation	60	77	68.00±5.48[b]
10 Hg	21	30	25.60±2.59[c]
15 Hg	33	44	38.30±3.33[d]
20 Hg	46	60	50.70±3.95[e]
10 Pb	13	21	16.90±2.64[f]
15 Pb	20	28	24.70±2.75[c]
20 Pb	40	49	43.60±2.99[g]

* One-hundred cells were analyzed per animal (10 animals/group, for a total of 1000 cells/treatment). All values are the mean±SD (n=10). Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Duncan as a post ANOVA test (P<0.05). Means with the same letter within the same parameter are not significantly different.

Body Weight Gain

To investigate the effects of γ–radiation and heavy metal ions applications on the body weight gain of mice, the initial and final weights of mice were calculated. The significant differences in body weight gain were not observed among the animals which survived 14 days. In control group, the weights of all mice increased about 5.57±1.06 g at the end of experimental period. In radiation, 20 µg/mL Hg and 20 µg/mL Pb groups, the weights of mice increased about 1.32, 0.70 and 2.47 g, respectively. There was a significant difference among control and treatment groups, and differences were statistically significant (p<0.05). Moreover, body weight less increased in Hg treated animals when compared to Pb treated animals and difference was statistically significant (p<0.05). The data for body weights of the animals belong to control and application groups was given in the Table 3.

Table 3. Mean Body Weight (g)

Treatment groups (µg/mL)	Initial	14th day	Difference
Control	33.63±1.53[c]	39.20±1.11[a]	+5.57[a]
Radiation	33.59±1.45[c]	34.91±1.51[b]	+1.32[b]
20 Hg	33.13±1.56[c]	33.83±1.46[b]	+0.70[b]
20 Pb	33.17±1.43[c]	35.64±1.35[c]	+2.47[c]

*All values are the mean±SD (n=10). Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Duncan as a post ANOVA test (P<0.05). Means with the same letter within the same parameter are not significantly different.

Pathology

In radiation group; the most consistent findings were observed in liver and intestine. In liver, paracentral and periacinar multifocal necroses, cytoplasmic vacuolar degenerations and nuclear changes characterized by hyperchromatic giant nuclei and twin nuclei in a hepatocyte (Fig. 4) were detected. In intestine, villous atrophy in the intestinal villi, vacuolar degenerations and necroses of the Lieberkuhn cript epithelia were seen (Fig. 5). In chemical group, diffuse cytoplasmic vacuolar degenerations and eosinophilic granular appearance in the hepatocytes (Fig. 6)

and epithelial necroses on the mucosa of the stomach were present.

IV. DISCUSSION

The frequency of *MN* was clearly increased in the γ–radiation, Hg and Pb treated animals, when compared with the control, and differences were found statistically significant. The frequency of *MN* was higher in mice exposed to γ–radiation than in mice treated with heavy metal ions. This difference can be explained by more damage (as breakage) at chromosomes of radiation. As well known, *MN* is occurring due to damages of the chromosomes leading to loss of genetic material. Ionizing radiation may take an important role in the creation of aberrations such as chromatid and chromosom breaks finally turned down to *MN* formation. There are several studies reporting the exact trigger role of radiation in *MN* formation. For example, Cole et al. [22] reported a dose-dependent increase in the frequency of *MN* in erythrocytes of mice after exposure to various doses of *x* or γ–radiation. In this study, histopathologic findings strongly confirming the chromosomal breakages theory suggested for the animals exposed to radiation. Because, the formation of new regenerated hepatocytes with giant nuclei and twin nuclei in a hepatocyte are indicating the cell division dysfunction during cell proliferation stages. Similarly, villous atrophy in the intestinal mucosae and necroses on the cript epithelia are resulting from hazardous effects of radiation on the chromosomes of labil epithelia having well regeneration capacity after birth.

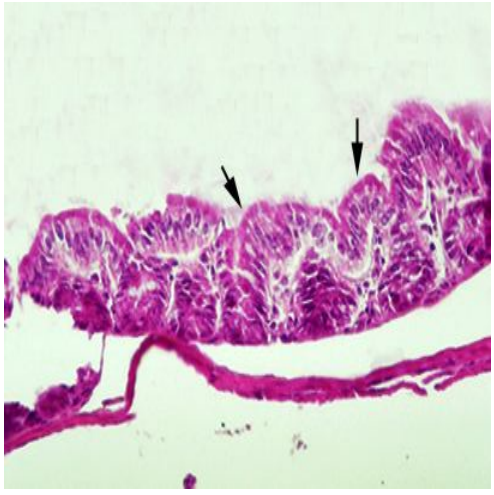

Figure 4. Dwarfed villi on the ileum mucosa in radiation group. Hematoxyline and Eosine staining (X120)

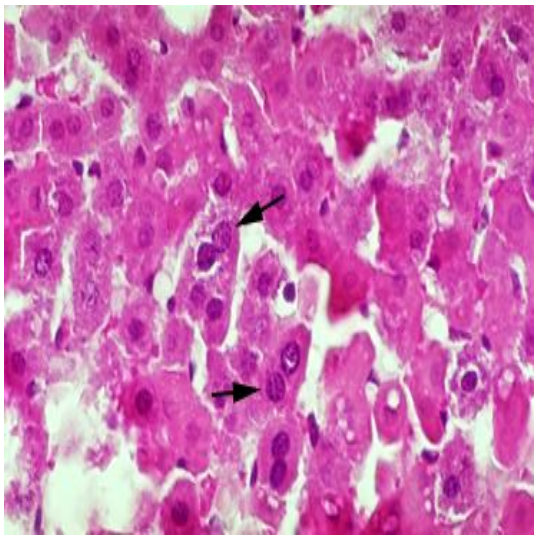

Figure 5. Focal necroses and numerous hyperchromatic giant nuclei of the hepatocytes in radiaton group. Hematoxyline and Eosine staining (X340)

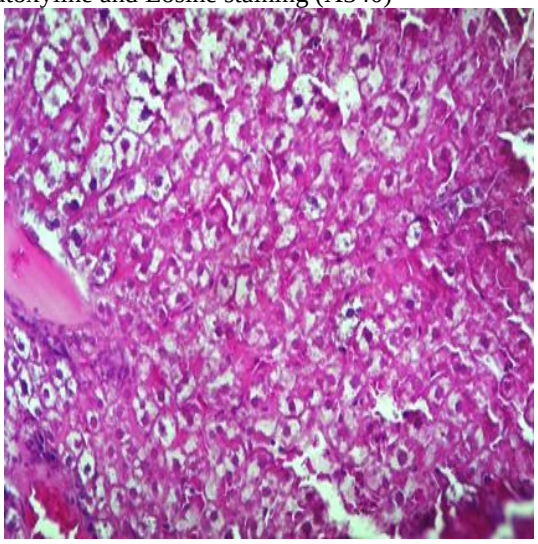

Figure 6. Diffuse hydropic degeneration in the cytoplasms of the hepatocytes and picnotic appearence of the nuclei in heavy metal groups. Hematoxyline and Eosine staining (X180)

At the same time, results from our study showed that there was a dose–depended increase in frequency of *MN* in Hg and Pb treated mice. The highest frequency of *MN* was observed at 20 µg/mL doses of Hg and Pb. All dosages also were observed statistically significant increases in the

number of micronucleated erythrocytes. Moreover, *MN* frequency elevated in Hg treated mice when compared to Pb treated mice and difference was statistically significant. This result may be related to a more toxic metal of Hg when compared to Pb. All these findings suggested that Hg and Pb had genotoxic activity induced *MN* formation in mice. This information is parallel with other genotoxicity data available so far. In most study, results indicated that, the test substances as chemical agents can be produce chromosomal or spindle damage and mitotic apparatus damage leading to the formation of *MN* in the erythrocytes [23]. For example, Korkmaz and Colak [24] found that a correlation between numbers of micronucleated erythrocytes with increased numbers of chromosomal breaks in *Mus musculus* var. *albinos*. Hg (especially methylmercury) and Pb metal ions interact with biomolecules and bind them via reactive groups such as hydroxyl and sulphydryl. Berces et al. [25] studied the genotoxic effect of mercury by using MN assay and they found that mercury ions increased micronucleus frequency linearly after incubation of whole blood with 10^{-3} M mercry ions for 30 min. So the biomolecule (protein or nucleic acid) conformation changes and the metabolic reactions are broken. Heavy metals may enter cell nucleus and bind to purine and pyrimidine bases or proteins such as spindle. The spindles may be denaturated and the interaction of spindles and chromosome decreased, so the chromosomes may have lagged.

In this study we investigated the changes in body weight gains of radiation, Hg and Pb heavy metal ions applied mice, as well. As a result, a significant difference was observed in body weight gain of radiation and heavy metal treatment groups compared with the controls. The mean body weights of mice in all treatment groups were lower than those of the controls. The highest level of body weight was observed in control group mice and least level of body weight was observed in mice Hg metal ions treated. In control groups, the weights of mice increased about 5.57 g according to the initial weight at the end of the 14^{th} day. In radiation, Hg and Pb treatment groups, the average weight increased about 1.32, 0.70 and 2.47 g according to the initial weight, respectively. Moreover, body weight less increased in Hg treated animals when compared to Pb treated animals and difference was statistically significant. These results may also relate to the metabolism rate of the mice. Namely, with exposure to γ–radiation, Pb and Hg metal ions in feeding period, the metabolism and diets of mice may be changed, and body weights of mice may be decrease. At result, the metabolisms of mice are enhancing for the removal of metal ions and free radicals generated by radiation from body. So the energy consumption is increase and the mice loss weight. The effects of radiation and chemical agents on body-weight were reported by biomonitoring studies. For example, Kennedy and Szuhaj [26] reported that related to decreased caloric intake/absorption and increased energy requirements as cause of weight loss due to radiation. Oshomah [27] was studied the effect of lead assimilation on albino mice and reported that the weight of albino mice was reduced by 12.9 g. Besides, Kim et al. [28] investigated the effect of simazine on body weight of male C57Bl/6 mice exposed to simazine 300 or 600 mg/kg daily during 4 weeks. At result, they showed that decreased of body weight in simazine-treated mice. And also, the cause another of this weight loss may be related with stomach damage. For example, in a previous study reported that the third of the mucosal surface of the stomach was thickened and necrotic in mice exposed to difference doses of Allyl lsothiocyanate [29]. In the present study, we observed severe epithelial losses on the intestinal mucosae that is characterised by villous atrophy. As well known, the intestinal epithelia play a great role on the absorbtion and enzymatic conjugation of the nutrients. In chemical group, severe gastric mucosae necroses may also adversely affect the weight gain, because of the incomplete chemical digestion of proteins and carbohydrates by pepsin and HCl.

Consequently, γ–radiation and heavy metal ions have a genotoxic effect in mice and the *MN* assay may be use for monitoring of this effect. So the results found in this study may be useful for understanding the corresponding mechanisms in humans or may be useful in the development of in vitro models in different cells of human. Moreover, these results may be helpful for method of treatment and monitoring of the response.

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