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Validation of Traditional Methods of Genotoxicity with Liver Tyrosine Aminotransferase Activity Induced by Metribuzin Treatment in Albino Rats

Mahmoud M Elalfy*, Mohamed S Aboumosalam and Fathy R Ali Sleem

Forensic and Toxicology Department, Mansoura University, Egypt

***Corresponding author:** Mahmoud M Elalfy, Faculty of Veterinary Medicine, Forensic and Toxicology Department, Mansoura University, Egypt, Tel: +201007178306; E-mail: mahmoudelalfy@mans.edu.eg

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Abstract

The genotoxicity of pesticides give a potential role in population health and ecosystem safety and here the we would like to explore genotoxicity of metribuzin herbicide. Metribuzin dosed orally in albino rats twice per week for 3 months at dose level 0, 110, 220, and 440 mg/kg. All rats were sacrificed and liver was preserved in liquid nitrogen and bone marrow cells was obtained from femur bone. It was founded that chromosomal aberrations were dose dependent with significant increase in all doses (1/20, 1/10 and ⅛ of the LD50% of metribuzin herbicide) specially higher doses represented by structural abnormalities as chromosomal break, fragments, gap, association and a centromeric chromosomes beside numerical abnormalities as polyploidy and hypoploidy. Additionally, DNA quantity was dose dependent significant increase. Notably, there was significant increase in micronuclei level in respect to control that considered as a clastogenic signal and defect in mitotic activity that founded to be decreased in bone marrow cell where the mitotic index had decreased drastically in comparison to control value. Notably, metribuzin enhanced expression of TAT gene at all doses when compared with control group. on conclusion, metribuzin herbicide had a genotoxic properties that constitute hazard and great concern to population and TAT gene expression could be a potential role in detection of toxicity.

Keywords: Metribuzin; Chromosomal aberration detection; Micronuclei and DNA concentration; TAT gene albino rats

Introduction

To enhance the agricultural production, too large amounts of Pesticides emitted into the environment and showing their effect through genotoxic effect or carcinogenesis, there are three cytogenetic end points micronuclei, sister chromatid exchange and chromosomal aberrations and micronucleus test considered most suitable one [1]. Genotoxic compounds considered as those causing direct or indirect DNA damage. Pesticides as one of those compounds considered as potential mutagenic chemicals with mutagenic properties as chromosomal alteration and DNA damage. The genotoxic effect depends on quantity and formulation of chemical used and tested at both *in vivo* and *in vitro* systems [2].

Pesticides testing for carcinogenicity and genotoxicity carried out before market authorization and some prove positive results as micronuclei, chromosomal aberration and sister chromatid exchange that considered as indicator of genotoxicity and increase cancer risk [3].

Metribuzin or 4-amino-6-{1,1-dimethylethyl}-3-{methylthio}-1,2,4-triazin-5(4H)-one is considered as a potent pre- and post-emergent herbicide used to remove a wide range of grass weeds from soybean, potato, sugar cane, tomato and other crops with high water solubility 1.22 mg/ L and has low to moderate persistence in the soil [4]. Also Kimberley et al. and James Morgan reported that LD50% of metribuzin in rats was 2200 mg/kg of body weight [5,6].

Metribuzin showed show significant increase in the pituitary and bile duct adenoma at dose level 14.4 mg in a chronic study for 2 years, and classified as class D carcinogen by EPA [7].

Metribuzin showed that significant increase in DNA damage after exposure to metribuzin also moderate genotoxic effect detected in modified SOS microplate assay further increase in DNA adduct observed in *in vitro* 32 P-post labeling study in wing spot test in wing of *Drosophila melanogaster* [8].

Notably, Alteration of intracellular levels of hydroperoxides and carbonyl proteins and in the activities of catalase, SOD and reduced Glutathione were reported after metribuzin exposure to human and rat spleen lymphocytes reflecting DNA damage and oxidative stress especially at high concentrations [9]. Moreover, the Vanillin had a protective effect of against metribuzin pesticide-induced toxicity and oxidative stress in rats [10].

After exposure to metribuzin in human lymphocytes directly for 24 hours not induce sister chromatid exchange only show cytotoxicity represented as cell death, However pre exposure of metribuzin to *Vicia faba* roots only for 4 hr (*in vivo* activation) followed by exposure to human lymphocyte show significant increase in SCE that are concentration dependent and indicator of genotoxicity [11].

In a micronucleus assay *in vitro* in rat and human spleen lymphocyte Medjdoub et al. declared that metribuzin causes DNA damage and significant increase in micronuclei (MN) frequency in both rat and human lymphocytes at high concentration (50 and 100 μM) [9].

Metribuzin is positive for the DNA adduct (adduct type 1 with increase 3 to 4 folds while adduct type 2 increase by 5 folds) formation *in vitro* by using 32P-postlabeling method according to Shah et al. [12].

There was potential correlation between metribuzin exposure and certain malignancies as lymphohematopoietic malignancies suggesting that metribuzin may act as epigenetic carcinogen but additional investigation still required [13]. Metribuzin showed significant increase in DNA damage that aren't dose dependent (13, 53 and 214 mg/l) out on erythrocytes of *Rana catesbeiana* tadpoles after application of comet assay [14].

Notably, borlak and elalfy found that diethylnitrosamine as a genotoxic significant increase of liver tyrosine aminotransferase activity [15] and also Dundjerski et al. reported that higher doses of Cadmium decreased both the hepatic glucocorticoid receptor binding of the hormone and to DNA, however, stimulated rat liver tyrosine aminotransferase activity (TAT) [16].

The aim of such study was to validate of traditional methods of genotoxicity with TAT gene expression induced by metribuzin treatment in albino rats.

Materials and Methods

Experimental animals and grouping

Male albino rats obtained from experimental unit, Faculty of pharmacy, Mansoura university; weighted from 95 to 115 gm. Animals were apparently healthy and housed in plastic cages contain wood shaving as a bedding material. Animals accommodated for 2 weeks before the experiment and maintained on a balanced ration also feed and water given ad libitum throughout the experiment. Rats divided into four groups each one contains eight rats weighed 115 ± 5 gm; First group gavaged with distilled water as control while the other three groups intubated with metribuzin orally dissolved in distilled water at dose 110, 220, and 440 mg/kg of Metribuzin (equivalent to 1/20, 1/10 and 1/5 of the LD50 of metribuzin (Kimberley et al. and James Morgan) orally twice per week for 3 months. Animals weighed twice per week before dosing to maintain constant dose throughout the experimental period [5,6].

Chromosomal aberration detection: Chromosomal aberration detection technique carried out according to Al-Joubori et al. rats intraperitoneally injected with colchicine (0.5 mg/kg body weight) 3 hours before sacrificing [17]. The femur removed immediately and bone marrow received in centrifuge tube by injection of 5 ml KCl (0.57%) hypotonic solution then incubated for 20 minutes at 37°C followed by centrifugation for 2 minutes at 2000 rpm and supernatant discarded and 5 ml of cold fixative solution (methanol and glacial acetic acid with ratio 3:1) added to precipitate and left at room temperature for 5 minutes followed by centrifugation for 2 minutes at 2000 rpm and such technique repeated twice, then from the 75 cm by using Pasteur pipette suspension dropped on a clean, moisten and cold slide followed by air drying and staining with giemsa stain solution 5% and at least investigation of 1000 metaphase cells per each group examined for chromosomal aberration.

DNA preparation, extraction and determination: DNA contents determined by the diphenylamine Procedure colorimetrically according to Karp [18], where after the animals sacrificed the liver removed, washed with saline solution and weighing 1 gm of liver tissue then homogenized with 4 ml cold distilled water, then 2 ml of liver homogenate suspended in 5 ml of 10% solution of trichloroacetic acid then centrifugation at 3000 rpm for 2 minutes followed by discarding the supernatant and repeating the same technique, Followed by resuspending the pellet in 10 ml ethyl alcohol 95% followed by centrifugation at 3000 rpm and discarding the supernatant to obtain the purified pellet and such technique repeated with the same way, after that purified pellet resuspended again in 5 ml of trichloroacetic acid TCA 5% and put in boiling water bath at 90°C for 15 minutes then centrifuged and 2 ml of supernatant put in centrifuge tube with 4 ml of diphenylamine reagent (1 g of diphenylamine+100 ml of the glacial acetic acid+2.5 ml conc. sulphuric acid) followed by putting the centrifuge tubes in boiling water bath for 10 minutes then cooled quickly and observe the change in color, Finally solution then transferred to cuvette for absorbance reading at 600 nm wavelength and takes the results.

Mitotic index detection: Mitotic index detected according to Sehgal et al. [19] where approximately 3000 cells for each group analyzed for the mitotic index (MI), calculated as the number of divided cells at metaphase per total number cells according to following formula:-

MI%=Number of the divided cells × 100/Total number of the calculated cells.

Micronuclei detection: Micronuclei assay detected according to Gebel et al. [20] where the bone marrow cells from the femur flushed by 5 ml saline solution by using syringe in a centrifuge tube and centrifuged at 4°C for 15 min, supernatant discarded, and the pellet was resuspended in 100 µl then one drop applied to a glass slide followed by air drying then fixed in methanol solution 95% for 2 minutes and stained with giemsa stain 5% stock solution, for counting, at least 1000 examined for each group and number of micronuclei detected from the total number of cells. The chi-square test used to determine the significance and the total chi-square.

RNA isolation, reverse transcription and RT-PCR

The trizol reagent kit was used for total RNA extraction from liver tissue. Reverse transcription was carried out using Superscript II reverse transcriptase. Primers that were used: Primer sequences for TAT were 5-TGGAGTTCACAGAGCGGTTG-3 (forward) and 5-GGTACTCGAAGCACGTTGCTG-3 (reverse) and b-actin 5-ggcattgttaccaactgggacg-3, 3- ctctttgatgtcacgcacgatttc-5 [21]. Moreover, Conditions for RT-PCR were as follows: 10 min at 95˚C followed by 40-50 cycles of 15 s at 95˚C, 15 s at 60˚C, and 15 s at 72˚C. RT-PCR was performed as previously described.

Statistical Analysis

The results were statistically evaluated using Student's "t" test. (Trial SPSS 2013). There was no fund shared in this article and all requirement paid by authors. There was no conflict of interest for all authors.

Results

Detection of chromosomal aberration in rats bone marrow exposed 110, 220, and 440 mg/kg (equivalent to 1/20, 1/10 and 1/5 of the LD50 of metribuzin) of Metribuzin given orally twice per week for 3 months.

Chromosomal aberration were dose dependent with significant increase in all doses (1/20, 1/10 and 1/5 of the LD50) specially higher doses represented by structural abnormalities as chromosomal break,

fragments, gap, association and a centromeric chromosomes beside numerical abnormalities as polyploidy and hypoploidy where the results illustrated in Figure 1 and Table 1.

Group	Total	Break	Fragment	Gap	Ring	Chromosomal association	Acentromeric chromosome	Hypoploidy	Polyploidy
G 1	2 ± 0.26^{d}	0.5 ± 0.18^{d}	0.63 ± 0.26^{d}	0.75 ± 0.16^{d}	0^{d}	0^{d}	0^{c}	0^{c}	0^{d}
G 2 (1/20 of LD 50%)	11.87 ± 0.51^{c}	2.37 ± 0.18^{c}	2.13 ± 0.13^{c}	2.63 ± 0.26^{c}	1.75 ± 0.16^{c}	0.75 ± 0.16^{c}	0.88 ± 0.22^{b}	0.75 ± 0.16^{b}	0.75 ± 0.16^{c}
G 3 (1/10 of LD 50%)	25.62 ± 0.23^{b}	4 ± 0.18^{b}	4.25 ± 0.35^{b}	4.13 ± 0.22^{b}	3.38 ± 0.32^{b}	2.13 ± 0.22^{a}	2 ± 0.26^{a}	2.75 ± 0.36^{a}	3 ± 0.26^{b}
G 4 (1/5 of LD 50%)	37.75 ± 0.88^{a}	7 ± 0.26^{a}	7 ± 0.42^{a}	6.63 ± 0.32^{a}	6.5 ± 0.26^{a}	1.63 ± 0.18^{b}	1.75 ± 0.25^{a}	2.25 ± 0.31^{a}	5 ± 0.26^{a}

Table 1: Detection of chromosomal aberration in rats' bone marrow exposed to 110, 220, and 440 mg/kg of A, b, c, d: Different letters are significantly different between groups ($p<0.05$).

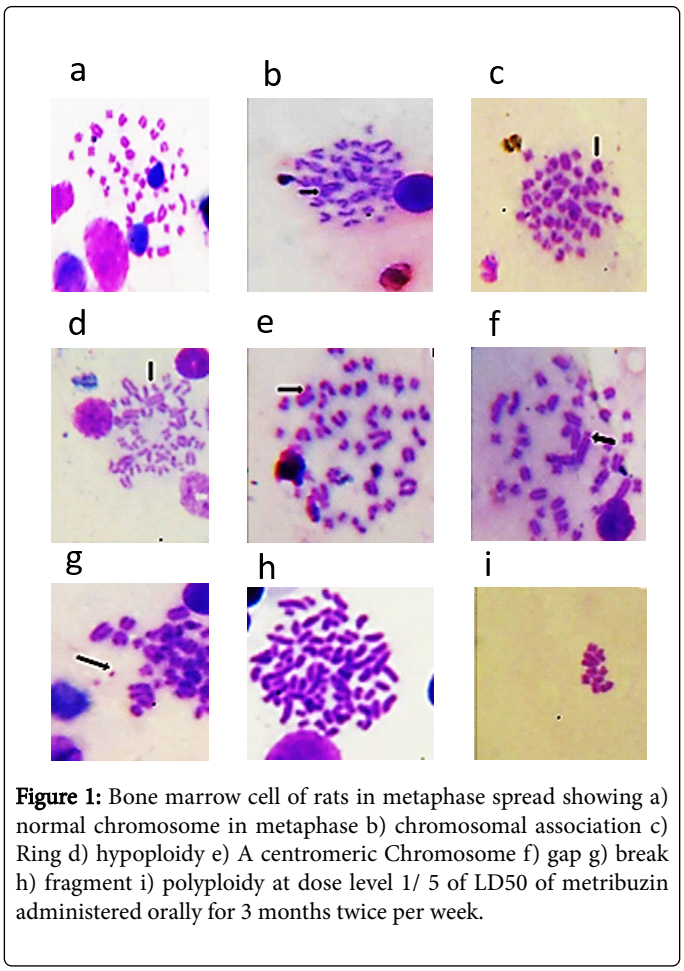

Figure 1: Bone marrow cell of rats in metaphase spread showing a) normal chromosome in metaphase b) chromosomal association c) Ring d) hypoploidy e) A centromeric Chromosome f) gap g) break h) fragment i) polyploidy at dose level 1/ 5 of LD50 of metribuzin administered orally for 3 months twice per week.

Estimation of DNA in liver tissue of rats administered metribuzin at dose level 110, 220, and 440 mg/kg of Metribuzin (equivalent to 1/20, 1/10 and 1/5 of the LD50 of metribuzin) given orally twice per week for 3 months.

Two doses of metribuzin (⅕ and 1/10 of LD50) showed significance increase in quantity of DNA after 3 months of administration compared to control group as illustrated in Table 2.

	Control	Group 2	Group 3	Group 4
DNA concentration (mg/kg)	13.4 ± 0.4	15.8 ± 0.8	18.8 ± 0.12a	20.6 ± 0.17a

Table 2: Showing Estimation of DNA in liver tissue of rats administered Metribuzin at dose level 110, 220, and 440 mg/kg (equivalent to 1/20, 1/10 and 1/5 of the LD50 of metribuzin) of Metribuzin given orally twice per week for 3 months.

Mitotic index detection in rats' bone marrow exposed to 110, 220, and 440 mg/kg (equivalent to 1/20, 1/10 and 1/5 of the LD50 of metribuzin) of Metribuzin given orally twice per week for 3 months.

The result according to chi square analysis showed that significant difference in mitotic index between treated and control groups where there is a dose dependent significant decrease in mitotic index in rats exposed to (1/20, 1/10 and 1/5 of the LD50) of Metribuzin equivalent to (440, 220 and 110 mg/kg) in comparison with the control group (Tables 3 and 4).

Group	Total no. of counted cells	No. of divided cells	No. of non-divided cells	M.I
G 1	3000	123	2877	4.1
G 2 (1/20 of LD 50%)	3000	92	2908	3.06
G 3 (1/10 of LD 50%)	3000	81	2919	2.7
G 4 (1/5 of LD 50%)	3000	75	2925	2.5
Total chi-square=15.23*		Degree of freedom=3	Probability=0.0016	

Table 3: Show Mitotic index detection in rats' bone marrow exposed to 110, 220, and 440 mg/kg of Metribuzin (equivalent to 1/20, 1/10 and 1/5 of the LD50 of metribuzin) given orally twice per week for 3 months.

	Control	G 1/20	G 1/10	G 1/5
G1				
G 2 (1/20 of LD 50%)	4.43*			
G 3 (1/10 of LD 50%)	8.53*	0.6		
G 4 (1/5 of LD 50%)	11.54*	1.58	0.16	

Table 4: Show chi square analysis of Mitotic index of groups treated with 110, 220, and 440 mg/kg of Metribuzin. *value means that there was significant difference between the control and the treated groups at the dose level of ($p<0.05$). ** Value means that there was a highly significant difference between the control and the treated groups at the dose level of ($p<0.001$).

Micronuclei assay detection in rats' bone marrow exposed to 110, 220, and 440 mg/kg (equivalent to 1/20, 1/10 and 1/5 of the LD50 of metribuzin) of Metribuzin given orally twice per week for 3 months.

The result according to chi square analysis showed that a highly significant change between the control and treated groups where there is a dose dependent highly significant increase in micronuclei in rats exposed to (1/20, 1/10 and 1/5 of the LD50) of Metribuzin in comparison with the control group (Figure 2, Tables 5 and 6).

Group	Total no. of examined cells	No. of micronuclei	No. of normal cells
G1	2000	187	1813
G 2 (1/20 of LD 50%)	2000	285	1715
G 3 (1/10 of LD 50%)	2000	322	1678
G 4 (1/5 of LD 50%)	2000	392	1609
Total chi-square=86.57*		Degree of freedom=3	Probability=000

Table 5: Show Micronuclei assay detection in rats' bone marrow exposed to 110, 220, and 440 mg/kg (equivalent to 1/20, 1/10 and 1/5 of the LD50 of metribuzin) of Metribuzin given orally twice per week for 3 months.

	Control	G 1/20	G 1/10	G 1/5
Control				
G 2 (1/20 of LD 50%)	22.60**			
G 3 (1/10 of LD 50%)	40.42**	2.51		
G 4 (1/5 of LD 50%)	83.92**	19.91**	8.07*	

Table 6: Show chi square analysis of Micronuclei assay detection in rats' bone marrow exposed to 110, 220, and 440 mg/kg (equivalent to 1/20, 1/10 and 1/5 of the LD50 of metribuzin) of Metribuzin given orally twice per week for 3 months. *value means that there was significant difference between the control and the treated groups at the dose level of ($p<0.05$). ** Value means that there was a highly significant difference between the control and the treated groups at the dose level of ($p<0.001$).

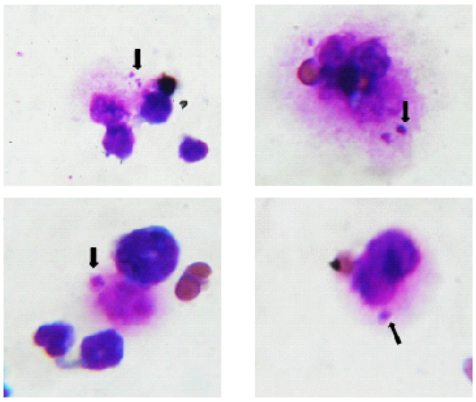

Figure 2: Showed micronuclei in rats bone marrow cells exposed to metribuzin at dose level 110, 220, and 440 mg/kg of metribuzin (equivalent to 1/20, 1/10 and 1/5 of the LD50 of metribuzin) given orally twice per week for 3 months.

TAT gene expression by Rt-PCR

It was found that TAT gene expression could correlate with genotoxicity of metribuzin when compared with control group (Figure 3).

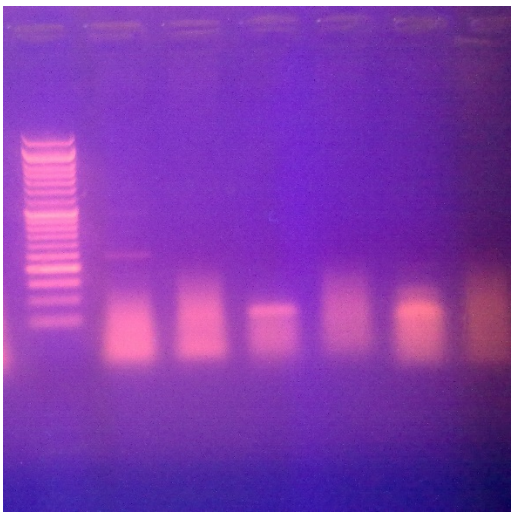

Figure 3: Show gel detection Rt-PCR of TAT gene expression in all doses of metribuzin compared with control in last lan.

Discussion

Chromosomal aberration were increased significantly in all doses specially at higher dose represented by both structural abnormalities and numerical abnormalities such results agree with Kaya et al. [8] who proposed that in a study in the wing of *Drosophila melanogaster* somatic mutation and recombination or wing spot test by using of four different concentration of the herbicide and results show that significant increase in DNA damage after exposure to metribuzin also moderate genotoxic effect detected in modified SOS microplate assay further increase in DNA adduct observed in *in vitro* 32 P-post labeling study. Also agree with Delancey et al. who reported that in a survey study to detect the correlation between the incidence of cancer and the exposure to metribuzin and potential correlation between metribuzin exposure and certain malignancies as lymphohematopoietic malignancies suggesting that metribuzin may act as epigenetic carcinogen [13]. Moreover, Clements et al. [14] declared that in Single cell gel electrophoresis or comet assay carried out on erythrocytes of *Rana catesbeiana* tadpoles to detect damage in DNA after exposure to metribuzin and other four herbicides, metribuzin showed significant increase in DNA damage that were not dose dependent. Notably, Shah et al. founded also that metribuzin was positive for the DNA adduct detected in *in-vitro* study by using 32P-postlabeling method [12].

Metribuzin at dose of ⅛ and 1/10 of LD50 resulted in significant increase in quantity of DNA after 3 months of administration compared to control group and such results inconsistent with Shah et al. where metribuzin was positive for the DNA adduct presence *in vitro* studies by using 32P-postlabeling method [12]. Also metribuzin considered as a pre-carcinogenic reported that in a study of rats at dose level 0, 1.3, 1.9, 5.3 and 14.4 mg metribuzin /kg/day in male rats showed significant increase in the pituitary and bile duct adenoma [7]. Additional, Gowri et al. found primary lesions as breaks, DNA protein crosslink and eventually loss of cell structure also proliferation with a total control loss of cellular mechanism may occur leads to increase quantity of DNA synthesis and mutagenicity [22]. On the other hand disagree with Soliman and Ghoneam, they founded that DNA content decreased than control and decrease the DNA synthesis and replication in *Vicia faba* after exposure to metribuzin [23].

Mitotic index was significant difference between treated and control groups in a dose dependent significant decrease in mitotic index in rats exposed to (1/20, 1/10 and 1/5 of the LD50) of metribuzin equivalent to (110, 220 and 440 mg/kg) in comparison with the control group and such results come in agreement with Haliem, [24] who reported that metribuzin cause a drastic decrease in mitotic index of *Allium cepa* meristematic cells and also according to Baszynski et al. attributed to inhibition of protein synthesis especially certain nuclear proteins that was essential in mitotic cycle so decrease the mitotic activity [25].

Micronuclei were increased in a dose dependent manner in rats exposed to (1/20, 1/10 and 1/5 of the LD50) of Metribuzin equivalent to (440, 220 and 110 mg/kg) in comparison with the control group and such results inconsistent with Medjdoub et al. who proposed that in a micronucleus assay *in vitro* in rat and human spleen lymphocyte metribuzin causes DNA damage and significant increase in micronuclei frequency in both rat and human lymphocytes at high concentration [9]. The micronuclei test considered as a potential test to assess genotoxicity also according to Maya et al. who reported that pre exposure of metribuzin to *Vicia faba* roots only for 4 hr (*in vivo* activation) followed by exposure to human lymphocyte show significant increase in SCE and micronuclei that were concentration dependent and indicator of genotoxicity.

Metribuzin enhanced expression of TAT gene at all doses of treatment and these results agree with unpublished data of Borlak and Elalfy [15] and Dundjerski et al. [16] who found correlation of expression of Dundjerski et al. with toxins like diethylnitrosamine or cadmium.

On conclusion, metribuzin herbicide had a genotoxic properties that constitute detected by traditional methods or expression of TAT gene as a new tools for genotoxicity.

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Conflict of Interest

Authors have no conflict of interest.

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