Genotoxicity of 1, 4 -Dioxane in Mouse Bone Marrow: I. Effect on the Micronucleus Assay

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Abstract

The genotoxicity of 1,4 dioxane (DX), was studied in Swiss albino male mice using the bone marrow micronucleus assay. Three doses of DX (0.57, 2.85 and 5.7 mg/kg body weight) were used in three different groups, (10 animals/group). Each animal received two intraperitoneal injections (i.p) of its respective dose level on two consecutive days and sacrificed 6 hours after the second injection. A control group of mice received water and the animals were subjected to the same procedure as the experimental groups. Bone marrow samples were obtained from the femurs of mice and processed for examination of the formation of micronuclei in polychromatic erythrocytes (PCEs). The results showed that treatment with large doses of DX caused a significant increase in the frequency of micronuclei (MN) in PCEs. The higher doses of 2.85 and 5.7 mg./kg were found to induce micronuclei at incidences of 12.35 and 13.9, respectively compared with 4.9 micronucleated polychromatic erythrocytes (MNPCEs) /1000 PCEs for the control. There is no apparent dose response relationship. Moreover, with these higher doses there was also a notable increase in the ratio of PCEs/normochromatic erythrocytes (NCEs) in the DX treated groups (1.26-1.39 respectively) compared with 0.89 for the control group which suggests a delayed maturation of the erythrocytes. The results demonstrate a possible genotoxic effect of Dioxane using the micronucleus assay.

Keywords: Dioxane, bone marrow cells, micronuclei & mice.

Introduction :

1,4-Dioxane (DX) is a chemical compound primarily used as an industrial solvent or solvent stabilizer that prevents the breakdown of chlorinated solvents during manufacturing processes. Industrial solvents are used in degreasing, electronics, metal finishing, fabric cleaning, pharmaceuticals, herbicides and pesticides, as antifreeze, for paper manufacturing and have many other applications (Rogozen *et al.*,1987; Sack and Steele,1989 and CTCP,1990). Dioxane is also used in paint and varnish strippers, as a wetting agent and dispersing agent in textile prcessing, dye baths,stain and printing compositions, and in the preparation

of histological slides. Additionally, Dx is used in cosmetics, deodorants, fumigants, automotive coolant liquid, and scintillation counters (Gelman 1988; Sitting 1991;Sax and Lewis 1993 and USITC 1994). Therfore ,exposure to DX may occur during its manufacture and its wide use as a solvent in a wide range of organic products.

Pervious research reports on DX (IARC,1976 , NCI, 1978 and IARC,1999) indicated that after oral administration, it increases the incidence of hepatocellular adenomas and carcinomas in mice,tumours of the nasal cavity, liver, subcutaneous tissues, mammary gland and peritoneal mesotheliomas in rats and tumours of the liver and gall bladder in guinea-pigs.

In mortality study of 165 workers who had been exposed to low concentrations of DX since 1954, seven deaths had occurred by 1975, two of which were from cancer (Buffler, *et al.*, 1978)

In a mouse-lung adenoma assay, DX produced a statistically significant increase in the incidence of tumors in males given an intermediate intraperitoneal dose; no such increase was noted in males given a lower or higher intraperitoneal dose or in females given three intraperitoneal doses or in either males or females given DX orally (Stoner *et al.*, 1983).

Kitchin and Brown (1990) reviewed the available mutagenicity, genotoxicity and tumor promoting data for this chemical and calculated that it was best described as a weak genotoxin which also possesses strong activity as a promoter of (liver) carcinogen.

DX induced DNA strand breaks in rat hepatocytes in vitro and it was reported to induce chromosomal aberrations in plants (IARC, 1987_a & IARC, 1987_b). On the other hand, DX was found to be negative in Salmonella assay genotoxic test and the CHO chromosome aberrations assay (Galloway *et al.*, 1987). The mouse liver micronucleus assay suggested that DX might be genotoxic and explained that the positive results were due to a non-genotoxic mechanism i.e., errors in genetic repair for hepatocyte proliferation (Morita and Hayashi, 1998).

In this study we are trying to throw more light into the disputed mutagenic role of DX using three different doses, assessed by the micronuclei formation in bone marrow cells of treated animals, generally recognized as a valid measure of genotoxicity, Heddle, *et al.* (1983) and Mavourin *et al.*, (1990).

Materials and Methods :

1,4-Dioxane:

1,4-Dioxane is $C_4H_8O_2$ and was purchased from (Sigma, USA). Three different concentrations were prepared in distilled water (0.57, 2.85 & 5.7) mg/kg body weight (b.w.).

Animals:

Adult male Swiss albino mice, *Mus musculus,* each weighing 20-25 g, were obtained from an inbred strain in the College of Veterinary Medicine, King Faisal University, Al-Hassa, Saudi Arabia. Mice were housed at room temperature (20-22C°) in different stainless steel cages, five animals/ cage. Animals in all groups were given a standard basal diet and water was given ad libitum.

Study Design:

Four groups of mice, each of 10 animals were assigned to a different treatment. Three groups were treated with DX and the fourth group used as a control. The DX treated groups were given 0.57, 2.85 or 5.7 mg of DX/ kg body weight. The different doses were selected on the basis of the maximum tolerated dose (MTD), (NTP, 1985). The MTD was estimated as 5.7 mg/kg body weight, based on survival rate of the animals 24 h after treating them with an intraperitoneal (i.p.) injection of DX over a wide dose range (10 animals / dose).

Micronuclear Assay:

Each mouse received 2 i.p. injections of the respective dose level on two consecutive days. The control group was given similar i.p. injections of equivalent amount of water. Six hours after the second injection, the animals were sacrificed and the femurs were dissected out. Bone marrow was obtained from the femurs and smears were prepared as described by Ledebur *et al.*, (1973). The slides were coded, stained with Giemsa and scanned for micronuclei according to the method described by Bali *et al.*, (1990). The slides were scored blindly by one investigator. For each animal, at least 1000 polychromatic erythrocytes (PCEs) were examined and then the number of micronucleated polychromatic erythrocytes (MNPCEs) and normochromatic erythrocytes (NCEs) in the same fields were counted. The ratio of PCE/ NCE was calculated. The results of the micronucleus test and the PCE/NCE ratios were analyzed statistically using ANOVA, Baily (1975).

Results:

All the three doses of DX increased the proportion of MNPCEs in the bone marrow (table 1 & fig. 1). Lower dose of 0.57 mg/kg b.w. showed 6.8±0.13 MNPCEs per 1000 PCEs as compared to 4.9±0.10 per 1000 in the control group, but the difference was statistically not significant (P>0.05). Higher doses of 2.85 and 5.7 mg/kg body weight significantly increased MNPCEs to 12.35±0.13 and 13.87±0.14 per 1000, respectively, as compared to controls.

Table (1)

The incidences of induced micronuclei in PCEs of control and 1,4-dioxane (DX) treated male mice*.

Treatment (DX)	Number of cells analyzed	Number of PCEs analyzed	Number of MNPCEs	Mean MNPCEs/ 1000 PCEs ± SD	P value (as compared to control)
Control	11106	5522	27	4.90±0.10	
Group 1 (DX , 0.57mg)	13848	8043	55	6.80±0.13	P>0.05
Group 2 (DX ,2.85mg)	14047	7848	97	12.35±0.13	P< 0.001
Group 3 (DX ,5.7mg)	12823	7137	99	13.87±0.14	P< 0.001

*10 animals/group P>0.05 = non-significant

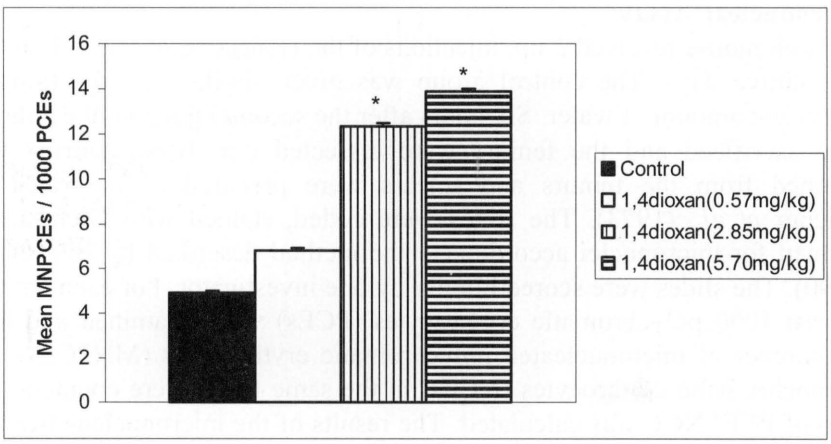


Fig. (1) Mean MNPCEs/1000 PCEs.

* Significant increase (P<0.01)

Moreover, there was a small but consistent increase in PCE/NCE ratios with all three doses of DX, i.e., 1.39±0.22, 1.27±0.20 and 1.26±0.28 for DX 0.57,2.85 and 5.7 mg/kg body weight, respectively (table 2 & fig.2). However, these changes were statistically not significantly (P>0.05) different from the PCEs/NCEs ratio of the control group (0.89).

Table (2)

The frequencies of PCEs, NCEs and the PCEs/ NCEs ratios in bone marrow cells of male mice* treated with 1, 4-dioxane (DX)

Treatment (DX)	Number of cells analyzed	Number of PCEs analyzed	Number of NCEs	Mean PCE/ NCE ratio ±SD	P value (as compared to control)
Control	11106	5522	5584	0.89±0.12	
Group 1 (DX, 0. 57 mg)	13848	8043	5805	1.39±0.22	P>0.05
Group 2 (DX, 2. 85mg)	14047	7848	6199	1.27±0.20	P>0.05
Group 3 (DX, 5.7mg)	12823	7137	5686	1.26±0.28	P>0.05

*10 animals/ group P>0.05 = non-significant

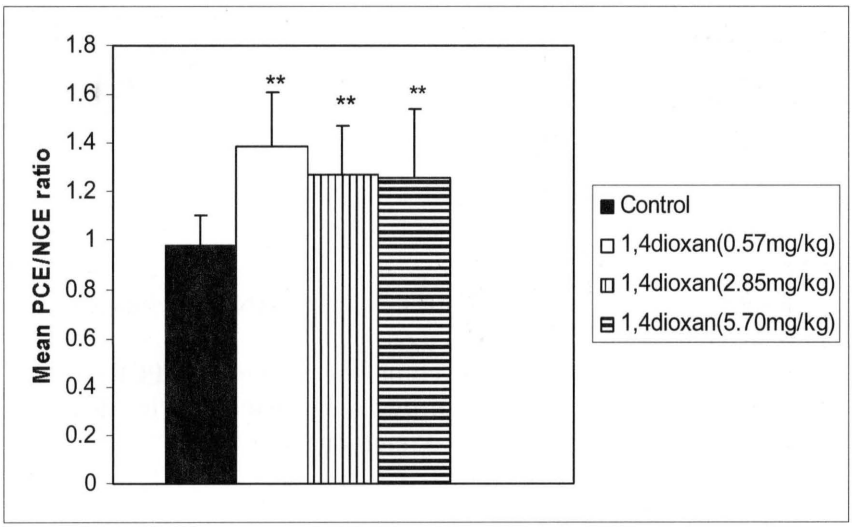
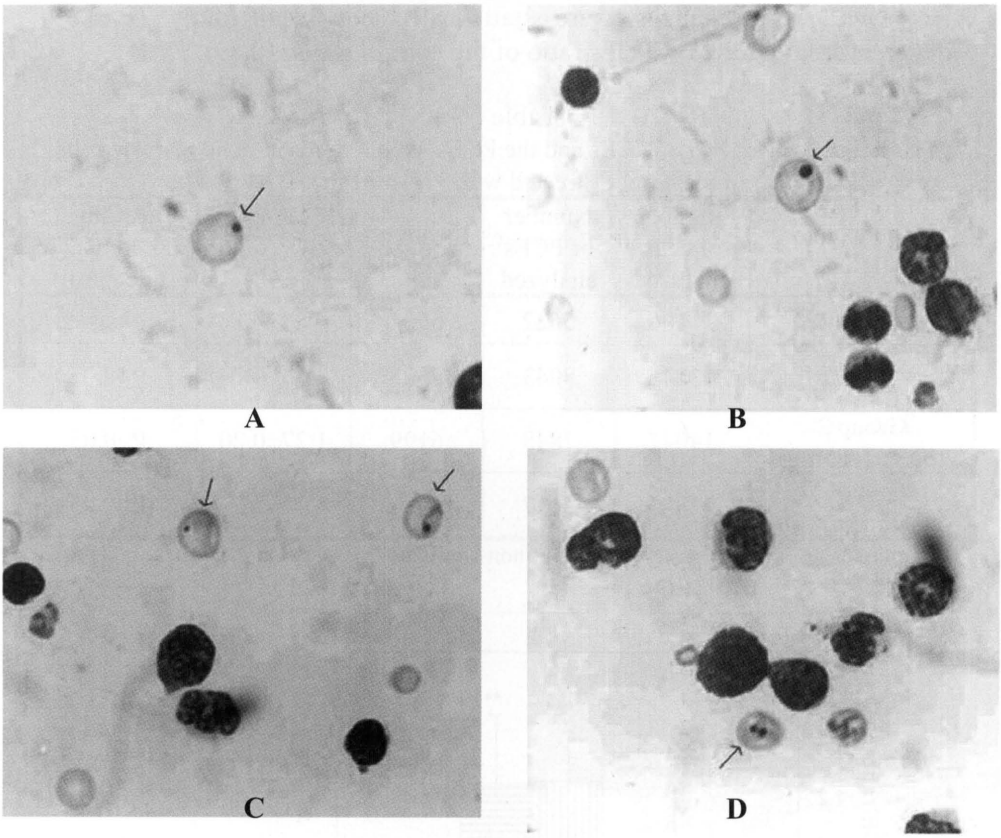


Fig. (2) Mean PCEs/NCEs ratio

** Non-significant increase (P>0.05)

Majority of MNPCEs had only one micronucleus (fig. 3- A, B &C) and only 1-2 micronucleated PCEs were noticed in any of the groups treated with DX that had more than one micronucleus (Fig. 3- D).



(Fig.3)

A - Bone marrow normochromatic erythrocyte (NCE) with single micronucleus from control male mouse.

B, C, & D – Micronuclei in polychromatic erythrocyte (PCE) from treated mice with Dioxane, C- (arrows indicate increase incidence of micronuclei) & D- PCE, with two micronuclei .

Discussion

The negative mutagenicity results of DX in the Salmonella assay (Galloway *et al*, 1987; Ashby & Tennant, 1988) and also in mammalian cells in vitro (Scott et al, 1981) lead to the conclusion that DX is not genotoxic (MAK-Dokumentation, 1996).

However, there has been other studies which suggest a potential effect of DX in the induction of clastogenic effect in hepatocytes micronucleus assay (Morita and Hayashi, 1998) and *in vivo* bone marrow assay in mice (Mirkova, 1994). The findings of Kitchin and Brown (1990) that DX caused DNA strand breaks in cultured rat hepatocytes, also suggests the possible mutagenic effect of DX. The data now available for DX in the micronucleus assay are inconsistent and scarce since Tinwell and Ashby (1994) and McFee *et al.*, (1994) were unable to obtain reproducible evidence for this agent in the micronucleus assay.

The present study shows significant increase in the incidence of micronuclei in bone marrow cells of mice treated *in vivo* with different doses of DX. The results suggest that DX has a clastogenic effect and can result in chromosomal damage. This in concordance with report of IRAC,1999, which has classified 1,4- dioxane in group 2B (possibly carcinogenic to humans) and states that most of the tests for genotoxic activity have produced negative results, but positive results were obtained in cell transformation assay and conflicting results were obtained in mouse bone-marrow cell tests for micronucleus induction. Also EPA, (1995) classifies 1,4- dioxane as B2, parable human carcinogen, based on the induction of nasal cavity and liver carcinomas in multiple strains of rats, liver carcinomas in mice, and gall bladder carcinomas in guinea pigs.

The doses and route of administration used in this study were different from those used by others in similar work. The very high doses (3000-5000 mg/kg) used by Mirkova (1994) and Morita and Hayashi (1998) were given orally or added to culture medium *in vitro.* The results presented in this study represent *in vivo* response to DX at relatively much lower doses (0.57-5.70 mg/kg) than the cytotoxic doses used in other studies and were given intraperitoneally.

The induction of micronucleated PCEs in bone marrow cells of mice treated with DX did not follow a dose response pattern as suggested by Mirkova (1994). Both sets of data obtained in this report and those of

Mirkova cannot be compared because of the magnitude of differences in the doses applied. However, DX has been shown in related studies that its carcinogenic property requires the accumulation of this agent in blood and tissues to induce the enzyme aniline hydroxylase after metabolic overloading suggesting a role conceived as a threshold related phenomenon (Young et al., 1978). The two large doses of DX (2.85 and 5.70 mg/kg) have resulted in a similar threshold effect which postulate a prerequisite accumulation of DX before exerting its clastogenic effect. Genotoxicity of DX seems to be achieved only after saturation and accumulation of the parent molecule (Hecht and Young, 1981).

The interaction of DX with the integrity of the genetic material assessed in this study by the formation of micronuclei is supported by the findings of Heil and Refferscheid (1992) where DX caused inhibition of replicative DNA synthesis in HeLa cells and the induction of DNA fragmentation in cultured rat hepatocytes (Kitchin and Brown, 1990). It is, therefore, not unlikely that under *in vivo* conditions, DX may exert clastogenicity expressed as chromosomal structural aberrations (micronucleus).

The inverse ratio of PCE/NCE after treatment of mice with DX, though not statistically significant, is in concordance with the established role of DX to interfere with mechanisms of cell proliferation and biotransformation (Goldsworthy et al., 1991; Miyagawa *et al.*, 1997). The observed decrease of PCEs in relation to NCEs suggests that DX may result in delay or inhibition of the maturation process of polychromatic erythrocytes to normocytes. Suzuki *et al.*, (1993) suggested that erythropoietin induction by DX may possibly cause an increase in PCEs, similar to the effect of cobalt and other mutagens observed in the micronucleus assay.

The issue of mutagenicity of DX stems its significance in relation to its carcinogenic capability. DX has been classified as class 4 carcinogen due to its cytotoxic mechanisn (Neumann *et al.*, 1998). The acknowledged lack of mutagenicity of DX in the Salmonella assay leads to its classification as a non-genotoxic carcinogen. Genotoxic carcinogens represent a group of agents with a known mechanism of action that involves induction of DNA damage, activation of oncogenes or inactivation of tumors suppressor genes. Genotoxic carcinogens are considered more serious than non-genotoxic carcinogens since the latter present less of a risk to humans (Ashby and Morrod, 1991).

It is generally accepted that micronuclei are induced via genotoxic mechanisms and therefore, DX might be considered as a genotoxic agent capable of inducig a genotoxic mechanism *in vivo*. Also the proportion of MNPCEs normally present in bone marrow may increase during increased erythropioesis due to blood loss or red cell destruction. Mice treated group with 1,4-dioxane showed very highly significant reduction of erythrocytes counts, accompanied by significant decrease in haemoglobin as well as haematocrite %, compared with control values (Moussa, 2004). This decrease in the total RBCs may be due to the destructive effect of the toxic 1,4-dioxane as supported by Linman (1975) or may be due to the circulating failure as a result of inability to maintain circulatory blood volume due to the decrease in the developing stages of RBCs in haemopoietic tissues (El-Feki , 1987). It is, however, well anticipated that the relationship between carcinogens and the induction of micronuclei is not fully understood and may not be a simple one. The results obtained in this study reopen the issue of genotoxicity of DX recommending the use of a battery of test systems, both *in vivo* and *in vitro* to help classification of dioxane either as a nongenotoxic carcinogen or a genotoxic carcinogen. Dioxane has been proven to induce chromosomal damages in the form of micronuclei in bone marrow cells of treated mice. Therefore dioxane may be considered as a potential clastogenic substance besides being established as carcinogenic.

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السمية الوراثية لمركب ٤,١- ديوكسان في نخاع العظم للفئران البيضاء: ١- التأثير علي اختبار النويات الدقيقة

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الملخص :

تم دراسة تأثير السمية الوراثية لمركب ٤,١- ديوكسان وهو مذيب واسع الاستخدام في عدد كبير من العمليات الصناعية وذلك على ذكور الفئران البيضاء . وذلك بفحص نخاع العظم لتقدير معدل تكوين الأنوية الدقيقة (micronuclei) باستخدام ثلاث جرعات من الديوكسان هي (٠,٥٧- ٢,٨٥ ، ٥,٧٠ ملجم/كجم) لوزن الجسم في ثلاثة مجموعات تتكون كل منها من عشرة فئران وذلك عند حقن الفئران مرتين من الجرعة المحددة في التجويف البطني البريتوني ليومين متصلين ثم الذبح بعد اليوم الثاني. كما تم عمل مجموعة ضابطة من الفئران البيضاء تم حقنها بالماء وتم أتباع نفس الخطوات معها. وقد تم الحصول علي نخاع العظم من عظمة الفخذ وتم إجراء اختبار تكون النويات الدقيقة (MN) لكريات الدم الحمراء عديدة الصبغة (PCEs) وكريات الدم الحمراء وحيدة الصبغة (NCEs).

وقد أوضحت النتائج أن المعاملة بالجرعات العالية من الديوكسان (٢,٨٥ ، ٥,٧٠ مج/كجم) تسببت في أحداث زيادة معنوية في معدل تكوين النويات الدقيقة في كريات الدم الحمراء عديدة الصبغة (MNPCEs) بنسبة (١٣,٩- ١٢,٣٥٪) مقارنة بالمجموعة الضابطة وهي (٤,٩٪) وقد وجد أنه لا توجد علاقة مضطردة بين الجرعة ونسبة تكون النويات الدقيقة. كما كان هناك أيضا أزدياد ملحوظ في النسبة بين نوعي الكريات الحمراء عديدة الصبغيات ووحيدة الصبغة (PCE/ NCE) وذلك في المجموعة المعالجة بالديوكسان (١,٣٩- ١,٢٦٪) مقارنة بالمجموعة الضابطة (٠,٨٩٪) والذي يرجح معه احتمال حدوث تأخر في عملية نمو كريات الدم الحمراء. هذا وتشير نتائج البحث إلى إمكانية حدوث سمية وراثية لمركب ٤,١- ديوكسان باستخدام اختبار النويات الدقيقة.