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Genotoxic Effects of the Anti-Cancer Drug Doxorubicin (Dxr) in the Bone Marrow Cells of Swiss Albino Mice (*Mus musculus*)

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ABSTRACT

The antineoplastic chemotherapeutic agent doxorubicin (DXR) is probably the most utilized drug for treating many human cancers. In the current study we aimed to evaluate the genotoxic effects of doxorubicin Swiss mice bone marrow cells using the toxicological endpoints chromosomal aberrations, mitotic index and micronuclei formation. Twelve male animals, aged 6-8 weeks and weighing 24±2g were divided into four groups. One group served as control was intraperitoneally injected only with distilled water. The three remaining groups were injected intraperitoneally with single doses of doxorubicin (0.2, 0.4 and 0.8 mg/g body weight) for two consecutive days. 24 hours later, animals were anaesthetized and killed by cervical dislocation. Colchicine (0.05 %) was injected to animals 90 minutes before sacrifice. After sacrificing the animals, both femurs were dissected out and the bone marrow cells obtained. All treatments with doxorubicin caused an increased significance in the incidence of chromosomal aberrations (CA) and micronuclei formation in polychromatic erythrocytes (PCEs) cells. Meanwhile, there was a gradual repression in the mitotic index (MI) percentages of the treated groups compared to that of the control. Different types of chromosomal aberrations (structural and numerical) were induced as a result of doxorubicin treatments. These includes: gaps, breaks, fragments, rings, centric fusions (CF) and polyploidy. The mean percentages of total chromosomal aberrations increased from 3.33 ± 0.28 in the control group to 12.67 ± 2.42, 29.00 ± 4.27 and 38.67 ± 2.82 for doses 0.2, 0.4 and 0.8 mg/kg B.W. respectively. The numbers of micronucleated PCEs observed in mice cells treated with doxorubicin were increased from 4.67±0.48 for the control group to 7.00±1.02, 10.67±2.10 and 17.67 for the three doses respectively. Meanwhile, the PCE / (PCE+NCE) ratio calculated in treated animals were 0.85 ± 0.02, 0.63 ± 0.08 and 0.54 ± 0.10 compared to 0.94 ± 0.12 for the control. Our results indicated that doxorubicin has genotoxic as well as cytotoxic effects in mice bone marrow cells.

Keywords: Doxorubicin, mice bone marrow, chromosomal aberrations, mitotic index, micronuclei.

INTRODUCTION

Cancer is a growing threat for human health. Despite the growing crises, research continue to focus on improving treatments and find a cure. In addition to surgery and radiotherapy, chemotherapy is commonly used in cancer treatment. Many chemotherapeutic drugs are nowadays known and used to combat with many forms of cancer. However, these antineoplastic drugs are a double-edged sword; they affect both healthy and malignant tissues. Like many other chemical drugs, they may be genotoxic and in the same time having clastogenic effects in various systems (Rodríguez-Arnaiz *et al*., 2004; Kusum Lata and Rudrama Devi, 2010&2012). It is therefore essential to evaluate that effective antitumor drugs for their cytotoxic potentiality and their ability to disturb genomic integrity (Tiburi *et al*., 2002). Plausibly, the most reliable evaluation of the risk of genotoxicity on human health is conducted in rodents by assessing the main endpoints of genotoxicity such as chromosomal aberrations and micronuclei formation (Okonko *et al*., 2016). Doxorubicin (also called Adriamycin), is probably the most utilized anti-tumor drug worldwide and is generally prescribed in combination with other drugs. It has widest spectrum of antitumor activity and is used with high degree of efficiency in many human cancers such as breast cancer, solid tumor, soft tissues sarcomas and aggressive lymphomas (Cortes-Funes and Coronado, 2007; Yang *et al*., 2014). Although, DXR has been extensively clinically utilized, the mechanisms responsible for its antiproliferative and cytotoxic effects are still uncertain (Buschini *et al*., 2003). It also has variable toxic adverse effects including cardiotoxicity, cytotoxicity and inducing chromosomal aberration (Rudrama *et al*., 2015). The current research was therefore performed to study *in vivo* the cytogenetic effect of doxorubicin in mice marrow cells utilizing the chromosomal aberration (CA), mitotic index (MI) and micronuclei (MN) formation as the toxilogical endpoints.

MATERIALS AND METHODS

Test drug:

Doxorubicin Hcl (Adricin^{R}, manufactured by EIMC united pharmaceuticals, Badr City- Cairo- A.R.E) purchased from local pharmacy at Assiut in the form of 10 mg/vial was used as the test drug.

Experimental animals:

Healthy adult male albino mice (Mus musculus), aged 6-8 weeks and weighing 24 ± 2 gm were purchased from the animal care unit of Faculty of Medicine, Assiut University. They were kept in capacious cages in the laboratory under standardized conditions and were provided with food and free access to tap water. The female estrous cycle hormonal effect was avoided by using male mice.

Experimental design:

For each of the two assays used (i. e. chromosomal aberrations and micronuclei formation) twelve mice were randomized, categorized into four groups of three animals each. Group one used as the control where animals were injected intraperitoneally only with distilled water, while the other three groups were injected with doses of 0.2, 0.4 and 0.8 mg/kg B.W. for two consecutive days. 24 h later animals were anesthetized and killed by cervical dislocation. Bone marrow cells were aspirated from control and treated mice**.**

Cytogenetic analysis:

Chromosomal aberration test:

Bone marrow cells were essentially prepared as prescribed by Preston *et al.* (1987). Mice were injected 2 h before sacrificing with 0.05 % colchicine dissolved in water, in order to arrest metaphase dividing cells. Immediately after the animals were sacrificed bone marrow from control and treated mice was taken off from both femurs into saline solution. The aspirated cells were

then divided into 2 parts; one was immediately processed for calculating the mitotic index (Cho *et al*., 2011), the second was used for studying chromosomal aberrations proceeded by hypotonic treatment with 0.075 ml kcl for 20 min at 37 ºC. **Then cells were centrifuged for 5 min. at 1000 rpm. the supernatant was shrug off, and the pellet re-suspended in freshly prepared chilled fixative methanol acetic acid (3:1v/v)** (Savage, 1993). The last step was repeated for two times. **Then cells were agitated and mixed thoroughly using a pasture pipette and drayed onto pre-cleaned chilled slides from a distance about 30-40 cm, air dried and stained with 5 % Giemsa stain** (Evans *et al*., 1964; Adler *et al*., 1984). **One hundred well spread metaphases per animal were examined and analyzed for different types of chromosomal aberrations using an Olympus research microscope at 1000x magnification** and tabulated according to Savage (1975). Photographs were taken.

Mitotic index (MI) estimation:

The MI was calculated from the slides used for assessing chromosomal aberrations**.** Randomly selected metaphases were examined to count the dividing cells and the total cell number. We examined 1000 cells per each mouse. The MI was calculated as: number of dividing cells/ total number of cells multiplied by 100**.**

Micronucleus Assay:

Animals were sacrificed, both femurs dissected, and marrow taken off with 2 ml of fetal calf serum. Smears were prepared on pre-cleans glass slides according to the procedure of Schmid (1975) and stained with Giemsa for 10 min (Krishna and Hayashi, 2000)**.** One thousand PCEs/ animal were scored to determine the number of micronucleated polychromatic erythrocytes (MNPCEs)**.**

Statistical analysis:

Data were analyzed using one-way analysis of variance (ANOVA) followed by two-tailed t test when the ANOVA test yielded statistical differences. The criterion for statistical significance used was $p \leq 0.05$. All data were expressed as the mean ± SD.

RESULTS

The data on the genotoxic effects of doxorubicin (DXR) evaluated from bone marrow cells of mice treated with 0.2, 0.4 and 0.8 mg/kg B.W. are furnished in Table (1). These data illustrate the changes observed in various types of chromosomal abnormalities.

Table 1. Frequencies and percentages of various types of chromosomal abnormalities (CA) recorded in bone marrow cells of mice after treated with three doses of doxorubicin

Group	No. of examined cells / 3 animals	Structural CAs					Numerical CAs polyploidy	Total Aberrations	% Aberration (mean± SD)
		Gap[º]	Break	Fragment	Ring	Centric fusion (CF)			
Control	300	6 (0.020)	6 (0.020)	4 (0.013)	0.00	0.00	0.00	10 (0.033)	3.33±0.28
0.2 mg/kg	300	9 (0.030)	18 (0.060)	16 (0.053)	4 (0.013)	0.00	0.00	38 (0.127)	12.67±2.42*
0.4 mg/kg	300	5 (0.016)	31 (0.103)	30 (0.100)	15 (0.050)	6 (0.020)	5 (0.016)	87 (0.290)	29.00±4.27*
0.8 mg/kg	300	6 (0.20)	38 (0.126)	35 (0.116)	25 (0.083)	12 (0.040)	6 (0.020)	116 (0.386)	38.67±2.82*

º Cells with Gaps were not included in the total chromosome aberration.
Values in the parentheses are percentages of aberration.
***significantly different from untreated control (G1) P<0.05.**

Doxorubicin caused a significant increase in chromosomal abnormalities at all tested doses. The mean frequency of chromosomally aberrated cells calculated for each dose with three animals per dose were 12.67±2.42, 29.00±4.27 and 38.67±2.82 at doxorubicin doses of 0.2, 0.4 and 0.8 mg/kg B.W. respectively. The control group had a mean percentage of 3.33 ± 0.28 (Table). These results indicated a DXR dose-dependent increase in the incidence of CAs observed (Figure1).

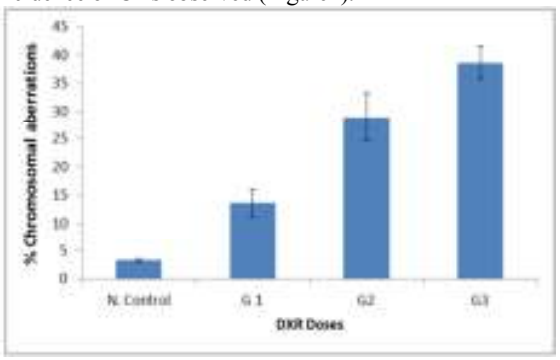

Figure 1. Effect of treatment mice bone marrow cells with different DXR doses on the incidence of chromosomal abnormalities.

The chromosomal aberrations induced included chromosomes with gaps, chromatid breaks, acentric fragments, rings and centric fusions (Robertsonian translocation). Cells with polyploidy were observed in mice treated with either dose of 0.4 and 0.8 mg/kg body weight. Figure (2) presents representatives of various types of chromosomal abnormalities observed in marrow metaphase chromosome spread of mice treated with DXR.

The type of structural aberration that occurred most frequently was chromatid breaks followed by chromosomal fragments with the least frequent being the centric fusion. Chromosomes with gaps were counted but ignored in the categories of the damaged cells, according to Alimba *et al.* (2006) that gaps are not good indicators of chromosome damage.

Mitotic index (MI):

In the current investigation the mitotic index was calculated in percentage to evaluate the effect of treatments with different doses of DXR on cellular proliferation in bone marrow cells of mice. The results obtained showed gradual decrease in the presence of dividing cells proportional to the dose tested (Table 2 and Figure 3).

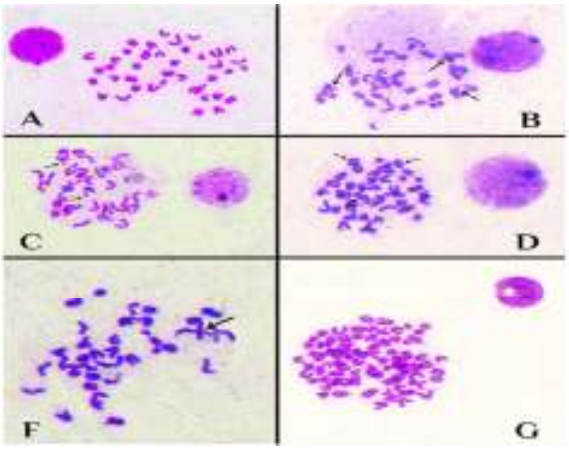

Figure 2 A-F. Representatives of bone marrow metaphase chromosome spreads of animals injected with different DXR doses. A) Normal metaphase . B) Chromatid break. C) Chromosomal fragment. D) Ring chromosomes. F) Centric fusion (Robertsonian translocation) . G) Polyploid cell.

Table 2. Counts of divided cells in bone marrow cells and its mean percentages in the control group and the three groups treated with doxorubicin.

Groups	Dose (mg /kg B.W.)	Total No. of scored cells / 3 mice	No. of divided cells / total cells	MI (mean ±SD)
G 1	Control	3000	259	8.63 ± 0.27
G 2	0.2	3000	198	6.60 ± 0.54
G 3	0.4	3000	139	4.63 ± 0.38
G 4	0.8	3000	93	3.10 ± 0.24

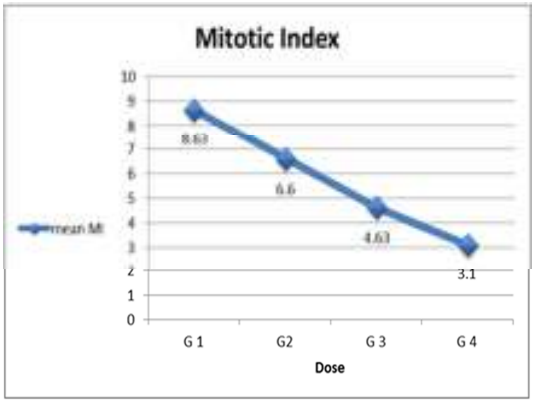

Figure 3. Mean percentages of divided bone marrow cells in the control group and the three groups treated with doxorubicin.

***In vivo* MN analysis:**

The results obtained in mouse bone marrow micronucleus test after treatment *in vivo* with different doses of the anticancer drug doxorubicin are presented in Table 3. Doxorubicin caused a significant ($p \leq 0.05$) increase in MNPCE in a dose-dependent manner (Figure 4). In fact, the increased number passed from a basal value of 4.67±0.48 in the control group to 7.00±1.02, 10.67±2.10 and 17.67±2.20 in groups treated with 0.2, 0.4 and 0.8 mg/kg body weight respectively. These results indicated that doxorubicin at the doses investigated had a genotoxic effects in mice marrow cells.

The cytotoxic potential of doxorubicin was evaluated by counting the number of PCEs among 1000 cells (PCEs + NCEs) per animal which is known as the PCE/NCE ratio. This number showed a mean value of 0.94±0.12 in the control group and decreased significantly to 0.85±0.02, 0.63±0.08 and 0.54±0.10 in mice treated with 0.2, 0.4 and 0.8 mg/kg B.W. respectively. This decrease reflected a dose-dependent response. Figure (5) shows the frequency of polychromatic erythrocytes (PCEs) in 1000 polychromatic and normochromatic erythrocyte (PCEs + NCEs) in animals treated with three doses of doxorubicin. Figure (6) showed the micronuclei induced in mice expressed to different doses of doxorubicin.

Table 3. Frequencies of micronucleated PCEs counted in mice marrow cells treated with different doses of doxorubicin.

Treatment	Total cells counted/mouse number	MNPCE/1000 PCEs mean ± S.D	PCE/ (PCE+NCE) mean ± S.D
Negative control	3000/3	4.67±0.48	0.94 ± 0.12
0.2 mg/kg	3000/3	7.00 ± 1.02	0.85 ± 0.02
0.4 mg/kg	3000/3	10.67 ± 2.10	0.63 ± 0.08
0.8 mg/kg	3000/3	17.67 ± 2.20	0.54 ± 0.10

All values are expressed as mean ± SD of three mice.
MNPCE statistical analysis one-way ANOVA.
PCE/ (PCE+NCE) statistical analysis chi-square (χ^2) test.

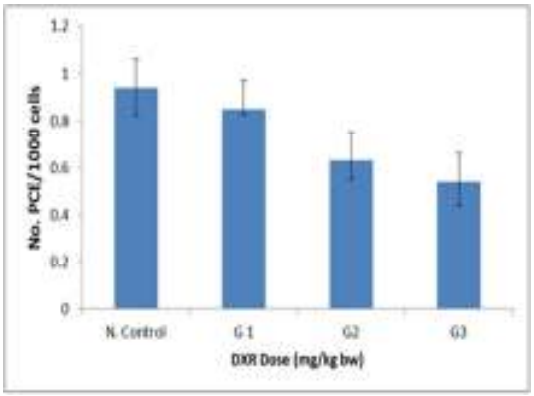

Figure 4. Number of polychromatic erythrocyte (PCEs) in 1000 PCE and normochromatic (NCEs) in animals treated with 0.2, 0.4 and 0.8 mg/kg dose of doxorubicin. Values are expressed as mean± SD, n=3.

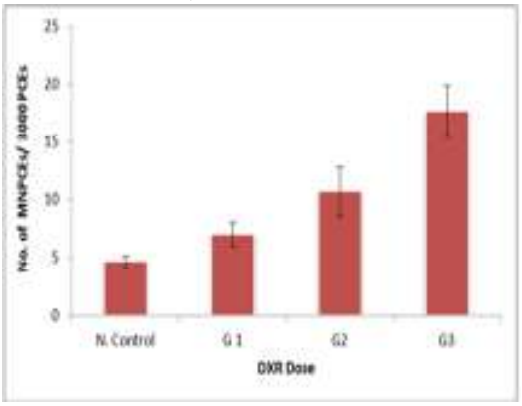

Figure 5. Frequency of micronucleated polychromatic erythrocytes (MNPCEs) in animals treated with increasing doses of doxorubicin. Values are expressed as mean± SD, n=3.

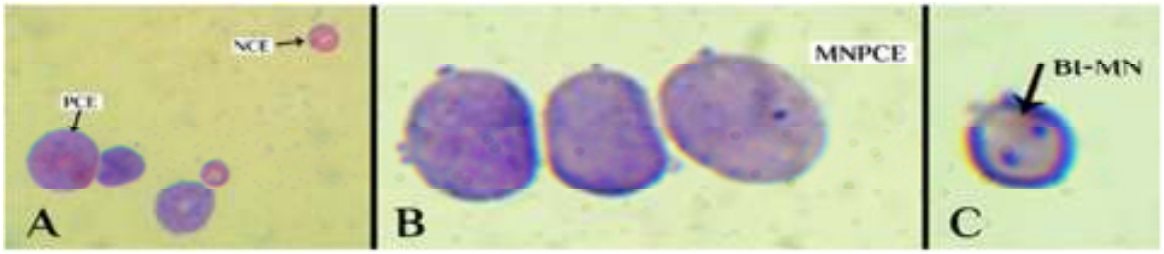

Figure 6. Micronucleus induced in mice exposed to doxorubicin. A) Polychromatic erythrocyte, (PCE), Normochromatic erythrocytes (NCE). B) Micronucleated Polychromatic erythrocytes. C) Two micronucleated polychromatic erythrocytes (Bi-MNPC).

DISCUSSION

Various in vitro and in vivo genotoxicity assays were proposed to evaluate the genetic damage caused by physical and chemical agents(Calik *et al*., 2005; Kato *et al*., 2013). Plausibly, the most reliable genotoxicity evaluation for human rich in cell proliferation is conducted in mammals through the induction of chromosomal abnormalities and micronuclei formation since they are highly correlated (Heddle *et al*., 1981). Particular attention is focused on chromosomal aberration induction as it represents an early warning signal for neoplasia (Hagmar *et al*., 1998). Additionally, the frequency of MN-PCEs in bone marrow is a reliable measure of both chromosome loss and breakage (Narayanan *et al*., 2002). The results obtained in the present investigation showed that cells of animals treated with the anticancer drug DXR had significantly increased the incidence of induced chromosomal aberrations while decreased the mitotic index. These results being consistent with the previous studies that revealed the ability of DXR to react with electron rich areas of susceptible molecular such as nucleic acid and proteins (Barton *et al*., 2003), and suggested that DXR target rapidly dividing cells and mitotic activity (Mishra and Bhiwgade, 2007). The results obtained support the earlier findings that DXR is capable of inducing mutations and chromosomal abnormalities in both normal and cancerous cells (Quiles *et al*., 2002; Abdella and Ahmed, 2009; Kusum Lata and Devi, 2010&2012) which showed that the incidence of chromatid-type aberrations correlated directly with Adriamycin dose. Among the types of structural chromosomal aberrations observed in the current study are centric fusion which produced metacentric-like chromosomes as a result of Robertsonian translocation between two acrocentric chromosomes. Similar results were reported by Au and Hsu (1980). The studies of Aydemir and Bilallug (2004) valuated the effect of DXR on inducing chromosomal aberrations in marrow cells of Wistar rats as well as that of Gülkaç *et al.* (2004) which coincide to the results obtained in the current study. In contradictory, Meistrich *et al.* (1990) failed to observe increases in chromosomal abnormalities in Adriamycin treated mice at 6 mg/kg BW or 8 mg/kg BW. With regard to the MN test, the results of our investigation showed that treatment of bone marrow cells caused a significant dose-dependent increase in MN-PCEs. This implies that doxorubicin is a genotoxic agent in mammalian cells and exposing human beings to it represents a human health risk. These results are comparable to Venkatesh *et al.* (2007) that DXR induced genotoxic effects in mice bone marrows. The results of the current investigation also revealed significant decline in P/N ratio in animals treated with 0.2, 0.4 and 0.8 mg/kg B.W. of doxorubicin compared to the control. Cicchetti *et al*. (1999) reported that the significantly decreased P/N ratio in treated animals suggests evidence of erythropoiesis depression with reduced nucleated erythrocyte precursors proliferation.

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التأثيرات السمية الوراثية لمضاد الأورام دوكسوروبيسين فى خلايا نخاع عظم الفأر السويسرى

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ربما يكون عقار دوكسوروبيسين المستخدم فى العلاج الكيميائى المضاد للورم هو أكثر العقاقير لعلاج العديد من أنواع السرطان البشرى عبر أنحاء العالم. أجريت الدراسة الحالية لتقييم التأثيرات السمية الوراثية لهذا العقار فى خلايا نخاع عظم الفأر بإستخدام استحداث الشذوذات الكروموسومية وتكوين النويات الصغيرة. استخدمت أربع مجموعات بكل منها ثلاث حيوانات ذكور ذات عمر 6 – 8 أسابيع ووزن 24 ± 2 جرام. تم حقن فئران المجموعة الأولى داخل الغشاء البريتونى بالماء فقط كمجموعة ضابطة (مقارنة). وحقنت المجموعات الثلاث الأخرى بجرعات دوكسوروبيسين 0.2، 0.4، 0.8 ملجم / كجم من وزن الجسم لمدة يومين متتاليين. بعد 24 ساعة من الجرعة الثانية تم تخدير الفئران وإعدامها بخلع العنق، وقبل إعدامها بساعة ونصف تم حقنها بالكولشيسين بتركيز 0.05 %. تم تشريح الفئران واستخلاص خلايا نخاع العظم من عظمتى الفخذ. أحدثت جميع المعاملات بالدوكسوروبيسين زيادة معنوية كبيرة فى تكرارات الشذوذات الكروموسومية التركيبية والعددية على السواء. وشملت هذه الشذوذات الفجوات والكسور الكروموسومية والشظايا والكروموسومات الحلقية والإلتحامات السنترومبرية والتعدد المجموعي. وقد ارتفع متوسط التكرار الإجمالى للشذوذات الكروموسومية من 3.33 فى المجموعة الضابطة الى 12.67، 29.00، 38.67 للجرعات 0.2، 0.4، 0.8 ملجم / كجم من وزن الجسم على التوالي. ازداد عدد خلايا PCE ذات النويات الصغيرة المشاهدة فى خلايا نخاع الفئران التي عوملت بجرعات الدوكسوروبيسين الثلاث من 4.67 للمجموعة الضابطة الى 7.00، 10.67، 17.67 على التوالي. فى ذات الوقت تناقصت نسبة P / N المحسوبة من 0.94 فى المجموعة الضابطة الى 0.85، 0.63، 0.54 في الحيوانات المعاملة بالجرعات الثلاث على الترتيب. تشير نتائج الدراسة الحالية إلى أن عقار دوكسوروبيسين له سمية جينية فضلا عن السمية الخلوية فى خلايا نخاع الفأر.