Evaluation of the Antimutagenic Effect of Vitamin C against DNA Damage and Cytotoxicity Induced By Trimethyltin in Mice

Ayman A. Farghaly and Mona A.M. Abo-Zeid

Department of Genetics and Cytology, Division of Genetic Engineering and Biotechnology, National Research Center, El-Behooth St. 31, Dokki 12622, Cairo, Egypt.
farghaly_5@yahoo.com, monaabozeid@yahoo.com

Abstract: The objective of this study is to investigate the utility of comet assay and chromosome aberrations analysis for detecting the possible antimutagenic activity of vitamin C to reduce the genotoxic effect of trimethyltin (TMT). TMT is one of the organotin compounds which is widely used as polyvinyl chloride heat stabilizers and marine biocides. In this study, male Swiss mice were treated interapretoneally (i.p.) with three tested doses 0.25, 0.50 and 1.0mg TMT/kg b.wt. for 1, 2 and 3 days. Alkaline comet assay in nucleated bone-marrow cells and chromosome analysis in spermatocytes were performed 24h after the last treatment. The amount of DNA damage in cells was estimated from comet tail length as the extent of migration of the genetic material. A significant increase in comet tail length indicating DNA damage was observed at all concentrations compared with control ($p<0.05$). The mean comet tail length showed a concentration- related and time-dependent increase. Also, the percentage of chromosome aberrations in spermatocytes was statistically significant ($p<0.05$) and showed dose and time dependent manner. Concurrent administration of vitamin C (VC) orally at 20mg/kg b.wt. with the highest dose of TMT for 1, 2 and 3 days reduced DNA damage in somatic and germ cells to a significant extent. In conclusion, our results indicated that vitamin C ameliorated DNA damage and genotoxicity induced by trimethyltin in mice somatic and germ cells *in vivo*. [Nature and Science 2009; 7(12):1-7]. (ISSN: 1545-0740).

Key words: trimethyltin, vitamin C, bone marrow, spermatocytes, comet assay, chromosome aberrations, mice.

1. Introduction

Among the various chemical agents that can induce damage in animals and humans, pesticides are a prime offender, chief among these are organotin compounds, which are used not only in agricultural pesticides, but also in many industrial processes, as material preservatives, and as components of antifouling paints **(Fent, 1996 and 2003)**. Organotins are widely dispersed throughout the ecosphere where they can be accumulated into the food chain **(Hodge *et al*, 1979)** via the tin geocycle **(Craig, 1988)**. Trimethyltin (TMT) is a potent toxicant that selectively kills cells in the central nervous system, immune system, spleen, lung, and kidney **(Brown *et al*, 1979; Snoeij *et al*, 1985; Philbert *et al*, 2000)**. TMT intoxication resulted in phenotypic changes indicative of apoptosis cell death, which includes chromatin condensation, nuclear fragmentation, mitochondrial dysfunction, reactive oxygen species production, membrane blebbing and caspase activation **(Stine *et al*, 1988; LeBel *et al*, 1990; Geloso *et al*, 2002; Jenkins and Barone, 2004)**. Administration of TMT at 2.8 mg/kg, i.p. induced neural damage seen in the dentate granule cells of mice **(Ogita *et al*, 2004)**. The induction of cytogenetic damage to mammalian cells by TMT has been indicated in a number of published reports *in vivo* and *in vitro* **(Ghosh *et al*, 1989; Ganguly *et al*, 1992; Ganguly, 1994; Dopp *et al*, 2007)**.

There is considerable evidence that the effects of mutagenic and carcinogenic agents can be altered by many dietary constituents. Vitamin C (VC) is an essential dietary nutrient required as a co-factor for many enzymes and a very efficient antioxidant, scavenging reactive oxygen and nitrogen species and protecting cells against free radical- mediated damage **(Sanchez-Moreno *et al*, 2003)**. Besides exerting antioxidant influence directly, VC can promote the removal of oxidative DNA damage from the DNA and/or nucleotide pool, through the upregulation of repair enzymes **(Cooke *et al*, 1998)**. The inhibitory effect of VC towards a number of mutagens/carcinogens was shown by many authors in humans and animals **(Mooney *et al*, 2005; Hassan *et al*, 2006; Fahmy *et al*, 2008)**. The goal of the present study is to evaluate the *in vivo* protective effect of VC against the DNA damage may be induced by TMT in mouse somatic and germ cells.

2. Materials and Methods:

2.1. Animals

Laboratory-bred strain Swiss albino male mice of 8-10 weeks old with an average weight of 27.5±2.5 g obtained from the National Research Center, Cairo, Egypt, were used. Animals were housed in groups (5 animals/ group) and maintained under standard conditions of temperature, humidity and light. The animals were given standard food and water *ad libitum*.

2.2. Chemicals

TMT was purchased from BDH Chemicals Poole, England. VC was purchased from Sigma, USA. All other chemicals used were of analytical grade.

2.3. Doses and experimental design

The experimental design involved: 1- Mice were treated i.p. with the doses 0.25, 0.5 and 1.0mg TMT/kg b.wt. for 1, 2 and 3 days. 2- Concurrent administration of VC at 20mg/kg b.wt. and 1mg TMT/kg b.wt. for 1,2 and 3 days. The samples were taken 24h after the last treatment from the bone-marrow cells for DNA damage detection by comet assay and spermatocyte cells for chromosome aberrations detection. In all experiments, the animals groups were treated with vehicle as a negative control, 20mg VC/kg b.wt. and 1mg mitomycin C/kg b.wt. as a positive control. All samples were collected after 24h.

2.4. Cell viability

To measure cytotoxicity, 15µl of each original cell suspension was mixed with15µl of 0.4% solution of trypan blue vital dye. Cells were analyzed with a light microscope and the percentage of viable cells was determined from 200 cells for each experimental group **(Zamorano-Ponce *et al,* 2004)**.

2.5. Comet assay

The comet assay was performed as described by **Tice *et al* (2000)**. Mice femurs were dissected out and bone marrow was aspirated from each femur into media solution. The cell suspension (25 µl) was mixed 1:10 with 250 µl molten low melting point (LMP) agarose, and samples of 75 µl of the mixture were rapidly spread on CometSlides. After gelling for 20 min at 4ºC in the dark, slides were put in a tank filled with lysis solution (2.5M NaCl, 0.1M EDTA, 10mM Tris base, 1% sodium lauryl sarcosinate and 1% Triton X-100) for 1h at 4ºC in the dark. Slides were then washed three times with neutralization buffer (0.4M Tris, pH 7.5) for 5 min and incubated in fresh alkaline buffer (0.3M NaOH and 1mM EDTA, pH>13) for 30min at room temperature to allow unwinding of DNA. Electrophoresis was then carried out at room temperature in fresh ice-cold alkaline electrophoresis buffer for 30 min (1V/cm; 300mA). After electrophoresis, slides were gently washed three times for 5 min in fresh neutralization buffer and exposed to 70% ethanol for 5 min. After drying at room temperature, slides were stained with 25 µl of ethidium bromide solution (20µg/ml) and covered with cover slip. Comets were examined at 200X magnification using a fluorescence microscope.

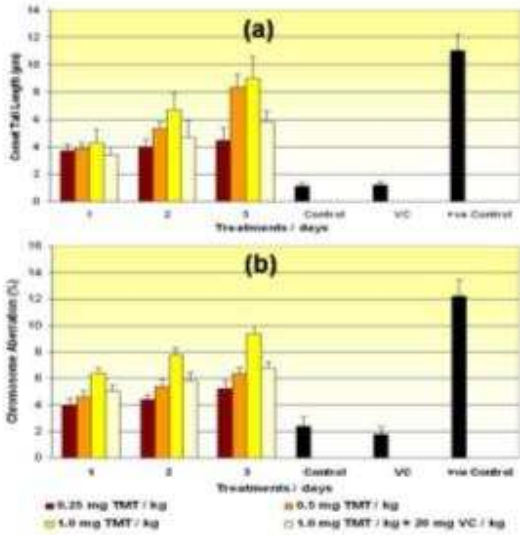

Figure 1: Diagram represents (a) Comet tail length (µm ± SE) (b) Chromosome aberration (% ± SE) induced by different doses of TMT for three times intervals compared with MMC as a positive control.

Table 1: The percentage of cell viability and comet tail length (µm) in mice nucleated bone marrow cells after treatment with TMT alone and in combination with vitamin C.

Treatments	Time of Treatment (Day)	(%) of Viable Cells	Comet tail length Mean ±SE
I. Control (vehicle)	-	93	1.14±0.23
II.VC 20mg/kg b.wt.	-	94	1.17±0.28
III. MMC (1mg/kg b. wt.) positive control	-	72	11.03±1.12[a]
IV. TMT 0.25mg/kg b. wt.	1	91	3.72±0.46[a]
	2	87	3.92±0.58[a]
	3	82	4.48±0.91[a]
0.5mg/kg b. wt.	1	85	3.90±0.48[a]
	2	80	5.31±0.52[a]
	3	78	8.37±0.97[a]
1.0mg/kg b. wt.	1	82	4.31±0.97[a]
	2	77	6.70±1.20[a]
	3	70	9.01±1.61[a]
V.TMT+V.C. 1.0mg/kg b. wt. + 20mg/kg b.wt.	1	84	3.42±0.55[a]
	2	81	4.71±1.19[ab]
	3	76	5.86±0.76[ab]

a) Significant compared to vehicle control ($p<0.05$)
b) Significant compared to TMT treatment at 1mg/kg b.wt. ($p<0.05$) (t-test)

Table 2: Number and mean percentage of diakinase metaphase I cells with chromosome aberrations in mice spermatocytes after treatment with TMT alone and in combination with vitamin C.

Treatments	Time of Treatment (Day)	XY un.	Auto. un.	XY+ Auto. un.	Chain (IV)	Total Aberrations	
						No.	Mean (%)±SE
I. Control (vehicle).	-	7	5	-	-	12	2.4±0.65
II.VC (20mg/kg b. wt.)	-	5	4	-	-	9	1.8±0.52
III. MMC (1mg/kg b. wt.) positive control	-	35	20	4	2	61	12.2±1.2[a]
IV. TMT							
0.25 mg/kg b. wt.	1	14	6	-	-	20	4.0±0.54
	2	15	7	-	-	22	4.4±0.40
	3	18	8	-	-	26	5.2±0.60 [a]
0.5 mg/kg b. wt.	1	16	7	-	-	23	4.6±0.42
	2	15	12	-	-	27	5.4±0.54 [a]
	3	18	13	1	-	32	6.4±0.48 [a]
1.0 mg/kg b. wt.	1	19	13	-	-	32	6.4±0.42 [a]
	2	22	15	1	1	39	7.8±0.56 [a]
	3	28	17	-	2	47	9.4±0.58 [a]
V. TMT+V.C.	1	20	5	-	-	25	5.0±0.50[a]
1.0mg/kg b. wt.	2	18	11	-	-	29	5.8±0.64[a]
+ 20mg/kg b.wt.	3	21	12	-	1	34	6.8±0.46[ab]

The total number of scored cells is 500 (5 animals/ group), XY un.: XY univalent; Auto. un.: Autosomal univalent
a) Significant compared to vehicle control ($p<0.05$)
b) Significant compared to TMT treatment at 1mg/kg b.wt. ($p<0.05$) (t-test)

Evaluation of DNA damage

Five good-quality slides were selected for each variable from the different treatment groups including controls. All slides were independently coded and scored. A total of 50 comets were scored for each variable. The comet tail length was measured with a calibrated ocular micrometer disk. The quantification of the DNA damage was made by calculating as comet tail length (µm) = total length of comet –head diameter. The significance of the effect of each treatment dose versus the solvent control and between treatments with protection versus treatment alone was evaluated by t-test.

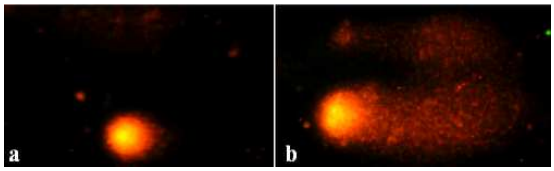

Figure 2: Photomicrographs of damaged DNA observed after the treatment of mice bone-marrow cells with TMT showing increase in tail moment. Comets were classified for qualitative evaluation into (a) Undamaged cell, (b) Highly damaged cell.

2.6. Chromosome aberrations

For spermatocytes preparation, animals from each group were injected i.p. with colchicine, 2-3h before sacrifice. Animals were sacrificed by diethyl ether at end of treatments. Chromosome preparations were made from testes according to the technique of **Evans *et al* (1964)**. 100 well spread diakinase-metaphase I cells were analyzed per animal (5animals/ group) for chromosome aberrations. Metaphases with univalents and chromosome translocations were recorded. The significance of treatment from the control data and treatment plus protective versus treatment alone was calculated using t-test.

3. Results:

3.1. Cell viability:

The cytotoxic effect of TMT was determined using three different doses for 1, 2 and 3 consecutive days. The percentage of viable cells reduced in a dose and time dependent. Oral administration of VC with the highest dose of TMT increased the percentage of cell viability for all the tested doses. The percentage of viable cells was always ≥70% after treatment for all experiments as recommended for comet assay by the International Workshop on Genotoxicity test Procedure (Tice *et al.*, 2000; Table 1).

3.2. Comet assay:

The amount of DNA damage in the cell was estimated from tail length as the extent of the migration of the genetic material in the direction of the anode. During electrophoresis, cell DNA was seen to more rapidly migrate towards the anode at the highest concentration than at the lowest concentration. Even the comet tail tended to increase when exposure was prolonged from 1 to 3 days. Mean tail length (µm) of comets obtained by TMT treatment is given in table (1). The trend of increase in comet tail length with increase in concentration and duration is depicted in figure (2). All the concentrations and their respective duration evoked significant DNA damage ($p<0.05$) compared with controls. The maximum DNA damage was recorded at the highest dose of TMT. This damage was reached to 9.01µm after treatment for 3 days with TMT in comparing to 11.03µm for positive control (MMC) and 1.14µm for negative control. The highest DNA damage reduced to 5.86µm after concurrent treatment of 1mgTMT/kg and VC (20mg/kg) for 3 consecutive days (Table 1, Figure 1a).

3.3. Chromosome aberrations:

The genotoxic effect of TMT on the germ cells was illustrated by examining the chromosome aberrations in mouse spermatocytes. Table (2) and figure (1b) represents the chromosome aberrations in mouse spermatocytes after treatment with different doses of TMT for the three time intervals.

It was demonstrated that the highest percentage of chromosome aberrations reached 9.4±0.58 after treatment with 1mgTMT/kgb.wt. for 3 days compared to 12.2±1.2 for positive control and 2.40±0.65 for negative control. This percentage was decreased to 6.8±0.46 after concurrent treatment of TMT (1mg/kg) and VC for the same period of time. The main types of chromosome aberrations observed were XY univalents and autosomal univalents (Figure 3).

4. Discussion:

The aim of the present study was to evaluate the protective effect of VC towards DNA damage induced by TMT in mouse nucleated bone-marrow cells and chromosome aberrations in mouse spermatocytes. DNA damage was carried out using comet assay, a molecular technique that has been increasingly employed to evaluate *in vivo* and/or *in vitro* DNA damage in individual cells **(Manas *et al,* 2009)**. Spermatocytes represent the only system in which the transmissible genetic damage from one generation to another takes place **(William and Hsu, 1980)**.

Our data showed that TMT induced statistically significant increase in DNA damage which represented by tail moment in bone-marrow cells and chromosome

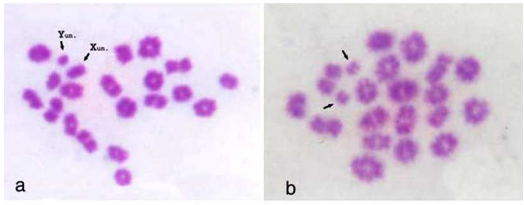

Figure 3: Metaphases spread from mice spermatocytes showing (a) XY univalents, (b) Autosomal univalents after treatment with 1mg TMT/ kg b.wt.

aberrations in spermatocyte cells in a dose and time response. The same observations showed by **Ghosh *et al* (1989)** and **Ganguly *et al* (1992)**. They demonstrated that TMT induced elevation in the frequency of micronuclei and sister chromatid exchanges in cultured human lymphocytes. Also, TMT had the ability to induce chromosome aberrations in mice bone-marrow cells *in vivo* 6h after treatment **(Ganguly, 1994)**. **Dopp *et al* (2007)** observed that di- and trimethyltin significantly induced micronuclei, chromosome aberrations, sister chromatid exchanges and DNA damage at non-cytotoxic concentrations in CHO-9 cells. It is worth to mention that other organotin compounds such as butyl- and phenyltin induced hyperdiploid cells (aneuploidy) and chromosome supercontraction even at low concentrations in cultured human lymphocytes **(Jensen *et al,* 1991)**.

With respect to the chromosome aberrations induced in mouse spermatocytes after 1, 2 and 3 days of treatments with TMT, it was observed that dissociated univalents dominated autosomal and XY-univalents. However, XY-univalents were more frequent. This type of abnormality has been discussed as an indicator of a mechanism of male sterility from radiations and mutagenic chemicals **(Cattanach *et al,* 1968; Rapp *et al,* 1977)**. Translocations in the form of chain IV were observed at low frequency. According to **William and Hsu (1980)**, chromosome translocations in germ cells have been observed in animals after treatment with ionizing radiation, but rarely in those treated with chemicals.

The DNA breaks that are detected in the comet assay and cytogenetic analysis may be resulted from: 1- The oxidative stress and the enhancement of the intracellular generation of reactive oxygen species (ROS) formed by TMT **(Sergent *et al,* 1999)**. These ROS can damage DNA and division of cells with unrepaired or misrepaired damage leading to mutations. Also, if these changes appear in critical genes, such as oncogens or tumor suppressor genes, initiation or

progression may result **(Loft and Poulsen, 1996; Pryor, 1997)**. 2- This DNA damage could be also originated from apoptotic cells. Several laboratories have reported that the onset of apoptosis can give comet images with cell aspect and tail parameter values of the same orders as those of cells with moderate DNA damages **(Florent *et al,* 1999; Choucroun *et al,* 2001)**. **Jenkins and Barone (2004)** reported that TMT had the ability to induce apoptosis in PC12 cells by initiating apoptotic pathway requiring oxidative stress, caspase activation and P38 protein kinase activity leading to cell death. Also, **Kawada *et al* (2008)** observed that i.p. injection of TMT at the dose 2.8mg/kg b.wt. led to a dramatic increase in the number of degenerating cells in the granule cell layer of the OB and AON of the mouse brain cells.

Interest in the chemopreventive functions of antioxidants has grown considerably in recent years. Evidence accumulated over the years shows that people with high dietary intakes of fruits and vegetables are less likely to develop cancer than people who have low dietary intake of these foods. While many chemopreventives in fruits and vegetables may have anticancer properties, much interest has focused on vitamin C **(Mayne, 2003)**. This study represents one of the premiere studies carried out to diminish the toxicity and the genotoxicity of the oxidative compound TMT by using the natural antioxidant compound VC. Vitamin C is a highly effective antioxidant. It acts as a reducing agent that can terminate free radical driven oxidation by being converted to a resonance-stabilized free radical. In this respect VC can protect indispensable molecules in the body, such as protein, lipids, carbohydrates and nucleic acids (DNA and RNA). VC also regenerates other antioxidants such as vitamin E **(Schneider *et al,* 2001**). Our results showed that concurrent administration of VC inhibited the DNA damage and chromosome aberrations induced by TMT in all tested doses. This ameliorative effect induced by VC may be resulted from enhancement of detoxification pathways that convert this reactive compound to less toxic and more easily excreted products **(Vijayalaxmi and Venu, 1999)** and/or through its action as the free radical scavenging efficiency (**Chaudiere and Ferrari-Iliou, 1999)**. In addition, numerous *in vitro* and *in vivo* studies have evaluated the protective effects of VC against several radical generating chemicals **(Blasiak and Kawalik, 2001; Blasiak *et al,* 2004; Robichova *et al,* 2004; Arranz *et al,* 2007)**.

In conclusion, our results demonstrated that VC could be a suitable agent for preventing TMT- induced DNA and chromosome damage in an *in vivo* mammalian system.

Correspondence to:
Ayman A. Farghaly and Mona A.M. Abo-Zeid Department of Genetics and Cytology,
Division of Genetic Engineering and Biotechnology,
National Research Center,
El-Behooth St. 31, Dokki 12622, Cairo, Egypt.
Emails: farghaly_5@yahoo.com,
monaabozeid@yahoo.com

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