Cytogenetic effects of albendazole on stem cells mice bone marrow

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التأثيرات الوراثية الخلوية للألبيندازول على خلايا نخاع العظم للفئران المختبرية

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المستخلص

تم حساب النسبة المئوية للتشوهات الكروموسومية وتكون النويات الصغيرة و معامل الانقسام في خلايا نخاع العظم للفئران المختبرية كطريقة لقياس قدرة الألبندازول على استحداث المتغيرات اعلاه, وعليه قسمت الدراسة وفقا لأوقات الحقن (البريتونى) والتركيز (200 و400 ملغم / كغم) للألبيندازول. قسمت الفئران إلى ثلاث مجموعات شملت: المراقبة السلبية، مراقبة إيجابية ومجموعة العلاج. قسمت المجموعة السلبية إلى مجموعتين، الاولى بدون اي معاملة والثانية عوملت مع 0.2 مل بمحلول ملحي. ,وقسمت المجموعة الإيجابية إلى مجموعتين، واحده منهم حقنت ABZ (200ملغم / كلغ) و الآخرى (400 ملغ / كلغ) لمدة 24 ساعة. شملت مجاميع المعالجة ست مجموعات: الأولى حقنت بالألبندازول(200 ملغم/كلغ) لمدة 72 ساعة ، الثانية (400 ملغ / كلغ) بنفس وقت المجموعة السابقة ، الثالثة حقنت بجرعتين (200 ملغم /كلغ) (الحقنة الأولى في اليوم صفر والحقنة الثانية في اليوم السابع)، الرابعة حقنت بجرعتين (400ملغ / كغ) بنفس الوقت ، حقنت الخامسة جرعتين (200 ملغ/كلغ,الأيام الثلاثة الأولى جرعة كل يوم/ و بعد سبعة أيام كرر ثلاث جرعات جرعة كل يوم) ،واخيرا حقنت السادسة بجرعتين (400 ملغ/كلغ) لنفس الوقت. بعد التحليل الإحصائي للبيانات أظهرت النتائج أن عقار الألبندازول أدى إلى زيادة كبيرة في التشوهات الكروموسومية، النواة الصغرى وانخفاض في معامل الإنقسام ($P < 0.05$). وترتفع هذه النسبة بالتزامن مع طول فترة العلاج.

الكلمات المفتاحية :اللألبندازول,معامل الانقسام,النوى الصغيرة,التشوهات الكروموسومية

Abstract

This experiment was estimated percentage of chromosomal aberration (CAs), micronucleus` (MN) and mitotic index (MI) in bone marrow cells of mie laboratory as a way to measure/e Albendazole (ABZ) ability of the above variables. This study divided according to times injection and concentration of ABZ (200 and 400 mg / kg). Mice were divided into three groups included negative control I&II (I no treated & II treated with 0.2 ml of normal saline), positive control split into two groups (one of them injected with ABZ 200 mg / kg, second with 400 mg / kg for 24 hours) and treatment groups. These groups are dividing to the sixth category according to concentration and period of treatment. Groups

I&II (I the mouse were treated with ABZ three days 200 mg/kg for every day and II same period, but dose400mg/kg). Groups III&IV (III the mouse treated with two injection of ABZ 200 mg/kg first dose at zero day, and the second at seventh day and IV same period but dose 400mg/kg). Groups V&VI (V the mouse treated with two doses ABZ 200 mg/kg first three days, with one dose/one day and after seven days repeat three doses one dose /one days and VI same period, but the dose is 400mg/kg). The results showed that the ABZ drug led to significant increase in CA, MN and decrease in MI at ($p<0.05$). This percentage increases in conjunction with the length of the treatment period.

Key words :Mitotic index, chromosomal aberrations, micronuclei, albendazole.

Introduction

Chemical drugs called Anthelmintic are used for controlling parasitic infections, which caused by helminthes (1).Albendazole is an Anthelmintic drug used in the treatment of many parasitic infestations (2,3). Albendazole (ABZ), which is a tubulin-disrupting benzimidazole (BZ) and a potent micro filicide when binds to β-tubulin, it will cause a structural impairment of cytoskeleton and worm death, can see extreme cellular disturbances in ABZ-treated worms which are characterized by nucleosomes DNA laddering and chromatin condensation(4). Albendazole (ABZ) possesses DNA damaging capabilities (5). It seems that the Albendazole exerts their effects by binding selectively, and they have a high affinity to the β-tubulin molecules, and in this case the disappearance of cytoplasmic microtubules suggests that Albendazole acts by preventing the microtubule-mediated transport of secretory vesicles in the helminthes absorptive tissues and the result will be releasing the digestive enzymes which cause tissue damage (6). ABZ has broad-spectrum activity, including helminthes as well as protozoa ABZ selectively bind to nematode ß-tubulin, inhibiting polymerization, thus preventing the formation of microtubules and so stopping cell division. Impaired uptake of glucose, leading to depletion of glycogen, and reduced stores of ATP has also been Resistance to ABZ has been attributed to specific amino acid changes in the tubulin protein, leading to reduced binding affinity for ß-tubulin (7).As known, bone marrow is considered as a major hematopoietic organ, which is composed of hematopoietic cells in various stages of ripeness, including erythrocytes, leukocytes and platelets (8).Many chemical compounds have been noted to be mutagenic because of the damage production and changes in the genetic material of DNA(9,10). The purpose of this study is evaluating the cytogenetic effects of Albendazole on mice bone marrow stem cell. The present study is designed to detect the genotoxicity of albendazole when it is treated at different times with concentrations (200, 400 mg/kg) and to clarify whether this drug can cause

chromosomal damages in the bone marrow of treated mice or not.The effect of the length period of the drug therapy on the bone marrow cells represented by chromosomal aberrations, micronucleus and mitotic index.

Materials and methods

Albendazole dose and concentration

Albendazole (200 mg /tablet) was a product of (Micro, India), it was solutes in normal saline and injected intraperitonlly (11).Two doses used (200,400 mg/kg) in different time.

Laboratory animals

Hundred albino Swiss male mice were gained from National Center for Drug Control and Research / Ministry of Health / Baghdad. Their ages ranged between (8-12) weeks and weighting (25 ±2) gm. They were divided into 12 groups; each group was put in a separated plastic cages. The cages left in a room with temperature (23-25ºC). The animals were given water and free excess to food (standard pellets) and care was taken to avoid stressful conditions.

Administration of laboratory animals

Mice groups (10 mice for every groups) were divided according to times of injection (intraperetonially) with concentration (200&400 mg/kg) of albendazole. All mice were killed after 24 hours from last treatment. The mouse bone marrow samples were taken from all animals for cytogenetic analysis [Mitotic index (MI) Chromosome aberration (CA) and Micronucleus (MN).

Negative control groups

Two types of control groups in the study:
Group I:
Negative control I, (an treated mouse).
Group II:
Negative control II, the mouse treated with 0.2 ml of normal saline

Positive control groups

There are two controls in this part
Positive control I, the mouse treated with albendazole 200 mg /Kg.
Positive control II, the mouse treated with of albendazole 400 mg /Kg.

Treatment groups

The treatment groups were divided according to treatment times and doses (200& 400 mg/kg) of albendazole as follow:
Treatment group I,
the mouse was treated (three days) with Albendazole (200mg/kg for every day).

Treatment group II,
The mouse was treated (three days) with Albendazole (400mg/kg for every day).
Treatment group III,
The mouse treated with two doses (first dose at zero days and second at seventh day) of albendazole 200mg /kg.

Treatment group IV,

The mouse treated with two doses (first dose at zero days and second at seventh day) of albendazole 400mg /kg.

Treatment group V,
The mouse treated with two doses (first three days one dos/one day and after seven days repeat three doses one dose /one days)of albendazole 200mg /kg

Treatment group VI,
the mouse treated with two doses (first three days one dos/one day and after seven days repeat three doses one dose /one days) of albendazole 400mg /kg

From all groups above the mouse bone marrow samples were taken for cytogenetic analysis (MI, CA, and MN).

Cytogenetic experiments

Chromosome preparation from somatic cells of the mouse bone marrow

The experiment was done according to Allen *et al* (12). Colchicine was injected 2hr. before sacrifice. Mice were sacrificed by cervical dislocation. It was dissected, and both of femur bones were excised. Bone marrow was aspirated by flushing with phosphate buffer saline (PBS) in the centrifuge tube. The suspension was flushed in the tube properly to get good cell suspension and centrifuged for 10 min at 2000 rpm. Supernatant was discarded and the pellet was treated with pre-warmed (37°C) KCl (0.56%) and shaken well. Suspension incubated in a water bath at 37°C for 20 min. Pellet was treated with freshly prepared fixative solution (Methanol: Glacial Acetic Acid, 3:1) and shaken well then centrifuged for 10 min at 2000 rpm. Fixative was repeated 3 times to get debris free white pellet. Few drops from the tube were dropped vertically on the slide. Slides were kept overnight to dry then stained with (Giemsa's stain) and observed under microscope in 40 x and then in 100x magnifications. A total of 100 well spread metaphase plates were scored for chromosomal aberrations) gap, chromatid break, polyploidy, acentric fragment, ring and fragmentation (were counted and data of scoring was expressed as percentage chromosomal aberrations.

Cytogenetic analysis

1-Mitotic index (MI) assay

The slides were examined under high power (40x) of light microscope, and (1000) of divided and non-divided cells were counted and the percentage rate was calculated for only the divided ones (metaphase cells) according to the following equation:-

$$\text{Mitotic Index} = \left(\frac{\text{Number of Metaphase Cells}}{\text{Total number of the cell(1000)}} \right) \text{x } 100$$

2-Chromosomal aberration (CA) assay

The prepared slides were examined under the oil immersion lens (100x) of light microscope for 100 divided cells per each animal, and the cells should be at the metaphase stage of the mitotic division where the chromosomal aberrations were clear and the percentage of these aberrations could be estimated.

3-Micronucleus MN assay

This experiment was done according to method of Schmid (13) as following:-

The femur bone cleaned from tissues and muscles, then gapped from the middle with a forceps in a vertical position over the edge of a test tube by a sterile syringe, (1 ml) of human plasma (heat inactivated) was injected so as to wash and drop the bone marrow in the test tube. Then the test tubes were centrifuged at speed of 1000 rpm (5 min).The supernatant was removed, and one drop from the pellet was taken to make a smear on a clean slide. The slides were kept at room temperature for (24 hr.). The slides were fixed with absolute methanol for (5 min.), then stained with Giemsa stain for (15 min), then washed with D.W and left to dry. Two slides for each animal were prepared for micronucleus test. The slides were examined under the oil immersion lens, and at least 1000 polychromatic erythrocytes (PCE) were examined for the presence of micronucleus. The micronucleus index was obtained using the following equation:

$$\text{Micronucleus Index} = \left(\frac{\text{Number of Micronucle i}}{\text{Total Count of PCE}} \right) \text{x } 100$$

Statistical Analysis

The values of the investigated parameters were given in terms of mean ± standard error, and the differences between means were assessed by analysis of variance (Two-sample T-test) using the computer program Minitab release (14.12) discovery Copyright 2004. The difference was considered significant when the probability value was less than $p<0.05$.

Results and Discussion

The results of mitotic index assay are presented in table (1). There are significant difference when we compare between negative control group II (0.2 ml of N. S.) with other groups (positive control I, II and treatment groups (I, II, III, IV, V and VI) and these differences were back to the toxic effect of albendazole (ABZ) alone by reducing the mitotic index (MI). All these results were significant at ($P<0.05$).

Table (1): Percentages of mitotic index in mice bone marrow for negative control group (I & II), positive control (I & II) and treatment groups (Mean ± SE).

Experimental Groups	MI%
Negative control I	6.6 ±0.3
Negative control II 0.2 ml of N.S	[a] 6.4±0.400
Positive control I ABZ 200 mg for 24 hours	[b]*4.02 ± 0.291
Positive control II ABZ 400 mg for 24 hours	[b]*3.52 ± 0.159
Positive control I ABZ 200 mg/kg for 24 hours	[c]*4.020 ± 0.291
Positive control II ABZ 400 mg/kg for 24 hours	[c]*3.520 ± 0.159
Treatment I	[c]*2.820± 0.146
Treatment II	[c]*2.520 ± 0.185
Treatment III	[c]*2.600±0.207
Treatment IV	[c]*2.320±0.177
Treatment IV	[c]*2.320±0.177
Treatment V	[c]*2.200 ± 0.130
Treatment VI	[c]*2.080 ±0.116

a Negative control II Vs. Negative control group I, b Positive control I&II Vs. Negative control group II, C treatment groups Vs. Negative group II, *Significant at ($P<0.05$).

Result of chromosomal aberrations in positive control I have (0.612%) and (0.882%) showed a frequency of total chromosomal aberrations in mice bone marrow cells. This finding has significant ($P<0.05$) when compared with negative control group II (0.1%).

In the treatment group (I, II, III, IV, V, V and VI), as showed in the table (2), that is treated with albendazole in different times gave a high significant different in mice bone marrow cells, when compared with negative control group II (0.1%). But, when the same dose of ABZ was given a

long time in treatment group (VI) with concentration (400 mg /kg), the result of total CAs showed a very high significant value (P<0.05) when compared with negative control group II.

Table (2): Percentages of different types of chromosomal aberrations (CA) in mice bone marrow for negative control (I & II), positive control (I&II) and treatment groups (Mean ± SE).

Experimental Groups	Types of chromosome aberrations %						Total %
	Acentric Fragment %	Ring %	Polyploidy %	Break %	Fragment %	Gap %	
Negative control I	0.0380±1.07	0.00±0.00	0.00±0.00	0.024±0.510	0.024±0.510	0.02.4± 0.40	0.11±1.05
Negative control II	0.032±0.374	0.00± 0.00	0.00 ±0.00	0.018± 0.374	0.018±0.374	0.032±0.200	[a] 0.1± 0.707
Positive control I	0.282±0.374	0.046±1.21	0.022± 0.860	0.058±0.663	0.094±1.33	0.11±1.84	[b*] 0.612±0.735
Positive control II	0.49±2.28	0.086±1.17	0.022± 4.17	0.072±0.735	0.122±2.06	0.09±0.316	[b*] 0.882±3.62
Treatment I	1.182±10.7	0.074± 0.92	0.02±0.707	0.114± 3.83	0.318±3.65	0.068±2.31	[c*] 1.776±0.735
Treatment II	1.246±11.4	0.046± 0.40	0.012±0.583	0.094±0.510	0.472±0.860	0.064±0.600	[c*] 1.934±10.6
Treatment III	1.518±16.9	0.088+4.22	0.01±0.775	0.138± 1.66	0.456±2.29	0.09+3.18	[c*] 2.292±15.8
Treatment IV	1.76±13.1	0.074±1.36	0.012±0.800	0.048±1.16	0.438±6.73	0.02±0.548	[c*] 2.3±19.8
Treatment V	1.986±3.22	0.044±0.510	0. 00±0.400	0.072±0.374	0.38±3.35	0.064±0.510	[c*] 2.55±5.51
Treatment VI	2.18±6.35	0.038±0.735	0.002±0.200	0.17±0.949	0.354±1.60	0.064±0.812	[c*] 2.8±6.72

a Negative control II Vs. Negative control group I, b Positive control I&II Vs. Negative control group II, C treatment groups Vs. Negative group II, *Significant at (P<0.05).

Table 3 showed the results of micronuclei (MN). The frequency of MN in negative control II showed a significant differences when compared with positive group and all treatment groups at (P<0.05).

Table (3): Percentages of micronuclei (MN) in bone marrow of mice for negative control group (I& II), positive controls and treatment groups (Mean ± SE)

Experimental Groups	MN %
Negative control I	3.1± 0.114
Negative control II	[a] 2.9±0.239
Positive control group I 200mg 24h	[b*]5.4 ± 0.210
Positive control group II 400 mg 24 h	[c*]6.6 ± 0.400
Treatment I	[c*]8.42±0.174
Treatment II	[c*]8.62±0.169
Treatment II	[c*]7.7±0.100
Treatment IV	*8.8±0.126
Treatment V	[c*]9.5±0.170
Treatment VI	[c*]12±0.316

a Negative control II Vs. Negative control group I, b Positive control I&II Vs. Negative control group II, C treatment groups Vs. Negative group II, *Significant at ($P<0.05$).

The positive control showed a significant reduction in MI and a high increasing in CAs and MN. The reason of these results was related to the cytotoxic impact of ABZ. The results showed that the Albendazole (ABZ) has ability to reduce cells proliferation (Mitotic index) in mice bone marrow. Albendazole has damaging effect on DNA of mice bone marrow cells. (14). Albendazole treatment leads to potent inhibition of cell proliferation (Mitotic index) in bone marrow. Chromosome aberration increases because of the ability of albendazole (ABZ) to act as mitotic spindle poisons, which leads to the potential risk of aneuploidy induction in exposed cells, albendazole inhibits tubulin polymerization, thus it will results in improper microtubule formation The

disturbance of the mitotic apparatus (spindle, kinetochores) and leads to micronucleus (MN) formation, either by chromosome fragments or by whole chromosomes (15,16). The results indicated to the Albendazole causes a high structural CAs instead of numerical CAs. The current study reveal that ABZ inhibits microtubule formation during the mitosis of parasites, in the same time it may also affect the genome of the host cell as shown in the study done by (17,18). A centric fragment increased when treated with albendazole, because the ABZ induces cell cycle arrest at G2, albendazole has well-defined effects as a microtubule disrupting agent, albendazole mechanism of action as a DNA damages and microtubule disrupting agent is assessed through analysis of histone H2AX phosphorylation and cell cycle progression (5). The chromatid type aberrations like breaks and gaps are produced during the S or G2 stage (i.e. during or after replication). Since albendazole affects the spindle filaments during the process of cell division so chromosome must first be broken to form any kind of rearrangement a chromosome may break at any stage of the cell cycle, if chromosome breaks during G1 and remains unrepaired through S phase the result will be visible in both chromatids as a chromosome breaks in the next metaphase. When a break occurs during G2 usually involves only one of the two chromatids and is therefore called a chromatid break a single break yields a deleted chromatid and an acentric fragment (1). Treatment group VI has given the highest proportion of chromosome aberration and shows significant differences, when compare with negative control. Albendazole has been reported to be oxdatively active, leading to strong reactive oxygen species (ROS) generation (19). This effect will add a further potential toxicity to this drug. It was reported that ABZ is poorly absorbed and rapidly metabolized, and the deleterious effects of ABZ when it is exposed individually may depend on the concentrations reached during the treatment period and on the tubulin binding capacity of the drug (20, 21). Therefore albendazole remains for a long time in the body.

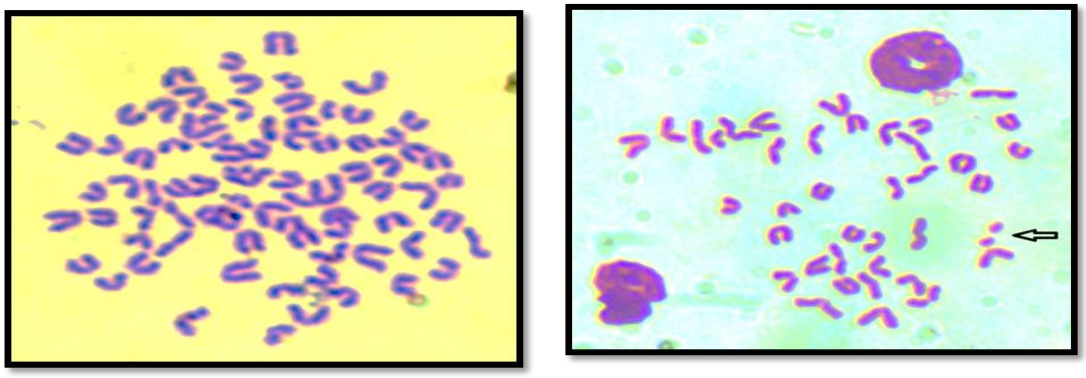
Albendazole is an inhibitor for the tubulin polymerization that is in charge of vital processes, including the motility and nutrient uptake in the parasite and chromosomal segregation at mitosis in exposed cells (22,16). The carbamate group has found in structural of albendazole it may be cause free radicals. The continuous and unsafe use of carbamate results the generation of reactive oxygen species (ROS) and the biological system's ability to detoxify the reactive intermediates resulting into oxidative stress. The biological antioxidant defense system plays a vital role in constituting natural defense against endogenous generated ROS and other free radicals (23,24).

Carbamate exposure is linked to free radical generation, so it causes oxidative stress and various types of damages to biomolecules such as DNA, lipids, proteins etc. (25).Albendazole cytotoxic effect involves tubulin binding. The

cytotoxic effect of ABZ usually involves binding to β-tubulin, it has been suggested that oxidative stress may also play a role in its parasitical mechanism. Reactive oxygen species (ROS) were induced by ABZ (26). The effects of ABZ and its metabolites on cell proliferation, as well as on the frequency of micro nucleated cells in bone morrow revealed that small nuclei are increased when dealing with drug ABZ. Since the absorption of ABZ in the body is slow that makes its availability in the body for longer duration. The formation of micronuclei may ultimately results in aneuploidy induction, an effect that could have severe consequences in humans.

In the framework of the results that have been obtained in the chromosomal tests showed increase the proportion of chromosomal aberration when treated with albendazole, and it works on the composition of the potential dangers of the cell, thus in humans genome. The chromosome aberration has been found acentric fragment, fragment. Therefore these aberrations transmitted over the generations and some of them cause cell death.

Figure (1) showed different chromosome aberrations in treatment groups(ABZ).



A B

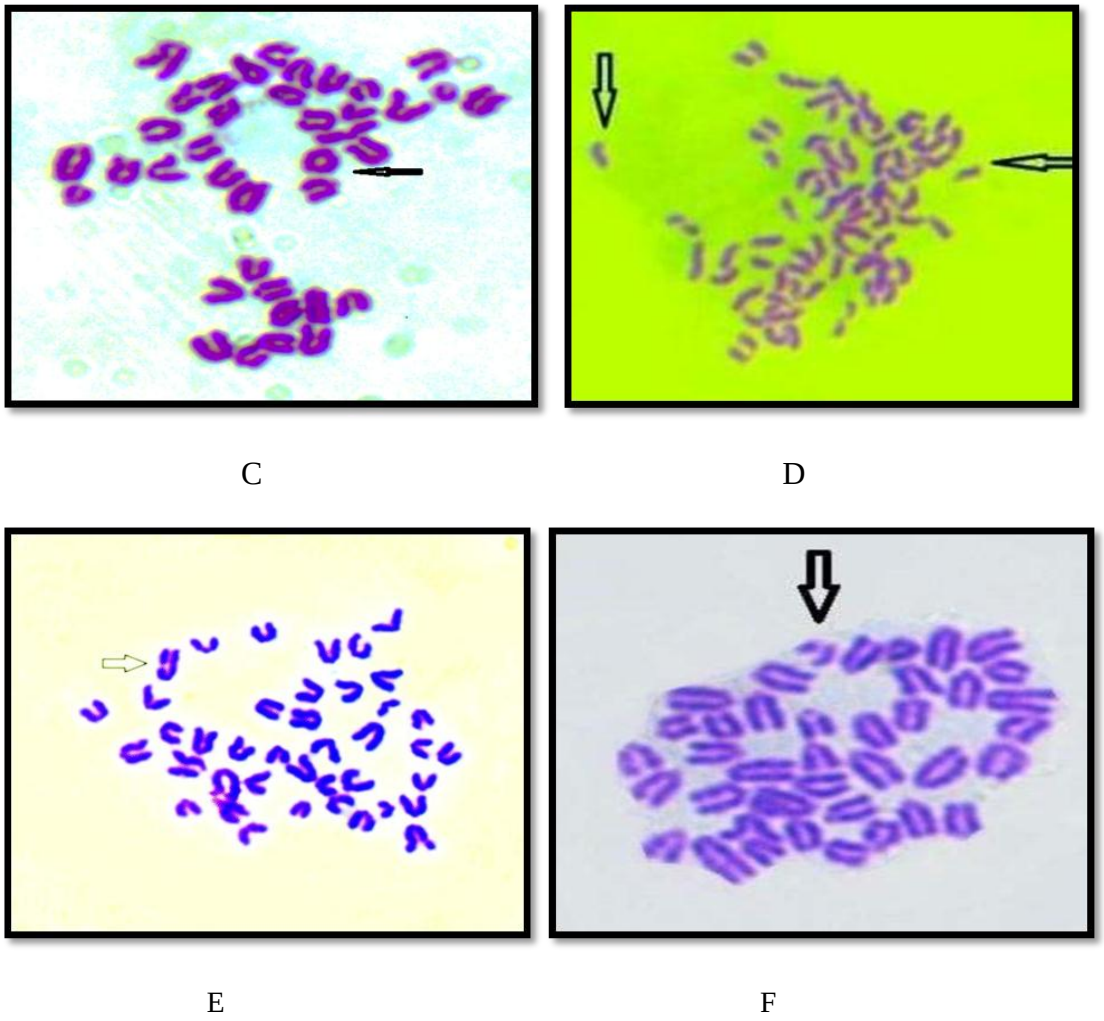


Figure (1): different chromosomal aberrations in mice bone marrow (100X) injected only with Albendazole. A: polyploidy B: acentric fragment C: ring D: fragment E: gap F: break.

In all three treatment groups, we can indicate the reducing of MI and increasing of CA and MN. MN can be formed during mitosis through the loss of either whole chromosomes or a centric chromosome fragments from the nucleus, which are separate from the nucleus of the cell (15,27).The difference in micronucleus (MN) rates was statistically significant in the comparison of the values before and after albendazole therapy ($p<0.05$). Therefore, we conferred that albendazole may potentially be a genotoxic agent for humans. The results of this study indicated that ABZ increase the MN either by chromosome fragments or by whole chromosomes at statistically significant levels in the treated cells compared with the negative control. According to the mode of action of ABZ and the previous genotoxicity studies of ABZ, the formation of MN may be possibly based on aneuploidy induction,

thus producing MN and this result compatible with study done by(15,22)

This study proved that albendazole increases the frequency of chromosomal aberration and therefore it increases the micronucleus as shown in figure (2), and decreasing mitotic index.

The increase in treatment groups may be caused by the mode of action of ABZ; gives rise to ideas about chromosomal damage. We suggested that MN formation can produced from centromeres damaged and chromosomes that fail to correctly attach to the spindle. DNA damage can be established on the chromosome non-disjunction as a result of improper microtubule formation and was not seen disturbance of the mitotic spindle due to the inhibition of tubulin polymerization by the interaction of the carbamate group of ABZ with micro tubular proteins (16). From this research, it is evident that the ABZ treatment increased the frequency of MN where there was a positive correlation between the increased drug concentration and the induction of MN in the bone marrow cells. Furthermore, the frequency of MN induced at the highest dose of ABZ was approximately three times higher than control value. The increase in MN between the two highest doses of ABZ was probably due to the absorption of the drug. This detect that albendazole encourage MN and produce non-disjunction events in human lymphocytes treated in vitro, and suggested that the mechanism is capable of inducing aneuploidy and the final consequence of MN formation, based on the chromosome non-disjunction for the period of cell replication as a result of disruption of the mitotic spindle (22).

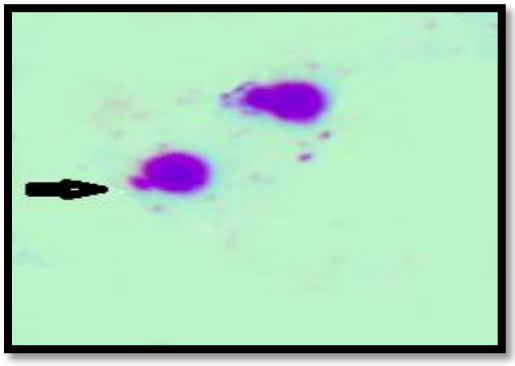


Figure (2): Albendazole formation MN at (100x).

Conclusions

Experiments conclude that albendazole has genetic toxicity effects on dividing cells in the bone marrow of mice. The genetic toxicity of albendazole leads to an increase in chromosomal aberration (CAs), an increase in the formation of micronucleus (MN) and reduction in the

mitotic index (MI).Different doses of albendazole and the time of treatment have various effects on dividing cells in the bone marrow. That the albendazole is a toxic contrast agent, which shows significant genotoxicity, effects on mouse bone marrow cells.

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