ORIGINAL ARTICLE

Modulatory Effect of L-Ascorbic Acid against Methyl Parathion Induced Mutation in *Salmonella enterica typhimurium* TA 100

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ABSTRACT

Aim: In view of many reports on the potential mutagenicity of methyl parathion, an organophosphate insecticide, the possible antimutagenic activity of L-ascorbic acid was evaluated by employing the Ames test. *Salmonella enterica typhimurium* TA 100 strain representing base-pair substitution was taken as the tester strain. **Material and Method:** Three concentrations of L-ascorbic acid, viz., 5, 10, and 20 mg/plate were selected against methyl parathion (0.5 mg/plate). Experiments were carried out for the antimutagenicity before and after metabolic activation of MP by incubating it with mouse liver homogenate (S9 fraction). **Observations and Discussion:** L-ascorbic acid significantly reduced the revertant colonies at its lower ($p<0.05$), moderate and highest concentrations ($p<0.001$). The magnitude of the protective effect was found to be higher in the case of methyl parathion after its metabolic activation ($p<0.05$). **Conclusion:** L-ascorbic acid is regarded as a potential antioxidant. Methyl parathion induced base-pair substitution can significantly be minimized with L-ascorbic acid.

Keywords: Methyl parathion, L-ascorbic acid, Free radicals, Antioxidant, Ames test, S9 fraction

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1. INTRODUCTION

In modern agriculture system, usage of pesticides became inevitable to control insect pests. Pesticides are considered as one of the highly polluting agents and widespread exposures to pesticides through contaminated air, water, and food lead to various adverse effects on non-target organisms. Methyl parathion, an organophosphate insecticide is used to control pests such as aphids, boll weevils, and mites that harm the commercial crops including cotton, soybeans, wheat, alfalfa, rice, lettuce, onion, sugarbeets, and artichokes[1]. Despite this insecticide has been restricted to certain commercial crops, its availability with low price and effectiveness made its utility in household control of insect pests as well[1]. World Health Organization classified it as Extremely Hazardous (Ia) and as per Rotterdam Convention, it is included under severely hazardous[2]. There are many epidemiological reports on MP exposures and health hazards[3]. The pharmacokinetics of MP exposed via dermal[4], inhalation and oral[5] have been studied in animal models. Earlier reports on vitro[6] and in vivo[7] indicate the mutagenic potency of MP. There are also some studies for the carcinogenicity of MP[8].

L-ascorbic acid, a water-soluble vitamin has an important nutritional role. Its potential antioxidant property has been exploited to overcome the free radical-mediated toxicities of certain agents[9]. Modulatory effect of L-ascorbic acid against methyl parathion induced toxicity in the small intestine of Swiss albino mice[10]; spermatotoxicity, hepatotoxicity[11]; DNA damage in human lymphocyte[6]; oxidative stress-mediated liver injury[12] have been reported. The protective effect of L-ascorbic acid against MP-induced DNA damage/genotoxicity has been reported in earlier studies[13].

Literature survey revealed that there are no reports for the possible modulatory effect of L-ascorbic acid against MP-

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induced using bacterial reverse mutation assay. Therefore, the present study was aimed at evaluating the modulatory effect of L-ascorbic acid against MP-induced base-pair substitution using *Salmonella enterica typhimurium* TA 100 by conducting Ames test with or without metabolic activation of the insecticide.

2. MATERIALS AND METHODS

2.1. Chemicals

Metacid-50 (CAS No. 268-00-0), manufactured by Bayers India Ltd., Mumbai, and L-ascorbic acid (CAS No.50-81-7), purchased from Central Drug House (P) Ltd., Mumbai (Batch No.10812), were used for the experiment. Sodium azide (NaN_3; CAS No. 26628-22-8) procured from Hi Media Laboratories, Pvt. Ltd., Mumbai, India was used as the positive mutagen. All other chemicals were procured from Merck, SRL, and Himedia, India.

2.2. Preparation of Mouse Liver Microsomal Enzymes (S9 Fraction)

A healthy mouse (Swiss albino mice: *Mus musculus*) was treated orally with phenobarbital (0.1%) for seven days. On the 6th day the animal was starved and sacrificed by cervical dislocation on the seventh day. The liver excised was washed with 0.1 M KCl and homogenized. The homogenate was centrifuged at 9000 x g for 10 minutes to extract out the supernatant and stored at -80 °C[14].

2.3. Bacterial Strains

Salmonella entrica typhimurium strains (TA100; MTCC Acc. No. 1252) were procured from Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology (IMTECH) Council of Scientific and Industrial Research (CSIR), Chandigarh, India. The strain was retrieved by culturing in Vogel-Bonner (VB) salt solution.

2.4. Strain Identification and Confirmation Assay

Analyses of the genetic integrity and the rate of spontaneous mutation is essential whenever Ames test is performed. A nutrient broth with overnight cultures is essential for performing the strain integrity assay[15].

Histidine Requirement: A loopful of overnight culture of *Salmonella enterica typhimurium* strain was streaked across the minimal glucose agar plate (GM agar plate) enriched with an excess of biotin and plates were incubated for 48 h at 37 °C.

Rfa Mutation: A loopful of overnight culture was streaked across the GM agar plates that was supplemented with an excess of biotin and histidine. A sterile filter paper dipped in 10 µl of 0.1% crystal violet solution was placed at the centre of the streak and incubated at 37 °C for 18 h.

UVrB Mutation: A loopful of inoculum was streaked over the GM agar plate. One half of the culture plate was covered with an aluminium foil and then exposed to the UV radiation for 8 to 10 seconds. The plate was incubated at 37 °C for 18 h.

2.5. Bacterial Mutagenicity Assay

The test for the mutagenicity and antimutagenicity was conducted by following the pre-incubation assay[16]. The mutagenicity and antimutagenicity of L-ascorbic acid was assessed at the concentration of 5, 10 and 20 mg/plate against the single dose of 0.5 mg/plate of methyl parathion. 0.05 ml of metabolic activation mix, 0.05 ml of the test chemical dilution (MP and L-ascorbic acid) and 0.1 ml of the overnight culture of *Salmonella* strains (TA100) were added to sterile glass tubes. 2 ml of molten top agar maintained at 43 °C to 48 °C was poured over the agar plates. Once the contents in the plates were hardened it was incubated at 37 °C for 48 hours, in an inverted position. The assay was carried out in triplicates, in the presence and absence of the metabolic fraction. The revertant colonies per plate were counted after 48 hours of incubation for all the doses. The number of colonies were expressed in the terms of percentage and calculated using the following formula:

Rate of antimutagenicity = [(M-S1)/(M-S0)] × 100, M being the number of revertant/plate induced by mutagen, S0 the number of spontaneous revertants, S1 being the number of revertants/plate in the presence of the extract and the positive mutagen[17].

2.6. Statistical Analysis

The mean ± standard deviation, where n = 3 were analysed for statistical significance. One-way ANOVA was used to assess the statistical difference with the significance criterion set at $p<0.05$.

3. RESULTS AND DISCUSSION

The absence of the culture growth after incubation in the media enriched with an excess of biotin shows that the strains are histidine dependent too and hence it can be confirmed that the tester strains are *Salmonella enterica typhimurium* TA100. The inhibitory zone surrounding the disc dipped in crystal violet indicates Rfa mutation. Rfa mutation leads to lessening of the lipopolysaccharide barriers and increased cell permeability. The absence of growth in the radiated region indicates uvrB mutation. Thus, the assays confirmed the presence of *Salmonella enterica typhimurium* TA100 strains.

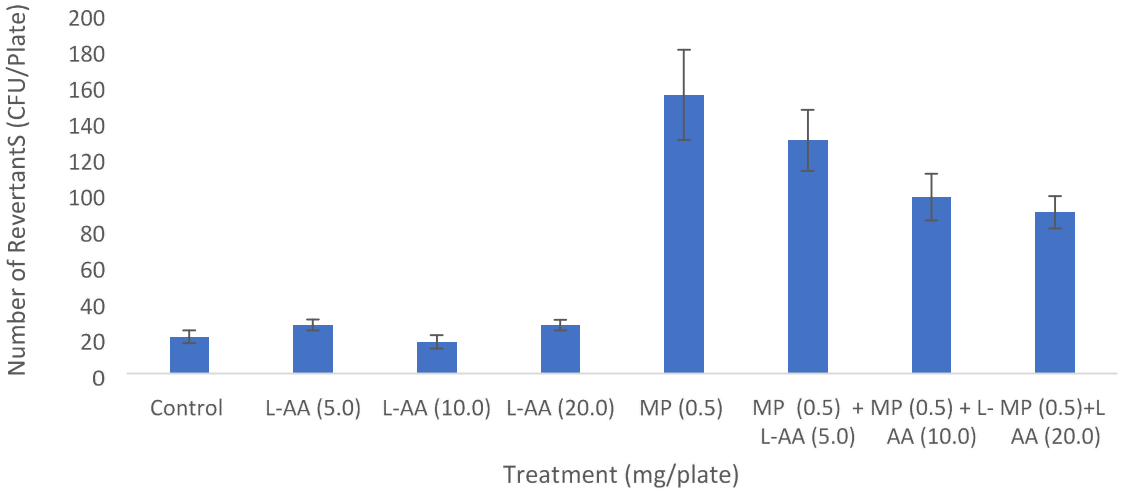
The results of the antimutagenic activity of L-ascorbic acid against MP are presented in the Table 1 and Figures 1 and 2.

Table 1: Results of the Antimutagenic Activities of L-Ascorbic Acid Against Methyl Parathion Without (-S9) and with Metabolic Activation (+S9)

Test Agents (mg/Plate)	*S. typhimurium* TA100 (- S9)		*S. typhimurium* TA100 (+S9)	
	Revertants (CFU/Plate) Mean±SD	Inhibition Rate (%)	Revertant (CFU/Plate) Mean±SD	Inhibition Rate (%)
10% DMSO	47.6±13.7	-	51.9±11.9	-
NaN_3 – 1.0	412.7±35.6*	-	569.5±41.3*	-
L-AA (5.0)	26.9 ±3.08	-	17.6 ±3.25	-
L-AA (10.0)	17.5 ±3.62	-	20.6±3.42	-
L-AA (20.0)	26.8±2.94	-	29.9±4.27	-
MP (0.5)	154.76±24.7[a]	-	257.6±35.41[b]	-
MP (0.5)+L-AA (5.0)	129.4±17.25*	16.88	193.6±26.24*	24.91
MP (0.5)+L-AA(10.0)	97.9±13.07***	36.36	121.0±19.32***	52.92
MP (0.5)+L-AA(20.0)	89.5± 9.29***	41.93	89.8±9.28***	64.98

Note: [a]p<0.01; [b]p<0.001 (MP Vs Solvent control); *p<0.05; **p<0.001 (MP (0.5) Vs MP co-treated with L-AA); CFU: Colony forming unit.

Figure 1: Concentration-Yield Effect of L-Ascorbic Acid Against MP-Induced Mutagenicity in the Absence of S9 Fraction



The selected concentrations of L Ascorbic acid did not show any increase in the number of revertant colonies both in the presence or absence of the S9 fraction, compared to the solvent control. It indicates that it is not mutagenic up to 20.0 mg/plate in the *Salmonella enterica typhimurium* TA 100. Taking into consideration the observations of the assays carried out for the assessment of the mutagenicity of L-ascorbic acid, its concentrations of 5.0, 10.0, and 20.0 mg/plate was used to assess its antimutagenicity against MP.

The concentration-dependent effect of MP and its lethality at higher concentrations were reported in previous report[18]. The mutagenic potency of the selected concentrations of MP (0.5 mg/plate) was evident by a significant increase in the number of CFU compared to the solvent-treated plate ($p<0.001$). Thus, the mutagenicity of MP in terms of base-pair substitution was confirmed. Further, MP was found to be capable of imparting base-pair substitution with or without S9 fraction. However, the observed effect was more in the presence of the S9 fraction compared to that of the culture plate inoculated with the MP without being treated with the S9 fraction ($p<0.05$). This indicates that the metabolism of MP released the products that further enhanced its mutagenic potency.

A comparison between MP (0.5 mg/plate) and co-treatment with L-AA showed a significant reduction in the number of revertant colonies compared to the MP alone inoculated culture

Figure 2: Concentration-Yield Effect of L-Ascorbic Acid Against MP-Induced Mutagenicity in the Presence of S9 Fraction

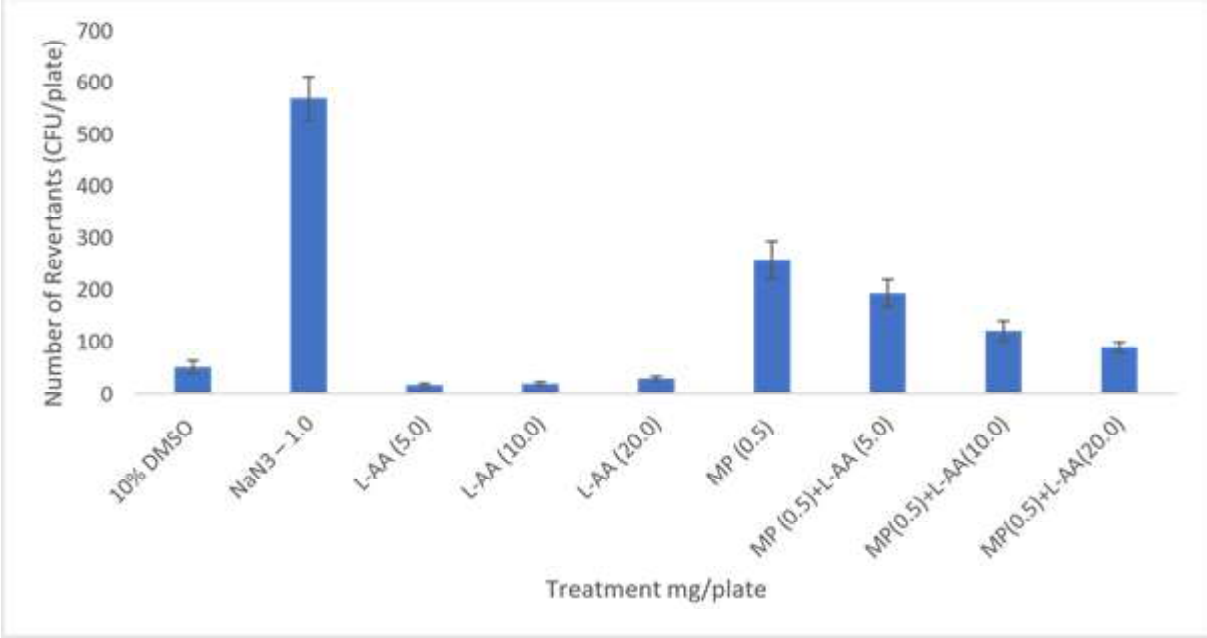


plate. The combined treatment significantly reduced the number of revertant colonies; L-AA at its lower concentration (5.0 mg/plate) reduced the number of colonies at a significant level ($p<0.05$). At a higher concentration of L-AA (10.0 mg/plate), the reduction rate was significantly enhanced up to 36.36% ($p<0.001$). Though the highest concentration of L-AA reduced the number of colonies compared to the MP alone treated plates, the rate of reduction was almost similar to that of the higher concentration ($p<0.001$). Thus, it can be understood that L-AA at its 10.0 mg/plate was the threshold concentration to yield the maximum antimutagenic activity against MP. A similar trend was also observed in the Ames test with the S9 fraction. The percentage increase in the inhibitory effect in the case of the S9 fraction compared to that of the culture plate without being added with the S9 fraction indicated that L-AA imparted antimutagenic activity with higher potency after metabolic activation of MP.

The exact mechanism(s) underlying the inhibitory effect of L-AA against MP induced mutagenicity is not fully understood. However, the antimutagenic potential of AA against different chemicals such as organophosphorus pesticides, including MP, has been demonstrated in many in vitro and in vivo systems[13, 6]. A number of diverse mechanisms of genoprotective action of AA against wide variety of chemicals have been suggested[19]. It has been advocated that AA exerts its effects through multiple inhibition mechanisms[20].

MP-induced mutagenic effects may be due to the higher alkylating potential of methyl esters. MP contains the phosphorothioate (P = S), which is oxidized to oxon (P = O), methyl-paraoxon, by liver microsome oxidase in the liver. The oxons are highly toxic compounds, which account for the profound cytotoxic effect of organophosphorus pesticides[21]. The heritable changes in the structure of DNA induced by treatment with electrophilic mutagens, such as powerful alkylating agents, are a direct consequence of the primary interaction between the electrophilic centres of the mutagens and the nucleophilic centres in the DNA[6]. Nucleophilic substitutions constitute the primary lesions resulting ultimately in cytotoxic or genotoxic effects[22]. Reports indicate that the alkylation reaction is directly related to the electron affinity of the substituents and therefore the reactivation of MP can be explained by methylation reactions of the phosphate esters. This is in accordance with the explanation given by Klopman *et al.*[23] that relevant structural features are responsible for the activity or inactivity in each category of the genotoxic effects of the pesticides. Under physiological conditions AA effectively competes with cellular nucleophilic sites, such as DNA, RNA, or proteins, thus masking these sites from binding by the mutagen and this mechanism has been proposed by Aidoo *et al.*[24] for ethylnitrosourea (ENU), where they suggested that the antimutagenic action of AA against ENU may be related to its ability to block the covalent binding of ENU to cellular DNA. This aspect of AA activity has been demonstrated in other studies where the vitamin prevented or decreased the binding of carcinogens to macromolecules including DNA[25]. The results of this type of work have been used to explain L-AA antimutagenicity against some indirect-acting mutagens

that are converted into electrophilic species against direct-acting mutagens[26]. Thus, the antimutagenicity of AA against MP can be related to its ability to block MP and/or its metabolites from covalent binding to cellular DNA as indicated from the present study. The pesticide undergoes a series of enzyme-mediated biotransformation in the liver. Therefore, one of the bases for mutation, it is these biotransformed products act as potent mutagens. Hence, the enhanced reduction in the mutagenicity of MP in Ames test in case of S9 fraction may be due to the L-AA-mediated hydroxylation of lipid/cholesterol, thus concomitantly minimizing the changes in the formation of mutagenic conjugates of the pesticide with them.

The mechanism of genoprotective effect of AA can also be justified in conjunction with the generation of free radicals/reactive oxygen species (ROS) from MP. The toxic manifestations induced by organophosphate insecticides are associated with enhanced production of ROS.

Among ROS, superoxide anion, hydroxyl radical and hydrogen peroxide enhance the oxidative process and induce peroxidative damage of cell membranes[27]. It is well-established fact that hydroxy radicals are potent mutagens. L-AA, since it is hydrophilic in nature, it is the most important free-radical scavenger in extracellular fluids, trapping the radicals in aqueous phase and protecting the biomolecules, including DNA[28].

Probably, the enhanced protective effect of L-AA as observed in the present study may be due to the scavenging of free radicals generated from MP during its metabolic activation. Thus, whatever the mode(s) of action of L-AA in minimizing the mutagenicity of the pesticide (MP), its protective role appears to be evident enough. Based on this, it can therefore be suggested that vitamin C is a good supplement to avoid/minimize the potential health risks due to exposure to toxic agents such as MP.

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