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Genotoxicity of Dactinomycin and Tramadol on Mice Bone Marrow

Muthana Ibrahim Maleek, Safaa A Faraj, Marwa Mahdi Khalaf

Department of Biology, College of Science,
University of Wasit, Wasit Province, AL- Kut, Iraq

Abstract: ***This study was established to investigate the genotoxic effects of tramadol 0.025 mg/kg on mice bone marrow before and after dactinomycin 0.4 mg/kg. Dactinomycinwas given alone to the mice intraperitoneally with 0.4 mg/kg for 24 hrs. Similarly, tramadol was injected with 0.025 mg/kg for 24 hrs, which were considered as positive controls. Moreover, different combinations of dactinomycin / tramadol were given in terms of different time points of treatment. Results showed high induction (p<0.05) of chromosomal aberrations (CAs) and micronuclei (MN) and reduction in mitotic index (MI) of following treatments: dactinomycin alone, tramadolaloneand tramadol /dactinomycin combination, compared to corresponding control groups. Interestingly, dactinomycin showed treatment with tramadol increase dose in three doses every 8 hours before and after chemotherapy highest urged all biological endpoints from other groups. And we concluded that the impact of higher mix may belong to an increase in the negative impact of dactinomycin that increase by tramadol and vice versa .in addition to the negative impact of the tramadol and dactinomycin especially when increasing doses tramadol during the day.***

Keywords: Mitotic index, chromosomal aberrations, micronuclei, Dactinomycin, Tramadol

1. Introduction

Dactinomycine is the greatest important member of actinomycines, which are a group of polypeptide antitumor antibiotics secluded from soil bacteria of the genus Streptomyces (1). Dactinomycin is a clear, yellow fluid managed intravenously and most usually used in treatment of a variation of cancers, including:Gestational trophoblastic neoplasia (2)Wilms' tumor (3)Ewing's sarcoma (4). In cell biology, dactinomycin is exposed to have the capacity to inhibit transcription. Dactinomycin does this through binding DNA at the transcription initiation complex and preventing elongation of RNA chain by RNA polymerase (5). dactinomycin have genotoxic special effects of its particular and similarly had preservative effects on sonication-induced genotoxicity in mice by parameters like chromosome aberrations (CA), mitotic index (MI), sperm head anomaly (SHA) and micronucleated erythrocytes (MNEs)(6). Tramadol is a version centrally substitute analgesic used for the treatment of mild to intense pain (7).It has been accepted in some nations since 1980 and become the most specific opioid worldwide (8). Much of the toxicity in tramadol overdose seems to be attributable to the monoamine uptake inhibition rather than its opioid effects (9). Tramadol overdose may make increase of creatinine phospho kinase (CPK). While CPK rise could be independent from seizure, in cases with seizure, CPK rise is more dramatic and may be related to acute renal failure (10) Rise in white blood cell count has been reported (11). Bleeding dangers due to tramadol interface with oral anticoagulants has similarly been stated (12).Tramadol effects can be through the overproduction of nitric oxide and oxidative stress (13).these meant tramadol does not affect directly the rate of cell division but tramadol ability to produce freeradicals, which in turn will affect the cell, including the nitricoxide (NO). The capability of an extra free radical to damage molecules like DNA, protein and lipids is frequently referred to the initiation and progression of carcinogenesis and abnormal vascular cell proliferation (14). This study was aimed to evaluate the effects of tramadol on increasing the effect of dactinomycin and vice versa.

2. Materials and Methods

Tramadol dose and concentration

Tramadol (100ml) was the product of Alexandria (Egypt); it's obtained from Al-Karamah Teaching Hospital as vial. For mouse injection, a dose of(1 mg/kg)was tested by dissolving the drug in distilled water to prepare the required dose and concentration Such concentration has been found to be genotoxic of bone marrow of mouse(15),which equivalent to (0.025 mg/mouse).

Dactinomycin dose and concentration

Dactinomycin (0.5 mg) was the product of Celon Laboratories LTD (India), its obtained from Al-Karamah Teaching Hospital as vial. For mouse injection, a dose of(0.015 mg/kg) (16).was tested by dissolving the drug in distilled water to prepare the required dose and concentration ,which equivalent to (0.4 *m*g/mouse)(17).

Laboratory animals

Eighty Albino Swiss male mice were gained from Iraqi Centre for Cancer and Medical Genetics Research / Mustansiriya University / Baghdad.

Their ages ranged from 8 to 12 weeks and weighting were (25 ±2) gm. They are divided into 8 groups; every group is put in a divided plastic cages. The cages are place in a room with temperature (23-25ºC). The animals are given water and free excess to food (standard pellets) and maintenance is taken to avoid stressful conditions.

Administration of laboratory animals.

All animals separated to groups (10 mice in every group) according to materials and times injected (intraperitoneally

0.2ml) and scarified after 24 hours of last treatment and mice bone marrow samples are taken for cytogenetic analysis as fallowing:

Control group.

Negative control group: animals injected 0.2 ml distilled water (10 mice).

Dactinomycin study Groups.

Positive control group I: animals injected dactinomycin (0.4 mg/kg).

Tramadol study groups.

Positive control group II**:** animals injected tramadol (0.025 mg/kg).

The interaction (Treatment) studies of tramadol and dactinomycin.

This study divided to five groups:

1. Treatment group I, animals injected tramadol (0.025mg/kg) and dactinomycin (0.4mg/kg) at the same time and sacrificed after 24 hrs.
2. Treatment group II, animals injected Dactinomycin (0.4 mg/kg) and after 24 hrs injected tramadol (0.025 mg/kg) and sacrificed after 24 hrs.
3. Treatment group III, animals injected tramadol (0.025 mg/kg) and after 24hrs injected dactinomycin (0.4 mg/kg) and sacrificed after 24 hrs.
4. Treatment group IV, animals injected dactinomycin (0.4 mg/kg) and after 24hrs injected tramadol (0.025 mg/kg) three doses every 8 hrs for 24 hrs and sacrificed after 24 hrs.
5. Treatment group V, animal's injected tramadol (0.025 mg/kg) three doses every 8 hrs for 24 hrs and after this time injected dactinomycin (0.4 mg/kg) and sacrificed after 24 hrs.
6. Cytogenetic experiments

Chromosome preparation from somatic cells of the mouse bone marrow

The experiment has been done dependent on Evan's *et al* (1964) (18) as follows. Firstly, the mice intraperitoneally (I.P.) were injected by (0.25 ml) of Colchicine with concentrating of (0.5mg/ml) is take 2 hours before sacrificing Mice are killed via cervical dislocation. The mice are fixed on its ventral side on the anatomy plate and the abdominal side of the animals, and its thigh area is swabbed with 70 % ethanol. The femur bone is taken and scour from the extra tissues and muscles, then gapped from the middle with a forceps in a vertical position above the edge of a test tube by a sterile syringe, 5 ml of Phosphate buffer saline (PBS) is injected consequently as to wash and drop the bone marrow in the test tubes. Then put these test tubes in centrifuge. For (10 min.) and at speed of (2000 rpm) **.**The supernatant is discarded and 5ml of potassium chloride (KCl) is additional as a hypotonic solution at 0.075 M, subsequently the tubes are put in water bath at 37º C for 20 min with shaking from time to time. Then we put these test tubes in centrifuge For (10 min.) and at speed of (2000 rpm)**.**The supernatant is discarded and the fixative solution is additional (as drops) on the inner wall of the test tube with continuous shaking, the volume is fixed to 5 ml and the substances are shaken well. To fix the cells keep the tubes in 4º C for (20 min) **.**put these test tubes in centrifuge for (10 min.) and at speed of (2000 rpm)this method is continual for three times and then the cells are suspended in 2 ml of the fixative solution. Few drops from the tube are dropped vertically on the slide from a height of 10 cm at a rate of 4-5 drops by a micropipette, to provide the chance for the chromosomes to spread well. Then the slides are kept to dehydrated overnight. Giemsa stain used to stain the slides then left for 15 min and then washed with Distall water (D.W.) and left over night to dry, and then covered via Tri sodium citrate (DPX).Many slides for every animal are arranged for cytogenetic assays.

The prepared slides are observed under the oil immersion lens for 100 divided cells per every animal, and the cells must be at the metaphase stage of the mitotic division where the chromosomal aberrations are pure and the percentage of these aberrations can be estimated (19).

3. Cytogenetic Analysis

1-Mitotic index (MI) assay

From similar slides that prepared overhead are examined by light microscope by magnification (40X), and (1000) of divided and non-divided cells are counted and the percentage rate has been calculated for only the divided ones (metaphase cells) according to the subsequent equation:

$$\text{Metaphase Index (\%)} = \left(\frac{\text{Number of Metaphase Cells}}{\text{Total Count}}\right) \text{x } 100$$

(King *et al.*, 1982; Shubber and Al-Allak, 1986). (20)(21).

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 must 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

The experiment was been done dependent on method of (Schmid, 1975)(22) .The animal is sacrificed by cervical dislocation, and then separated to obtain the femur bone. After cutting both ends of the bone, it is gapped with a forceps washed the femur bone with 1ml of human plasma (heat inactivated) to accumulate the cellular content inside the test tube. Discarded the supernatant after put the test tube is centrifuged (1000 rpm) for five minutes. The cellular precipitate is gently mixed, and a thin smear is form on a clean slide. The slide is left for (24 hr.) at a standard room temperature to dry. The slides are fixed by absolute methanol for (5 min.), then stained by Giemsa

stain for (15 min), then washed by D.W and left to dry. Five slides for every animal are prepared for micronucleus test.

The slides are examined under the oil immersion lens, and at least 1000 polychromatic erythrocytes (PCE) are examined for theappearance of micronucleus. The micronucleus index is got using the following equation:

Micronucleus Index =

$$\left(\frac{\text{Number of Micronuclei}}{\text{Total Count of PCE}}\right) \text{x } 100$$

(Schmid, 1976) (23).

4. Statistical Analysis

The data obtained were statistically analyzed using a 2 * 2 contingency table (X2) (24).The difference is considered significant when the probability value is less than $p<0.05$

5. Results and Discussion

The results of metaphase test are presented in table (1). There are significant differences when we compare between negative control and positive control (I and II) and this differences were due to the toxic effect of dactinomycin alone and tramadol alone too by reducing the mitotic index (MI). And also there is a significant different when we compare the treatment groups (I, II, III, IV and V) with negative control. All these results were statistically significant ($p<0.05$).

Table 1: Percentages of mitotic index in bone marrow of mice for negative control, positive control groups and treatment groups

Groups	No. of animal	No. of cell examined	Mitotic index	%
Negative control	5	25000	1271	**5.084**
Positive control I (Dactinomycin for 24 hr.)	5	25000	867	**a*3.468**
Positive control II (Tramadol for 24hr.)	5	25000	1113	**a*4.452**
Treatment group I (Dactinomycin and Tramadol for 24 hr.)	5	25000	711	**b*2.844**
Treatment group II (Dactinomycin 48 hr. – Tramadol 24 hr.)	5	25000	718	**b*2.872**
Treatment group III (Tramadol 48 hr. –Dactinomycin 24 hr.)	5	25000	783	**b*3.132**
Treatment group IV (Tramadol 3 dose every 8 hr for 48 hr. – Dactinomycin 24 hr.)	5	25000	883	**b*3.532**
Treatment groupV (Dactinomycin 48 hr._ Tramadol 3 dose every 8 hr for 48 hr.	5	25000	782	**b*3.128**

[a] Positive control groups vs. Negative control, [b]Treatment groups vs. Negativecontrol,*Significant at ($p<0.05$)

Table 2: Percentages of different types of chromosomal aberrations (CA) in bone marrow of mice for negative control, positive control groups and treatment groups

Groups	No. of animal	No. of cell examined	Chromosome Aberration													
			Acentric Fragment		Gap		Break		Fragment		Ring		Poly ploide		Total	
			NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%	NO.	%
Negative control	5	500	102	20.4	52	10.4	18	3.6	2	0.4	0	0	1	0.2	175	**35**
Positive control I (Dactinomycin for 24 hr.)	5	500	375	75	62	12.4	33	6.6	26	5.2	11	2.2	7	1.4	514	**a*102.8**
Positive control II (Tramadol for 24hr.)	5	500	171	34.2	102	20.4	21	4.2	7	1.4	1	0.2	1	0.2	303	**a*60.6**
Treatment group I (Dactinomycin and Tramadol for 24 hr.)	5	500	379	75.8	135	27	37	7.4	15	3	6	1.2	7	1.4	579	**b*115.8**
Treatment group II (Dactinomycin 48 hr. – Tramadol 24 hr.)	5	500	365	73	107	21.4	42	8.4	10	2	5	1	5	1	534	**b*106.8**
Treatment group III (Tramadol 48 hr. – Dactinomycin 24 hr.)	5	500	311	62.2	23	4.6	16	3.2	8	1.6	0	0	3	0.6	361	**b*72.2**

Treatment group IV (Tramadol 3 dose every 8 hr for 48 hr. – Dactinomycin 24 hr.)	5	500	244	48.8	17	3.4	24	4.8	39	7.8	7	1.4	1	0.2	322	**b*64.4**
Treatment groupV (Dactinomycin 48 hr._ Tramadol 3 dose every 8 hr for 48 hr.	5	500	402	80.4	58	11.6	47	9.4	32	6.4	4	0.8	4	0.8	547	` **b*109.4**

[a] Positive control groups vs. Negative control, [b] Treatment groups vs. Negative control, *Significant at ($p<0.05$)

The results of chromosomal aberrations present in table 2. Animals treated with dactinomycin positive control I with dose (0.4 mg/kg) showed a high frequency of total chromosomal aberrations (102.8%) respectively in mice bone marrow cells, these findings were significant ($p<0.05$) when compared with negative controls (35%) As well as, the animals that treated with tramadol positive control II with dose (0.025 mg/kg) demonstrated a significant differences(60.6%) respectively in mice bone marrow cells, when compare with negative control.

When the same dose of dactinomycin was given together with tramadol, chromosomal aberrations were increased, chromosomal aberrations were increased, these results showed significant value ($p<0.05$), there was an induction for all treatment groups when compared with negative control. Also when the same dose of dactinomycin was given for 48hr and tramadol 3 dose every 8 hr. for 24 hr.,these results showed significant value ($p<0.05$) when compared with negative control.

Table 3: Percentages of micronuclei (MN) in bone marrow of mice for negative control, positive control groups and treatment groups

Groups	No. of animal	No. of cell examined	micronucleus	%
Negative control	5	25000	675	**2.7**
Positive control I (Dactinomycin for 24 hr.)	5	25000	1048	**[a]*4.192**
Positive control II (Tramadol for 24hr.)	5	25000	1019	**[a]*4.076**
Treatment group I (Dactinomycin and Tramadol for 24 hr.)	5	25000	1164	**[b]*4.656**
Treatment group II (Dactinomycin 48 hr. – Tramadol 24 hr.)	5	25000	1143	**[b]*4.572**
Treatment group III (Tramadol 48 hr. –Dactinomycin 24 hr.)	5	25000	1003	**[b]*4.012**
Treatment group IV (Tramadol 3 dose every 8 hr for 48 hr. – Dactinomycin 24 hr.)	5	25000	1136	**[b]*4.544**
Treated group III Dactinomycin 48h before Tramadol three doses every 8h for 24h	5	25000	1390	**[b]*5.56**

[a] Positive control groups vs. Negative control, [b] Treatment groups vs. Negative control, *Significant at ($p<0.05$)

Table 3 shows the results of micronuclei (MN). The frequency of MN in negative control showed a significant differences when compared with positive groups and also treatment groups at ($p<0.05$).

The positive control I showed a significant reduction in MI and a high increase in CAs and MN. The reason for this result was due to the toxic effect of dactinomycin that cause DNA damage .dactinomycin was tested for potential genotoxicity in male mice. The mice were formerly assessed at different intervals, against appropriate control, using the occurrence of micronucleated erythrocytes and serum protein electrophoresis assay as the end points. A rise in the micronucleus incidence did not occur until 6 h post-treatment, probably as a result of cell cycle elongation or mitotic delay. Though, the frequency of micronucleated PCEs increased between 2 and 36 hr. then reduced rapidly and reached a minimum level at 48 h. Most of the cells had one micronucleus, but a few cells with large-size micronuclei were also encountered(25).Alsowhen Indian muntjac and Chinese hamster cells in culture were treated with dactinomycin, the sister chromatids, especially the distal segments, seemed to have difficulty separating in anaphase. The separated proximal parts progressively became stretched. The nucleolus arranger sections appeared to be most susceptible to stretching, and breaks in these sections were often observed. Electron microscopic observations indicated that the sticky chromatids (and less frequently sticky chromosomes) contain connecting submicroscopic chromosome strands. When the treated cells were allowable to grow in a drug-free medium for several days, a high incidence of endoreduplicated mitotic figures was found. Chromosome and chromatid breaks and further aberrations were common, mainly localized at G band negative areas mainly nucleolus organizer regions (26).

Figure below showed different chromosome aberrations from mice bone marrow for positive control I.

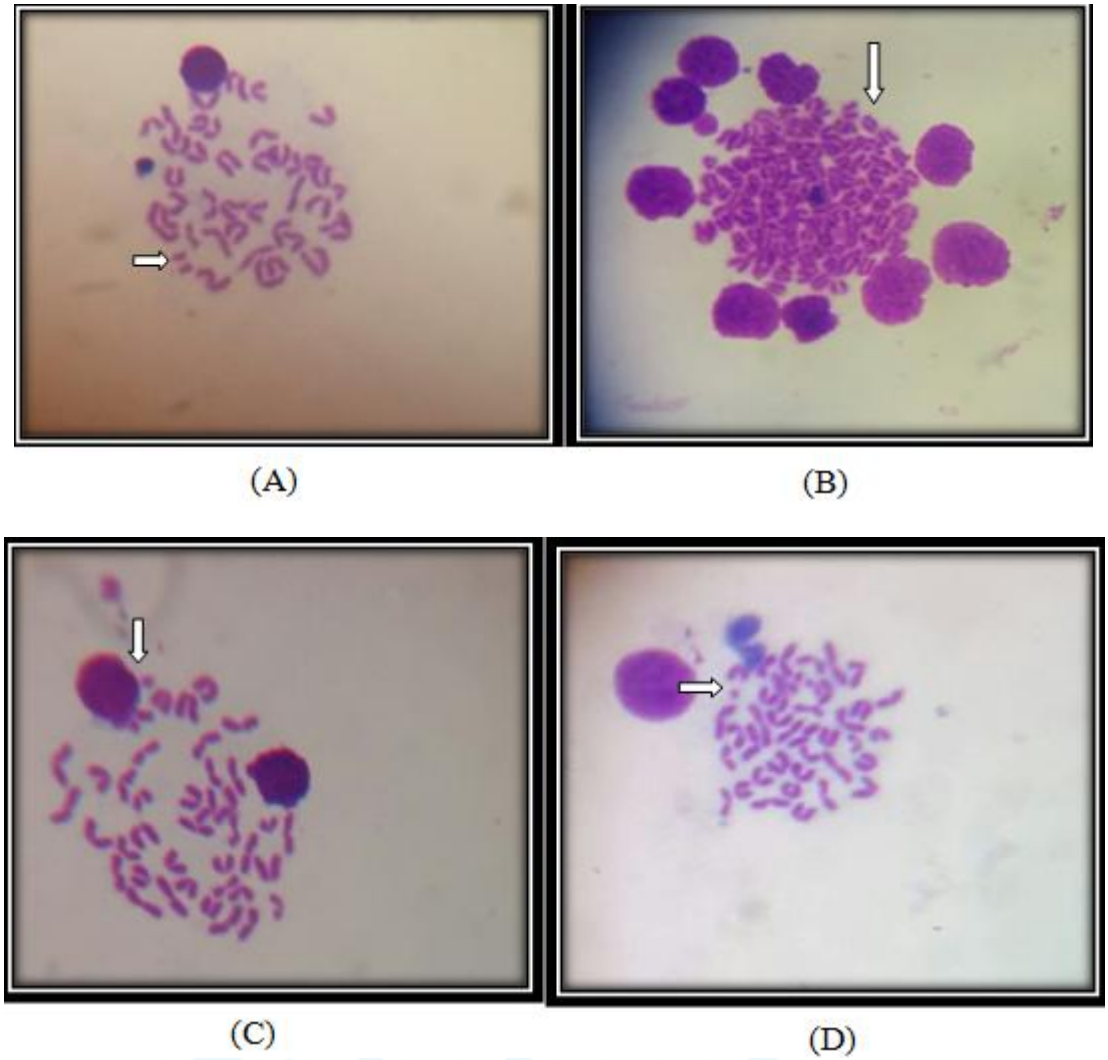

Figure 1: Chromosomal aberrations in mice bone marrow at (100x) injected only with Dactinomycin. A: acentric fragment B: polyploidy C: fragment D: break

In the positive groups II, There is an increase, but few noticeable in the value of CAs and MN and a significant reduction in MI when compared with negative control. The reduction of MI for mice that treated with single dose (0.025 mg/kg)Many of researches and studies that explain these findings, including the study of (Marwa A Ahmed and Adel Kurkar, 2014) (13).That showed that tramadol treatment affects the testicular function of adult male rats, and these effects can be through the overproduction of nitric oxide and oxidative stress induced by this drug. I can conclude that Tramadol does not affect directly the rate of cell division but tramadol ability to produce a free radicals, which in turn will affect the cell, including the nitric oxide (NO) .This effect explained to nitric oxide (NO) by study (Samar Burney *et al*., 1999)(27) whose shown that Nitric oxide able to react with DNA via several pathways. Once produced, following conversion of nitric oxide to nitrous anhydride and/or peroxynitrite can cause the nitrosative deamination of DNA bases such as guanine and cytosine. Complex oxidation chemistry can also happen causing DNA base and sugar oxidative modifications.

Tramadol similar other opioids makes a decrease in plasma antioxidant levels, which may replicate a failure of the antioxidant defense mechanism against oxidative damage (28) This means increasing oxidative stress, which in turn will affect the Mitotic index , Micronucleus and form of Chromosome aberration. Oxidative stress may have an essential role in DNA fractions made by irradiation or any genotoxic materials (28).

The significant differences between treatment groups with negative control and positive control groups corresponding to them, may to the nested effect of dactinomycin and tramadol also effect due to increase dose of tramadol 3 doses every 8 hr. in two treatment before and after dactinomycin, which tramadol induce micronuclei and dactinomycin cause an increase in MN because it's effect on the of DNA and cause chromosome damage (CAs), thus induce MN formation.

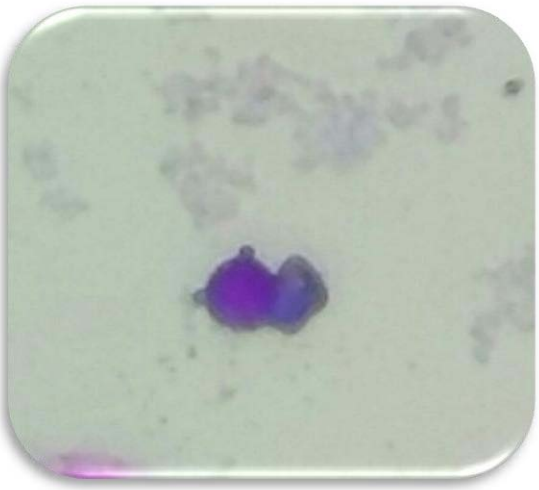

Figure 2: Dactinomycin formation MN at (100x)

6. Conclusions

Data conclude that the dactinomycinmay as genotoxic contrast agent, which shows significant genotoxic effects on mice bone marrow stem cells. Moreover there is a significant reduction in MI and significant increasing in CA and MN caused by tramadol , which gives evidence for the genotoxic effect of tramadol in mouse bone marrow stem cells. As well as, both dactinomycin and tramadol as a combined treatment and tramadol increasing dose treatment demonstrated a very aggressive effects on mice stem cells, which may belongs to the interact action of both chemicals.

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