# Journal of Pharmacology and Toxicology

ISSN 1816-496X

Academic Journals Inc.

www.academicjournals.com

Journal of Pharmacology and Toxicology 7 (7): 330-337, 2012
ISSN 1816-496X / DOI: 10.3923/jpt.2012.330.337


# Cytogenetic Effects of Technical and Formulated Tribenuron-methyl on Rat Bone-marrow Cells

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## ABSTRACT

World Health Organization emphasized the necessity of evaluating toxic hazard of the formulated pesticides. So, in current study, the genotoxic and cytotoxic potential of technical and formulated tribenuron-methyl herbicide were investigated in male rat bone-marrow cells, using the Structural Chromosomal Aberration (SCA) and micronucleus (MN) test systems. Technical and formulated tribenuron-methyl were administrated to rats as single or repeated oral doses of 5 (NOAEL), 25, 50 and 100 mg $kg^{-1}$ b.wt. for 21 days at 48 h intervals. Results showed that repetitive dose of formulated tribenuron-methyl (100 mg a.i. $kg^{-1}$ b.wt.) induced significant decrease in the mitotic activity. After repetitive doses, the frequency of total chromosomal aberrations was statistically significant ($p \leq 0.05$-$0.01$) at two higher doses, 50 and 100 mg a.i. $kg^{-1}$ b.wt. of formulated tribenuron-methyl as well as significant increase ($p \leq 0.05$) at high dose (100 mg a.i. $kg^{-1}$ b.wt.) of technical tribenuron-methyl. The frequency of MN was statistically significant at two higher doses, 50 ($p \leq 0.05$) and 100 ($p \leq 0.01$) mg a.i. $kg^{-1}$ b.wt. of formulated tribenuron-methyl. Our results reveal that tribenuron-methyl has a clastogenic/genotoxic potential as measured by the bone marrow CA and MN tests in rats. The partial differences of the genotoxic effects obtained with pure and commercial tribenuron-methyl indicate that commercial formulations may contain additional hazardous compounds. Therefore, it is important in assessing the real human hazard from pesticides to investigate not only the active principle but also the commercial formulations used in agriculture.

**Key words:** Tribenuron-methyl, bone-marrow, mitotic activity, chromosomal aberration, micronuclei

## INTRODUCTION

Pesticides have made valuable contribution to human health by increasing food and fiber production and by reducing occurrence of vector-borne diseases (Blindauer *et al*., 1999). Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, including non-target species, air, water, bottom sediments and food. Unfortunately, while the acute toxicity of most pesticides is well documented (Ecobichon *et al*., 1990), information on chronic human illness resulting from pesticide exposure is not as sound (Wilkinson, 1990). It has been estimated that occupational exposure accounts for 4% of all human cancers (Doll and Peto, 1981).

Genotoxic are considered among the most serious of the possible potential side effects of agriculture pesticides. Data of genotoxicity and carcinogenicity of the tested insecticides are rather controversial, depending on the genetic or the assay used (Moretti *et al*., 1997).

In fact, many modern herbicides kill weeds selectively by impairing metabolic processes that are unique to plant life, through its effect on biochemistry branched chain amino acid synthesis (ALS or AHAS) as an inhibitor. For this reason, their systemic toxicities in mammals are generally low. Although selective toxicity toward target organisms is a desirable quality, it is not absolute and most pesticides are toxic to a greater or lesser extent toward non-target organisms, including humans (Ernest and Patricia, 1997; Mansour *et al*., 2008; Mansour and Mossa, 2009, 2010a, b). Tribenuron-methyl initially registered in EPA, 1989 and is commonly used as herbicide in Egypt, against broad leaf weeds in wheat, within IPM program of wheat in reclaimed area (Soliman *et al*., 2000) and its side effect on wheat plant defense enzyme (Sabra and Houssien, 2004). By the very nature of their use in weed control, they are common contaminations of the environment, food, water and domestic structures.

Pesticides are usually applied in their formulated forms, where the active ingredient is combined with organic solvents, emulsifying and wetting agents, which affect the pesticide penetration and performance. These additives may synergize or antagonize the toxicity of the active ingredient (Abo-Zeid *et al*., 1993; Mansour and Mossa, 2005; Marzouk *et al*., 2005; Sabra *et al*., 2005; Mansour *et al*., 2008; Mossa and Abbassy, 2012). Consequently, World Health Organization emphasized the necessity of evaluating toxic hazard of the formulated pesticides (WHO, 1991). Therefore, this study aimed to evaluate the genotoxic effects of sub-lethal doses of pure and commercial formulation of tribenuron-methyl on male rats.

## MATERIALS AND METHODS

**Chemicals:** Tribenuron-methyl (technical 95% and Granstar® 75% DF) were obtained from Du Pont de Nemours and Company, Inc. Giemsa was obtained from S.d. fine-chem. Ltd, Colchicine from BDH, England; May-Grünwald's from S.D. Fine Chem Limited, Mumbai, India; Fetal Calf Serum (FCS) from Biosource Intenational, USA and potassium chloride from HMRZEL laboratories Ltd., Netherlands. All other chemicals were of reagent grades and obtained from the local scientific distributors in Egypt.

**Animals and experimental protocol:** Male albino rats (*Rattus norvegicus*) weighting 90±10 g were obtained from Animal Health Research Center, Cairo, Egypt. Animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals." Animals were housed in clean plastic cages with free access to food (standard pellet diet) and tap water *ad libitum*, under standardized housing conditions (12 h light dark$^{-1}$ cycle, temperature was 21±2°C and a minimum relative humidity of 45%) in the laboratory animal room. After one week of adaptation to laboratory conditions, the animals randomly divided into eighteen groups each comprising of six animals. The rats of the first 8 groups received single oral dose equals 5 No Observable Adverse Effect Level (NOAEL) (Tomlin, 2004), 25, 50 and 100 mg kg$^{-1}$ b.wt. of technical and formulated tribenuron-methyl using corn oil and water as solvents, respectively. The second 8 groups were given (48 h intervals) orally ten repetitive doses of 5 (NOAEL), 25, 50 and 100 mg kg$^{-1}$ b.wt. of technical and formulated tribenuron-methyl, respectively. The others two groups (control) were received an equivalent volume of distilled water or corn oil (0.5 mL rat$^{-1}$).

**Chromosomal abnormalities in rat bone-marrow cells:** Cytogenetic analysis was performed by the direct method of rinsing marrow of long bones (femur and tibia) according to Adler (1984). Experimental animals were injected (i.p.) with colchicine (4 mg $kg^{-1}$) 1.5 h prior to sacrifice. Both femurs were dissected out and cleaned of any adhering muscle. Bone marrow cells were collected from both the femurs by flushing in KCl (0.075 M, at 37°C) incubated at 37°C for 25 min. Cells was centrifuged at 2000 rpm for 10 min, fixed in aceto-methanol (acetic acid : methanol, 1:3 v/v). Centrifugation and fixation were repeated five times at an interval of 20 min. The cells were resuspended in a small volume of fixative, dropped onto chilled slides and allowed to dry. They were stained the following day with freshly prepared 2% Giemsa stain for 3-5 min and washed in distilled water to remove excess stain. One hundred metaphases per animal were screened to 600 metaphases for each treatment and control to obtain the total number of chromosomal aberrations.

**Mitotic index determination:** Investigation the effects of the compounds used upon cell proliferation were carried out. The slides those were prepared for the chromosome aberration assay were used to calculate the mitotic index. The mitotic indices were obtained by counting the number of mitotic cells in 6000 cells per treatment (1000 cells/animal) and control. The mitotic index was calculated as the ratio of the number of dividing cells to the total number of cells.

**The micronucleus assay:** The method described by Schmid (1975) was used for analysis of micronuclei (MN) in polychromatic erythrocytes (PCEs) of rat bone marrow. After the rats were killed, bone marrow was flushed into a test tube containing calf serum (3 mL) and then centrifuged at 1500 rpm. Smears were made on the slides, air dried and stained with May-Grunwald Giemsa method (D'Souza *et al*., 2002). Micronuclei were identified as dark-blue staining bodies in the cytoplasm of polychromatic erythrocytes. Per animal, 2000 PCEs from all the four randomly selected slides were scored for the presence of MN at a magnification of 1000x with a light microscope. The frequently of micronucleated cells was expressed as a percent of micronucleated cells to total polychromatic erythrocytes.

**Statistical analysis:** The data were analyzed by using SPSS (version 11.0) for Windows and expressed as Means±SE. Paired samples t-test was used to compare between the data of the control and those of treatments.

## RESULTS

**Cell proliferation:** Statistical analysis of results in Table 1 revealed that all of treatments as single and repetitive doses causes insignificantly decreased in the mitotic activity of rat bone marrow cells except repetitive treatment with 100 mg a.i. $kg^{-1}$ b.wt. of formulated tribenuron-methyl caused significant decreased ($p \leq 0.05$). The mitotic index (%) recorded 8.63% in the control group and decreased to 7.76% of formulated tribenuron-methyl (100 mg a.i. $kg^{-1}$ b.wt.) treated-group. Also, our results reveled that formulated form was most effective than technical form of tribenuron-methyl.

**Analysis of chromosomal aberrations:** The metaphase analysis of the bone marrow cells revealed various types of chromosomal aberrations, which consisted of deletion, fragment and

Table 1: Effect of single and repetitive oral doses of technical and formulated tribenuron-methyl on mitotic index in bone marrow cells of male rats

| | Single dose | | | | Repetitive doses | | | |
|---|---|---|---|---|---|---|---|---|
| | Technical | | Formulated | | Technical | | Formulated | |
| Doses (mg kg$^{-1}$ b.wt.) | No. of dividing cells | Mitotic index (%) | No. of dividing cells | Mitotic index (%) | No. of dividing cells | Mitotic index (%) | No. of dividing cells | Mitotic index (%) |
| 0 | 265 | 8.83 | 265 | 8.83 | 265 | 8.83 | 265 | 8.83 |
| 5 | 264 | 8.80 | 263 | 8.76 | 260 | 8.66 | 259 | 8.63 |
| 25 | 263 | 8.76 | 260 | 8.66 | 256 | 8.53 | 253 | 8.43 |
| 50 | 261 | 8.70 | 258 | 8.60 | 250 | 8.33 | 244 | 8.13 |
| 100 | 259 | 8.63 | 254 | 8.46 | 245 | 8.16 | 233 | 7.76* |

Total number of examined cells: 1000/animal, Statistical difference from the control *significant at p≤0.05 and **highly significant at p≤0.01

Table 2: Effect of single and repetitive doses of technical and formulated tribenuron-methyl on chromosomal aberration in male rat bone marrow cells

| | Single dose | | | | | | | | Repetitive doses | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Technical | | | | Formulated | | | | Technical | | | | Formulated | | | |
| Doses (mg kg$^{-1}$ b.wt.) | D | B/F | S | Total aberration[1] | D | B/F | S | Total aberration[1] | D | B/F | S | Total aberration[1] | D | B/F | S | Total aberration[1] |
| 0 | 2 | 2 | 2 | 6 | 2 | 2 | 2 | 6 | 3 | 2 | 2 | 7 | 3 | 2 | 2 | 7 |
| 5 | 1 | 2 | 3 | 6 | 2 | 2 | 3 | 7 | 4 | 3 | 3 | 8 | 4 | 4 | 3 | 9 |
| 25 | 2 | 2 | 3 | 7 | 2 | 3 | 3 | 8 | 4 | 5 | 2 | 11 | 4 | 6 | 2 | 12 |
| 50 | 2 | 4 | 3 | 9 | 3 | 3 | 3 | 9 | 4 | 5 | 3 | 12 | 5 | 6 | 2 | 13* |
| 100 | 3 | 3 | 4 | 10 | 3 | 4 | 4 | 11 | 5 | 5 | 4 | 14* | 5 | 6 | 6 | 17** |

D: Deletion, F: Fragment, B: Breaks, S: Stickiness, [1]A number of 100 metaphases/animal were counted, Statistical difference from the control, *significant at p≤0.05 and **highly significant at p≤0.01

breaks (Table 2) and also, stickiness. In single treatment studies, tribenuron-methyl did not enhance significantly the frequency of total chromosomal aberrations compared to control. After repetitive doses, the frequency of total chromosomal aberrations was statistically significant (p≤0.05-0.01) at two higher doses, 50 and 100 mg a.i. kg$^{-1}$ b.wt. of formulated tribenuron-methyl as well as significant increase (p≤0.05) at high dose (100 mg a.i. kg$^{-1}$ b.wt.) of technical tribenuron-methyl compared to control (Table 2). The mean of the induced chromosomal aberrations reached 13 and 17 at the doses of 50 and 100 mg a.i. kg$^{-1}$ b.wt. of formulated tribenuron-methyl compared to 7 in control group, respectively. It was observed that tribenuron-methyl induced a dose-related increase in total chromosomal aberrations frequency in rat bone marrow cells.

**Micronucleus test:** The percentage frequency of MN in tribenuron-methyl treated and control groups are presented in Table 3. There was no significant (p<0.05) increase in the frequency of micronuclei in single-treated groups in any of the doses of tribenuron-methyl compared to the control. After repetitive doses, the frequency of MN was statistically significant at two higher doses, 50 (p≤0.05) and 100 (p≤0.01) mg a.i. kg$^{-1}$ b.wt. of formulated tribenuron-methyl compared to control. In both the doses, the MN was increased to 0.80 and 0.95% compared to 0.62% of control group.

Table 3: Effect of single and repetitive doses of technical and formulated tribenuron-methyl on micronucleated polychromatic erythrocytes in male rat bone marrow cells

| | Single dose | | | | Repetitive doses | | | |
|---|---|---|---|---|---|---|---|---|
| | Technical | | Formulated | | Technical | | Formulated | |
| Doses (mg kg$^{-1}$ b.wt.) | Micronucleated polychromatic | MN (%) | Micronucleated polychromatic | MN (%) | Micronucleated polychromatic | MN (%) | Micronucleated polychromatic | MN (%) |
| 0 | 20 | 0.50 | 20 | 0.50 | 25 | 0.62 | 25 | 0.62 |
| 5 | 22 | 0.55 | 23 | 0.57 | 26 | 0.65 | 27 | 0.67 |
| 25 | 22 | 0.55 | 24 | 0.60 | 28 | 0.70 | 29 | 0.72 |
| 50 | 23 | 0.57 | 25 | 0.62 | 30 | 0.75 | 32 | 0.80* |
| 100 | 25 | 0.62 | 25 | 0.62 | 31 | 0.77 | 38 | 0.95** |

Total examined cells: 2000/animal, MN: Micronucleus, Statistical difference from the control *significant at $p \leq 0.05$ and **highly significant at $p \leq 0.01$

## DISCUSSION

In fact, occupational exposure occurring at all stages of pesticide formulation, manufacture and application involves exposure to complex mixtures of different types of chemicals, active ingredients and by-products present in technical formulations such as impurities, solvents and other compounds produced during the storage procedure (Bolognesi, 2003). Although inert ingredients have no pesticidal activity, they may be biologically active and sometimes the most toxic component of a pesticide formulation. Moreover, absolute selectivity of pesticides, however, is difficult to achieve and most pesticides are a toxic risk also to humans (Ernest and Patricia, 1997). So, the current study was conducted to evaluate the genotoxic effects of technical and commercial formulation of tribenuron-methyl on male rats.

Genetic damage at the chromosomal level entails an alteration in either chromosome number or chromosome structure and such alterations can be measured as CA or MN frequency. Micronuclei are acentric chromosomal fragments or whole chromosomes left behind during mitotic cellular division and appear in the cytoplasm of interphase cells as small additional nuclei. Moreover, the increase levels of CA have been associated with increased cancer risk (Hagmar *et al*., 1994, 1998). Hagmar *et al*. (1994) reported that an increased level of chromosomal breakage appears to be relevant biomarker of future cancer risk.

In single treatment studies, tribenuron-methyl did not enhance significantly the frequency of total chromosomal aberrations, MN and MI% compared to control. In repetitive doses, high dose (100 mg a.i. kg$^{-1}$ b.wt.) of formulated tribenuron-methyl caused significant decrease in mitotic activity and two high doses (50 and 100 mg a.i. kg$^{-1}$ b.wt.) of formulated caused significant increase in micronucleated polychromatic erythrocytes (MN) in rat bone marrow cells. Also, repetitive doses of formulated tribenuron-methyl (50 and 100 mg a.i. kg$^{-1}$ b.wt.) and technical (100 mg a.i. kg$^{-1}$ b.wt.) caused significant increase in the percentage of Chromosomal Aberration (CA) in rat bone marrow cells.

However, USEPA (1996) published that tribenuron-methyl was classified a Category C (possible human carcinogen) under EPA's cancer assessment guidelines. A gene mutation assay in *Salmonella* Typhimurium and Chinese hamster ovary cells *in vitro*; structural chromosomal damage, including a micronucleus test in mice; a cytogenetics assay in rats and an unscheduled DNA synthesis assay in rat primary hepatocytes *in vitro* were negative for genotoxicity USEPA (1996). Also, no information was available on the mutagenicity profile of the technical and

formulated tribenuron-methyl in male rats. Peluso *et al.* (1998) reported that mice intraperitoneally injected with 400, 500, or 600 mg $kg^{-1}$ of Roundup (commercial formulation containing glyphosate and the surfactant polyoxyethyleneamine (POEA) formed DNA adducts in the liver and kidneys in a dose-dependent manner, but no adducts were observed when only glyphosate was given. These results appear to indicate that POEA and not the active pesticide ingredient, may have genotoxic potential.

Genotoxic damage related to pesticide exposure has been reported (Yoder *et al.*, 1973; De Ferrari *et al.*, 1991; Rupa *et al.*, 1991; Kourakis *et al.*, 1992; Joksic *et al.*, 1997; Brega *et al.*, 1998) in agriculture workers exposed to pesticides (e.g., chlorothalonil, cypermethrin, deltamethrin, endosulfan, fenamiphos, fosetyl, iprodione, profenofos, propineb, vinclozolin, aldicarb, benomyl, captan, carbendazim, carbofuran, cartap, etc.). Others studies have shown that prolonged exposure to pesticides may induce genotoxic effects in occupationally exposed human populations (Dulout *et al.*, 1985; Nehez *et al.*, 1988; Abbassy *et al.*, 2007), the evaluation of the genotoxicity of pesticides in use is of immediate concern. In fact, many of these chemicals are mutagenic (Galloway *et al.*, 1987; Garaj-Vrhovac and Zeljezic, 2000; Mossa, 2004; Abbassy *et al.*, 2004, 2005; Mansour *et al.*, 2008), linked to the development of cancers (Leiss and Savitz, 1995).

In conclusion, our results reveal that tribenuron-methyl has a clastogenic/genotoxic potential as measured by the bone marrow CA and MN tests in rats. The partial differences of the genotoxic effects obtained with pure and commercial tribenuron-methyl indicate that commercial formulations may contain additional hazardous compounds. Therefore, it is important in assessing the real human hazard from pesticides to investigate not only the active principle but also the commercial formulations used in agriculture.

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