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Research Article

Assessment of micronuclei formation for genotoxicity and hematological indices after exposure to commercial malathion and ascorbic acid: *In vivo* study

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ABSTRACT

Impurities in commercial formulations of Malathion cause pronounced toxicity and they interact with DNA, causing mutagenicity. Ascorbic acid is a potent water soluble antioxidant that protects the cellular compartment from oxygen and nitrogen radicals. In the present study effect of ascorbic acid was studied on 14 days of repeated treatment of commercial grade Malathion (CGM) induced toxicity assessed by micronuclei (MN) formation for genotoxicity and hematological indices in Swiss albino mice. The treatment with 50% of LD_{50} and 5% of LD_{50} of CGM (LD_{50} :1000 mg /Kg body weight) resulted in a significant dose dependent increase in the frequency of MN. Significant decrease was observed in a dose dependent manner in the RBC, hematocrit, thrombocyte count as well as a significant increase in WBCs and reticulocytes. The treatment with 250 mg/Kg body weight of ascorbic acid did not have any effect on the MN induced by 50% LD_{50} CGM treatment but arrested 5% LD_{50} induced MN. A similar dose dependent result was obtained with ascorbic acid treatment with a normalization of hematological indices in 5% LD_{50} treated animals. CGM treated animals in both the groups showed a significant decrease in body weight which was restored by ascorbic acid. The results of the present study suggest a protective effect of ascorbic acid against CGM induced genotoxic damage as well as altered hematological indices and bodyweight in a dose dependent manner.

Keywords: Ascorbic acid; Body weight; Hematological indices; Malathion; Micronuclei.

INTRODUCTION

Organophosphorus pesticides (OP) are one of the most widely and indiscriminately used pesticides which in large amounts cause environmental pollution (Yehia et al, 2007). Malathion (O,O-dimethyl-S-1,2, bis ethoxy carbonyl ethyl phosphorodithioate) is one of the most widely used organophosphate pesticides used for pest control of crops, for mosquito eradication and is available in different commercial formulations (Vidyasagar et al, 2004). The primary mechanism of action of Malathion is inhibition of acetylcholinesterase (AchE) by phosphorylation of serine residues in the active center of the enzyme (Eto M, 1974) causing accumulation of acetylcholine at cholinergic synapses leading to increased activation of nicotinic and muscarinic receptors. (Kalender et al, 2006).

Some mutagenicity studies have demonstrated the genotoxicity of technical grade malathion (TGM) but the reports are controversial (Edwards, 2006). TGM in experimental animals has shown that it has a potential to produce chromosomal aberrations and micronuclei (MN) (Dulout et al, 1982, Salvador et al, 1988). Flessel et al (1993) reviewed five *in vivo* animal studies and found that exposure to TGM gave positive results for chromosomal damage whereas pure Malathion is not known to produce chromosomal aberrations (Garry et al, 1990). Kasprzak (2002) reported that TGM is said to cause mutagenic and carcinogenic effect in time and dosage dependent manner.

Commercial grade formulations of Malathion are reported to contain about eleven chemicals that could be created during the production process, the major impurity being isomalathion and malaoxon which are said to decrease the LD_{50} of commercial formulations (Ryan and Fukoto, 1984). Impurities in commercial formulations of Malathion cause pronounced toxicity (Flessel et al, 1993).

Malathion and its impurities interact with DNA, mechanism of action unknown, causing mutagenicity. Imamura and Talcott (1985) demonstrated that Malathion and its impurities alkylate 4-*p*-nitrobenzylpyridine, a model nucleophile for alkylation reactions. Alkylating agents are known to cause DNA damage (Ferguson and Denny,1995). Electrophilic sites of Malathion makes it a mutagen capable of binding to nucleophilic sites in DNA. Genotoxic carcinogens are

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electrophiles or activated to electrophilic intermediates and bind to critical macromolecules (Schoental,1984). Genotoxicity leads to carcinogenicity and hereditary defects via germ cell defects, teratogenecity (Mitchelmore and Chipman, 1985).

Hematological and biochemical parameters alter in experimental animals exposed to OP pesticides (Kalender et al, 2005). Alteration in the hematological parameters such as reduced RBCs and haemoglobin (Hb) are seen due to OP exposure (Kalender et al, 2006). Baselt et al (1989) also report that TGM causes disturbance in functions of blood cells.

TGM brings about alteration in the immune system (Handy et al, 2002) and hematological system (Durak et al, 2009). Malathion brings about oxidative stress due to its effects on the defense enzymes such as superoxide dismutase, glutathione peroxidase causing lipid peroxidation all of which could contribute to genotoxicity and altered haematological indices (Rosenstock et al, 1990). Due to the above cited reasons utilization of an antigenotoxic agent could play a vital role in protecting from CGM.

Evaluation of an antioxidant that could attenuate the subchronic doses of CGM induced genotoxicity and toxicity on hematological system is required. Antioxidants help in counter acting the effects of free radicals (Foy et al, 1998). Ascorbic acid is a potent water soluble antioxidant that protects the cellular compartment from oxygen and nitrogen radicals (Jurczuk et al, 2007). Dietary ascorbic acid helps in reducing oxidative stress and could attenuate pesticide induced toxicity (Yavuz et al, 2004). Ascorbic acid appears to be a promising agent for protection against the CGM toxicity. In the present study effect of ascorbic acid was studied on CGM induced toxicity assessed by MN formation for genotoxicity and hematological indices in Swiss albino mice.

MATERIALS AND METHODS

Animals

Swiss albino mice, 8- 9 weeks' old, *Mus musculus* species, (weighing approximately 21 – 24 grams) obtained from Venkateshwar Enterprises, Laboratory animal suppliers, Bangalore, were used for the experiment. The animals were housed in plastic cages, fed with a standard laboratory diet and water ad libitum and maintained at a laboratory temperature of 28°C ± 2°C. All mice were handled in accordance with the standard guidelines for the care and use of laboratory animals as outlined in the Declaration of Helsinki and the study was conducted after obtaining ethical clearance.

Chemicals

Commercial grade 50% Malathion manufactured locally was obtained.

Ascorbic acid (L-ascorbic acid) was supplied by Himedia, Mumbai, India. All other chemicals used for the experiments were purchased from Merck or Hi Media and were of analytical grade.

Animal treatment schedule

A LD_{50} value for cumulative doses (14 days) of the CGM was calculated and was determined to be 1000 mg /Kg body weight (bw). Ascorbic acid was evaluated for its safety effects and a dose of 250 mg /Kg body weight was selected.

After an adaptation period of one week the mice were randomly divided into two groups, namely the control (n = 12) and the experiment groups (n = 24). Control group was randomly assigned as groundnut oil control and ascorbic acid control group with 6 animals each.

Control group was randomly assigned as groundnut oil control (Vehicle Control- VC) and ascorbic acid(AA) control groups with 6 animals each. VC received groundnut oil 2 ml/kg bw and AA group received ascorbic acid 250 mgs/Kg bw in water both for 14 days at 12 hours interval.

The mice in the experimental groups were divided into two groups, namely the CGM treated group (n = 12), and the CGM plus ascorbic acid group (n = 12). Each of these groups were divided into 2 subgroups with 6 animals each and were treated with 2 different doses of CGM.

Malathion-treated group

The test compound CGM was administered to the mice once a day in groundnut oil by oral gavage for 14 days at 24 hours interval. The first study sub group treated with 2 different doses of CGM alone was designated as Group 1a - 50% of LD_{50} and Group 2a - 5% of LD_{50}.

Malathion plus ascorbic acid treated group

The Malathion plus ascorbic acid treated group received ascorbic acid after one hour of CGM treatment for 14 days at 24 hours interval. The second sub groups were designated as Group 1b - 50 % of LD_{50} + ascorbic acid and Group 2b-5 % of LD_{50} + ascorbic acid.

Measurement of body weight

Measurement of body weight of the control and study mice were measured at the end of 14^{th} day with the help of a digital weighing balance.

Bone marrow micronucleus assay

After 24 hours of last treatment the animals were anesthetized and the bone marrow was aspirated from the femur and the tibia into one ml of 5 % bovine serum albumin in phosphate buffered saline (pH 7.2). The cells were centrifuged for 5 minutes at 1000 rpm, Smears were prepared from the pellet on clean glass slides and then stained with May-Grunwald's stain, followed by Giemsa stain. Smears were dried and analyzed under oil immersion using Olympus CH20*i* microscope at 100x magnification. Presence of MN was assessed in 2000 polychromatic erythrocytes (PCE) per

each animal (Schmid, 1975). The number of PCE having micronuclei (MNPCE) in the study group and control groups were recorded. Corresponding normochromic erythrocytes (NCE) to 2000 PCEs were also recorded and P/N (Polychromatic erythrocyte/Normochromatic erythrocyte) ratio was determined (Gonzalez Borroto et al, 2003).

Blood sampling and hematological assays

Blood was collected in tubes containing anticoagulant (EDTA) at the end of 14th day from the retro orbital plexus and tail vein of each animal 24 hours after the last treatment. Hematological parameters were analyzed with the freshly collected blood using Sysmex KX21 analyzer. Parameters studied were RBC count, haemoglobin, hematocrit, WBC Count and platelet count.

Reticulocyte count

Reticulocyte count was evaluated following procedure of Brecher (1949) where new methylene blue was used for vital staining of the reticulocytes. Equal volume of blood and new methylene blue was mixed and incubated for 10 minutes. This allows the RNA to precipitate following this smear was prepared and the reticulocytes were counted using a light microscope.

Statistical analysis

The mean ± SD were determined for each study group. Data were analyzed by one-way ANOVA and Tukey multiple comparison procedure to calculate the significance. p <0.05 value between study groups was taken as statistically significant. Data analyzing for one group, before and after treatment, was done using Student's t-test.

RESULTS

No compound- related mortality occurred during the study as the doses of CGM and Ascorbic acid selected were non-toxic to the mice. No significant difference in any of the study parameters were observed between study groups and ascorbic acid treated groups (p value >0.001).

Statistical comparison was made between control (VC) and ascorbic acid treated groups and VC and groups 1a and 2a, between groups a and b and between group b and VC whenever required for data interpretation.

Evaluation of body weight

The change in body weight in the experimental animals after CGM and ascorbic acid treatment is presented in Table 1. Body weight of both groups 1 and 2 animals showed a significant decrease when compared to control groups (p value < 0.001). Ascorbic acid showed a restoration of decreased body weight both in groups 1 and 2 (p values 1b vs 1a and 2b vs 2a < 0.001). The effects of ascorbic acid was seen better in 5 % LD_{50} treated animals (p value VC vs 1b < 0.001 and VC vs 2b >0.05).

Changes in Micronucleus formation

Genotoxic effect of CGM and the protective effect of ascorbic acid were evaluated by detection of the micronucleated cells in the proliferating haematopoietic cells in the bone marrow by scoring 2000 PCE / animal.

The results of micronucleus assay are presented in Table 2. The MN induced after administration of CGM and ascorbic acid in PCE and the PCE: NCE ratio were statistically analyzed. The frequencies of the MN formed in 50% LD_{50} animals were high (p value VC vs 1a < 0.001). MN was found to be higher in number on 5 % LD_{50} exposure as well but lower than that the micronucleus formed during 50% LD_{50} (p value VC vs 2a < 0.01). The results suggest that the genotoxic effect of CGM is dependent on the dosage of CGM.

The ratio between PCE and NCE is reported in Table 2. The ratio is a useful index that reveals the genotoxicity of the test compound in the bone marrow cells. A significant decrease in PCE: NCE (treated vs control) gives an evidence of erythropoietic depression and would indicate a direct effect on production of erythrocytes in the bone marrow. An increase in the ratio of PCE/NCE would indicate greater production of erythrocytes, possibly due to loss of mature erythrocytes. The mean values of PCE: NCE ratio decreased significantly (p<0.001) when treated with 50% LD_{50} and also 5% LD_{50}. A significant increase in the frequency of MNPCEs was associated with a significant decrease of the PCE: NCE ratio in group 1a as compared to the vehicle control.

The treatment of mice with ascorbic acid did not have any effect on the MNPCE induced by 50% CGM treatment but arrested 5% LD_{50} induced MNPCE (VC vs 2a p value < 0.01 whereas VC vs 2b p value > 0.05). Ascorbic acid also arrested the CGM induced decline in the PCE: NCE ratio in 5% LD_{50} treated groups (VC vs 2a p value < 0.001 whereas VC vs 2b p value > 0.05).

Changes in hematological parameters

Results of the alterations in hematological parameters are shown in Table 3. Significant decrease was observed in RBC, hematocrit, thrombocyte count at the end of CGM treatment in group 1a (p value <0.001). In group 2a only Hb values were found to be decreased (p value <0.001) but the decrease was lesser compared to group 1a. WBCs and reticulocytes increased significantly in group 1a, and in group 2a there was increase in reticulocyte count (p value <0.001).

Ascorbic acid was found to increase the RBC levels in group 1a suggesting its ameliorating role (group 1a vs 1b , p value <0.05). Ascorbic acid ameliorated the decreased Hb levels in group 2a (VC vs 2a, p value <0.001), but VC vs group 2b (p value >0.05, not significant). WBC levels which were increased in group 1a was ameliorated by ascorbic acid (p value 1a vs 1b<0.001) and ascorbic acid also ameliorated the WBC levels in 2b (p value VC vs 2a <0.001 and VC vs 2b>0.05

Table 1: Depicting change in body weight of Swiss albino mice with ascorbic acid on CGM treatment (Mean ± SD)

Groups	Body weight(g) Before Treatment	Body weight(g) After Treatment
VC	22.8 ±1.8	23.3 ±1.6
Ascorbic Acid	22.6 ± 1.6	23.6 ±1.0
Group 1a	22.6 ± 1.5	***14.5 ±1.5
Group 2a	22.8 ± 0.9	***14.5 ±1.3
Group 1b	21.5 ± 1.3	$^{+++,***}$18.8 ±1.1
Group 2b	23.1 ± 1.1	$^{+++}$22.3 ±1.2

n= 6; *Comparison with control; +Comparison of vitamin C treated groups with CGM treated group; $^{+++}$ or ***p value < 0.001; Tukey Kramer multiple comparisons test

Table 2: Effect of CGM and Ascorbic acid for 14 days on bone marrow MNPCEs and on the

Parameters	VC	Ascorbic Acid(AA)	50% LD_{50} CGM	5% LD_{50} CGM	50% LD_{50} CGM + AA	5% LD_{50} CGM + AA
MN (Per 2,000 PCE's)	3.66±1.03	3±0.89	21.3±4.17***	9±1.26**	20.5±3.6	6±0.63
PCE/NCE Ratio	1.11±0.05	1.18±0.11	0.79±0.03***	0.89±0.05***	0.78±0.02	1.12±0.04

n = 6; p value ***< 0.001, **< 0.01 compared to VC; Tukey Kramer multiple comparisons test

ns). The reticulocytes that were increased due to both 50% and 5% LD_{50} CGM exposure were ameliorated by ascorbic acid (p value 1a vs 1b<0.001, 2a vs 2b<0.01). But in group 1b the reticulocyte count differed significantly when compared to normal suggesting that ascorbic acid could not ameliorate the effect of 50% LD_{50} CGM so as to normalize the values.

DISCUSSION

CGM due to its impurities can be a potent mutagenic and genotoxic agent. Indiscriminate exposure to this pesticide may increase the risk of cancer. The results of the present work revealed that CGM induces increase in MN frequency, alters hematological parameters even at doses far below toxic levels and the treatment of ascorbic acid along with CGM shows protection against anemia, increased WBC and genotoxicity. Ascorbic acid plays a protective role against damage induced by CGM.

Use of pesticides in agriculture continues though they cause environmental pollution that leads to health hazards (Abdollahi et al, 2004). Many of these pesticides can be mutagenic and are linked to cancer (Zeljezic and Garaj-Vrhovac, 2004). Therefore agents that can protect against the effect of pesticides are required.

CGM exposure induced decreased body weight which can be due to decreased food consumption or fluid and electrolyte imbalance as a consequence of reduction of cholinesterase activity (Kalender et al, 2006).

Dose dependent increase in frequency of MNPCE has been reported by others who have studied the effects of different technical grade pesticides on bone marrow cells (Salah et al, 1998). Micronucleus test is a useful method for screening chemical clastogens *in vivo* (Schmid, 1975). This test observes whether the test compound can damage or inhibit the mitotic apparatus. Micronuclei are formed when the chromosome fragments or whole chromosomes lag behind during cell division. Dermal exposure to single dose of TGM causes increased frequency of MN (Dulout et al, 1982). Dose dependent increase of MNPCE during intraperitoneal exposure to OP pesticide has been demonstrated by Cicchetti et al (1999). Genotoxic effects of CGM can be attributed to Malathion acids, the hydrolytic products of Malathion produced during metabolism, these acids being alkylating in nature, alkylate DNA causing damage (El-Dib et al, 1996). Malathion and its impurities interact with the DNA causing mutagenicity (Wild, 1975). Malathion and its impurities are electrophilic capable of binding to nucleophilic sites in DNA. Malathion, has two potential electrophilic sites—the alkyl group(s) and the phosphoryl group (Schoental, 1984). Malathion can induce cell cycle delay and increase in the frequencies of sister chromatid exchanges leading to genotoxicity (Chen et al, 1981).

Generation of free radicals by Malathion contribute to its genotoxicity (Possamai et al, 2005). Impurities in Malathion bring about an increase in the generation of reactive oxygen species (ROS) by changing the antioxidant defenses such as superoxide dismutase (SOD), glutathione peroxidase and increased lipid peroxidation. ROS cause oxidative DNA damage which is an early cause for carcinogenicity (Rosenstock et al, 1990).

CGM induced decrease in RBC, Hb and hematocrit that lead to anemia may be due to decreased rate in erythropoiesis along with increased destruction of erythrocytes. Free radicals produced by OPs interfere with the biosynthesis of Hb and also shorten the life cycle of circulating RBCs (Patil et al, 2003). Enhanced MDA levels in the erythrocytes lead to their intravascular

Table 3: Effect of CGM and ascorbic acid for 14 days on hematological parameters (Mean ±SD)

Parameters	VC	Ascorbic Acid (AA)	50% LD_{50} CGM	5% LD_{50} CGM	50% LD_{50} CGM + AA	5% LD_{50} CGM + AA
RBC (millions/cumm)	8.15±0.36	8.0±0.15	***6.38±0.51	7.5±0.4	6.8±0.4	8.08±0.22
Hemoglobin(g/dl)	13.5±1.1	13.81±0.7	***9.2±0.7	***11.35±0.5	10.21±0.6	12.6±0.7
Hematocrit (%)	39.4±4.9	38±5.8	***28.35±2.68	34.5±2.6	31.8±3.1	38.1±2.4
WBC (10^3 mm^{-3})	4.9±0.5	5.3±0.4	***14.6±0.8	7.2±1.2	$^{+++}$10.4±1	5.9±1.0
Thrombocytes (lakhs/cumm)	4.0±1.0	4.2±0.8	***0.9±0.5	5.6±0.4	1.24±0.4	$^{+}$4.12±1.4
Reticulocyte count (%)	2.28±0.19	2.4±0.11	***8.41±0.2	***3.4±0.3	$^{+++}$7.75±0.4	$^{+}$2.81±0.31

n = 6; *Comparison with control; +Comparison of ascorbic acid treated groups with CGM treated group; + or *p < 0.05, ++ or **p < 0.01 and +++ or ***p < 0.001; Tukey Kramer multiple comparisons test

haemolysis causing anaemia (Gabbianelli et al, 2002). Increased WBC count seen in 50% LD_{50} treatment could be attributed to the effects of OPs on causing bone marrow abnormalities and their effects on immune cells in the bone marrow due to free radical challenge (Kalender et al, 2006). The increase in the leukocytes indicates an alteration in immune system, tissue damage and necrosis caused by pesticide induced oxidative stress (Halliwell and Gutteridge, 1999). Lipophilic nature of OPs (Grajeda-Cota et al, 2004) allows it an easy entry into the cell membrane which may also contribute to alteration in hematological parameters during CGM treatment. Neurotoxic effects of OPs greatly influence the immune system. Esterases, such as AchE, are vital membrane bound proteins that aid the immune system to interact with and destroy foreign organisms (Pruett et al, 1992). Increase in WBC along with decrease in RBC suggests that bone marrow is not completely depressed (Kalendhar et al, 2005). Garg et al (2004) report decreased total leucocytes due to OP exposure. CGM induced a dose dependent decrease in the platelet count. Similar results are observed by Köprücü et al (2006). CGM bought about a dose dependent increase in the reticulocyte count. Reticulocyte production increases in response to loss of RBCs. Decreased reticulocyte count indicates that the RBCs are being destroyed prematurely (Campbell, 2005). CGM induced anemia may be responsible for increased reticulocyte count.

Antioxidants help in counter acting the effects of free radicals. Vitamin, ascorbic acid is an exogenous chain-breaking antioxidant which inhibits free-radical-mediated chain reactions (Foy et al, 1998) and is a part of the redox buffer system scavenging harmful free radical metabolites / ROS (Sato et al, 1990). Ascorbic acid inhibits cell and DNA damage induced by ROS, inhibits the oxidative metabolism of compounds and prevents the production of mutagenic electrophilic metabolites (Goncharova and Kuzhir, 1989). This prevents attack on nucleophilic sites on DNA and blocks adduct formation and decreases genotoxicity (Liehr et al, 1989)

Ascorbic acid is present in the extracellular matrix defending the cells from the free radicals by not allowing them to pass across the membrane to interact with extracellular components. Free radicals damage the membrane first causing lipid peroxidation of the membrane, this could be one of the mechanisms involved in attenuation of the altered hematological parameters and genotoxicity due to CGM exposure (Verma et al, 2007). Dietary ascorbic acid helps in reducing oxidative stress by restoring levels of SOD and CAT (Rana et al, 2010). Ascorbic acid was found to increase the platelet count in 5% LD_{50} CGM treated animals. The effect of ascorbic acid on WBC and platelet formation may be due to its free radical scavenging activity. It was observed that ascorbic acid is beneficial in attenuating the MN induced by the lower dose of CGM. Ascorbic acid at an appropriate dose may be able to attenuate the genotoxic effects of the CGM.

The selected dosage of ascorbic acid treatment showed no effect on the decreased RBC, Hb or hematocrit but ascorbic acid could significantly decrease the WBC count in 50% LD_{50} treated animals however not to normal levels. Our study reveals that ascorbic acid reduces genotoxicity and altered hematological indices, however the dosage of ascorbic acid has to be standardized for efficient attenuation.

CONCLUSION

Evaluation of an antioxidant that could attenuate the effects due to the sub chronic doses of CGM induced genotoxicity and altered hematological indices are required. Thus a study to evaluate the ameliorating effect of Ascorbic acid on the above mentioned CGM induced toxicity was of our interest. Sufficient supplementation with vitamins by those who regularly come in contact with pesticides is beneficial in combating the adverse effects. As the use of antioxidants to treat oxidative stress continues, our observation is that ascorbic acid can be used to attenuate CGM genotoxicity and toxicity on hematological parameters. Further studies to determine the effective dose and to duplicate the effects of ascorbic acid in CGM exposed humans needs to be performed.

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