Minimization of Cytogenetic Toxicity of Malathion by Vitamin C

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Summary Genotoxic effect of agriculture-used concentration of organophosphorous pesticide, Malathion, is decreased by the dietary concentration of sodium salt of L-ascorbic acid for parameters like mitotic-index and clastogeny in onion root-tip cells, clastogeny and meiotic-index in mice, and lethal mutation rate in Drosophila melanogaster. The vitamin itself is not genotoxic, and its concurrent administration is more effective than pretreatment with it. Possible mechanism of such vitamin C-mediated minimization of pesticide-genotoxicity is discussed.

Key Words Malathion, ascorbic acid, genotoxicity, mitotic-index, meiotic-index, clastogenicity, mutagenicity

With the large-scale proliferation of chemical mutagens in our environment, the genetic load of non-target populations is bound to increase. Sincere efforts, therefore, were made to minimize the genotoxicity of these xenobiotics. The use of antioxidant-like vitamin C (ascorbic acid) has given some positive results in this direction, and information in this regard started trickling in about a decade ago when Pauling (1) noted that vitamin C is capable of converting cancerous cells into “normal” ones. In subsequent years, the similar role of this vitamin was observed on neuroblastoma cells in culture (2), or on retardation of pace of growth in human tumor cells (3). Minimization of toxicity of methylmercury chloride (4), PCB (5), diaziquone (6) and many other substances by vitamin C is well documented.

Studies related to the antigenotoxic effect of this vitamin against pesticides are few (7–9) as compared to those against other genotoxicants like aflatoxin (10, 11), cyclophosphamide (12), etc. It is, therefore, desirable that the so-called protective effect of this vitamin be checked in a wide variety of test-systems using more parameters (13). The present work is an endeavor in this direction. Here the antigenotoxic effects of vitamin C have been studied over pesticide-induced mitotic and meiotic inhibitions, chromosome clastogeny, and lethal mutation rate in plant, Drosophila, and mammalian systems.
MATERIALS AND METHODS

Pesticide. Organophosphorous pesticide Malathion (O-O-dimethyl phosphorodithioate of diethyl mercaptosuccinate, Cyanamid India), widely utilized in Afro-Asian countries for control of aphids, bugs, scales, moths, and similar sucking insect pests in fruits, cereals, corns, leguminous crops, cotton, vegetables, etc. (14), was used. The generally recommended dose for its use (depending upon pest and plant) ranges widely from 0.2 to 1.5 liters of 20 to 30% pesticide solution per hectare (15). The doses/concentration(s), therefore, chosen for the present work were in close proximity to those recommended for field use. The mode of administration, and doses used, are given in Table 1.

Vitamin. The source of vitamin C was synthetic injectable preparation of the sodium salt of l-ascorbic acid (Rosch India Ltd.) with the tradename "Redoxon." The dose of the vitamin used in the present study was proportional to the human dose (500 mg/day), and its mode of administration is given in Table 1. The vitamin was administered concurrently with the pesticide (M + VC) and also as a pre-treatment (VC → M) before exposure to the pesticide.

Test-systems. i) Onion root-tip cells: Mitotic-index and frequencies of structural changes in mitotic chromosomes as well as of mitotic disruptions were studied. Onion bulbs were grown over jars in five groups, each containing five bulbs. When healthy young roots could come out to a length of about 1 cm, the bulbs were subjected to two different treatments each of 24-h duration (Table 2). After termination of the treatment, three roots per bulb were cut, fixed in acetomethanol (1:3) for 24 h and then preserved in 70% ethanol. Acetocarmine-stained squash preparations were made and the slides were screened for counting the number of dividing cells and locating abnormalities in them. The randomness in counting was achieved by following the method suggested by Bhalla et al. (16).

Table 1. Dose, concentration, and mode of administration of Malathion and vitamin in different test-systems.

| Test-systems | Dose/concentration | Mode of administration       |
|--------------|---------------------|------------------------------|
| 1. Onion root-tip cells: Malathion | 50, 100, 200, 400 and 800 ppm | Bulbs grown on water solution |
| Vitamin C    | 0.005%              |                              |
| 2. Mice:     |                     |                              |
| Malathion    | 0.2 μg/kg body wt./day | By intubation                |
| Vitamin C    | 0.25 ml (1%)/day    | By intraperitoneal injection |
| 3. Drosophila: |                    |                              |
| Malathion    | 0.006%              | Mixed in food medium         |
| Vitamin C    | 0.005%              |                              |

J. Nutr. Sci. Vitaminol.
Table 2. Mode of treatment with vitamins and pesticides.

| Initial material | 1st treatment (24h) | 2nd treatment (24h) | Abbreviated in the text as |
|------------------|---------------------|---------------------|----------------------------|
| 5 groups (I–V) of bulbs grown on tap water | Group I to IV on fresh tap water | I. Tap water | Control (C) |
|                  |                     | II. Vitamin C       | VC                         |
|                  |                     | III. Malathion      | M                          |
|                  |                     | IV. Malathion+vitamin C | M+VC                     |
|                  |                     | V. Malathion        | VC→M                      |
| Group V on vitamin C |                   |                     |                            |

**ii) Mice:** Seven- to eight-week-old laboratory inbred Swiss albino mice (of both sexes 1:1), *Mus musculus* (parent stock supplied by CDRI, Lucknow) were sorted into five groups, each with $n=10$. They were subjected to treatment for 10 days to obtain VC, M, and M+VC group of animals. The VC→M group of mice were kept for 10 days on vitamin-supplemented food prior to being subjected to the pesticide treatment for the next 10 days. The control group of animals received neither vitamin nor pesticide. On the 11th day, the animals were sacrificed. Extraction of bone marrow, its pretreatment with sodium citrate, incubation, centrifugation, flame-drying, and staining with Giemsa were performed by following the method suggested by Kilian et al. (17). Screening of chromosomal abnormalities, if any, was performed in 300 well-spread metaphase plates selected randomly at the rate of 30 metaphases per animal per group of the experiment by following the scoring protocol suggested by Preston et al. (18).

For studying the effects on meiotic-index in testes cells, the methodology of Adler and El-Tarras (19) was followed. The mice were subjected to different treatments in the manner similar to that followed for mitotic-studies in bone marrow preparations.

**iii) Drosophila:** The laboratory inbred Oregon-R strain of *Drosophila melanogaster* was used. 36±12-h-old male flies were chosen in five groups for this purpose. One group was kept on vitamin-supplemented food for 5 days and the remaining four groups on normal food. Thereafter, the VC, M, and M+VC groups were obtained by maintaining the flies for 5 days on food supplemented with vitamin C, Malathion, and Malathion+vitamin C respectively. On the following day, virgin females were put with these treated males @1 male : 2 female per vial for 48 h for mating and laying of eggs. Thereafter the same flies were transferred to fresh sets of vials for another 48 h. In this manner the $M_1$ generation was obtained in five 2-day egg-laying broods. The total number of eggs laid in each brood, and the larvae hatched therefrom were counted. The loss of eggs, *i.e.*, those from which larva could not come out even after 48 h of their being laid, was deemed to be due to the incidence of dominant lethal mutation in the zygote/developing embryo (20). The results were expressed in the form of relative mortality rate (RMR):
The Muller-5 (Bw<sup>sc3</sup>) method was used for the detection of recessive lethal mutations in the X-chromosome of males.

RESULTS

Effects on mitotic-index (MI)

Significant decreases in MI were noted (Fig. 1) in pesticide-treated bulbs, and the mitosis completely halted at 800 ppm dose. The vitamin alone did not produce any appreciable (significant) change in MI. When pesticide and vitamin were administered concurrently, decreases in MI were still observed, but with lesser intensity. Pretreatment with the vitamin also could produce significant protective effect though only against the two lower doses (50 and 100 ppm) of the pesticide.

Mitotic chromosome clastogeny

i) In onion root-meristem cells. Pesticide-induced abnormalities were broadly put into two categories: a) chromosome/chromatid breaks and their subsequent rearrangements manifested as fragmentation, laggard, clumping, and anaphase bridges; and b) mitosis-disrupting types, such as multipolar spindles, polyploidy, disturbed cytokinesis, multinucleated cells, and precocious separation of sister

![Graph](image-url)
Table 3. Abnormality rate (%±SE) cumulatively for all concentrations of pesticide and vitamin administration in root-tip cells of onion.

| Variants   | Total   | Break-related | Mitosis disruptive |
|------------|---------|---------------|-------------------|
| Control    | nil     | nil           | nil               |
| M          | 4.68±0.91| 4.11±0.85     | 0.57±0.32         |
| M + VC     | 1.56±0.37***| 1.32±0.34*** | 0.24±0.14         |
| VC→M#      | 2.52±0.60*  | 2.52±0.60     | nil               |

* and *** indicate significant difference at 5 and 0.1% levels from the corresponding value in the M-variant. # The difference between M+VC and VC→M groups of animals was not significant.

chromatids. The incidence of abnormalities significantly increased upon pesticide treatment: nil in the control, 2% at 50 ppm, and 5.5% at 100 ppm dose; at the two higher concentrations, the dose-dependence was not noticed (Fig. 1). Upon concurrent administration of the vitamin and the pesticide, no abnormality was seen at the 50 ppm (lowest) dose of the pesticide; and at the next two higher doses, significant decreases in the abnormality frequency were observed. The pretreatment with the vitamin also showed some protective effect though only against the lower doses of the pesticide.

In order to understand the relative minimization rate of break-related as well as mitosis-disruptive abnormalities by the vitamin, the sum total of the incidence of these abnormalities at all the doses of the various treatment and control groups were compared (Table 3). It appears that most (around 80%) of the abnormalities caused by the pesticide were break-related, and very few were of the mitosis-disruptive type. The vitamin-mediated (concurrent or pretreatment) decrease in abnormality frequency was mainly due to a decrease in the incidence of break-related damages. These results are suggestive of an anticlastogenic nature of vitamin C.

ii) Bone marrow cells of mice. The salient features of the result obtained are: (a) the vitamin alone did not cause any significant (even at the 5% level) increase over the spontaneous rate of abnormal metaphases; (b) the pesticide significantly (at the 0.1% level) increased (almost four times) the abnormality incidence; and (c) the concurrent administration of the vitamin was more effective than its pretreatment in minimizing (significant at the 1% level) the pesticide-induced damages. The frequency of total abnormalities on many occasions was found to be higher than that of the corresponding abnormal cells (Table 4). This was due to the fact that more than one type of abnormality were sometimes coincidental in the same cell. The most common combinations appear to be those of chromatid breaks or gaps with acentric fragments, ring chromosomes, and metacentric chromosomes. Needless to say that all these are pre- or post-restitutional manifestations of the chromosome/chromatid breaks themselves. The
Table 4. Rate (%±SE) of incidence of chromosome/chromatid abnormalities in bone marrow cells of pesticide-fed mice subjected to vitamin C administration.

| Experiment groups | Abnormal metaphases (No.) %±SE | Abnormality types | Individual (No.) %±SE | Mitosis disrupting (No.) %±SE | Total (No.) %±SE |
|-------------------|---------------------------------|-------------------|------------------------|-------------------------------|-----------------|
| Control           | 5.33±1.3 (16)                  |                   | 4.00±1.1 (12)          | 1.33±0.7 (04)                | 5.33±1.3 (16)   |
| VC                | 6.33±1.4 (19)                  |                   | 5.00±1.3 (15)          | 1.33±0.7 (04)                | 6.33±1.4 (19)   |
| M                 | 21.33±2.4 (64)                 |                   | 18.66±2.2 (56)         | 7.33±1.6 (22)                | 26.00±2.5 (78)  |
| M+VC              | 9.66±1.7 (29)                  |                   | 8.00±1.6 (24)          | 3.66±1.1 (11)                | 11.66±1.9 (35)  |
| VC→M              | 19.00±2.2 (57)                 |                   | 16.33±2.1 (49)         | 7.66±1.5 (23)                | 24.00±2.4 (72)  |

No. of animals and metaphases screened in each experiment is 10 and 300 respectively. a,b,c, and d indicate significant difference with the corresponding value in the control, VC, M, and M+VC experiment groups at 5% (*), 1% (**), and 0.1% (***) levels respectively.

Table 5. Meiotic-index (MeI) in primary spermatocytes of mice and the phase-distribution of dividing cells.

| Experimental groups | MeI %±SE | Prophase I %±SE | Diakinesis-metaphase-I %±SE | Ana-telophase-I %±SE |
|---------------------|----------|-----------------|-----------------------------|-----------------------|
| Control             | 60.77±0.49 | 58.64±0.49 | 1.27±0.11                   | 0.85±0.09             |
| VC                  | 58.86±0.43 | 56.32±0.51 | 1.48±0.16                   | 1.06±0.08             |
| M                   | 54.90±0.53 | 50.98±0.54_b | 3.13±0.18_b                 | 0.78±0.09_b           |
| M+VC                | 67.12±0.49 | 64.26±0.50_b,c | 2.17±0.15_b,c             | 0.68±0.08_b           |
| VC→M                | 61.09±0.48 | 57.23±0.48 | 2.65±0.15_a,b              | 1.20±0.16_c           |

a,b,c indicate significant difference (at the 5% level) with the corresponding value in the control, vitamin, and Malathion-treated groups respectively.

mitosis-disrupting abnormalities were comparatively less frequent in the control as well as in the treated groups of animals. The vitamin could minimize both types of abnormalities with almost uniform magnitude.

Meiotic index

The pesticide caused a minor, though significant, decrease in the meiotic-index among the primary spermatocytes of mice (54.90%, in comparison to 60.77% in the control) (Table 5). This decrease was mainly due to a significant decrease in the proportion of cells in prophase I (a slight increase in metaphase I frequency was J. Nutr. Sci. Vitaminol.
Fig. 2. Relative mortality rate (RMR) of eggs in the M₁ generation of males that had received Malathion treatment with various modes of Vitamin C administration.

induced by the pesticide). The vitamin C annulled this minimization by increasing the frequency of cells in prophase I. Concurrent administration of the vitamin led to an increase in this index to an extent which was more than that in the control (67.12%). In animals pretreated with the vitamin, the meiotic index was equal to the control value. Vitamin C itself could not bring any significant change (from the control).

**Drosophila**

1) Dominant lethal mutation rate. The pesticide increased the hatching rate (decreased RMR) among the eggs laid during the 5th to the 8th day (Fig. 2), but decreased the same during the last brood (9–10th day). No significant change in the hatchability rate was observed during the first 4 days (broods 1 and 2). According to the chronological history of spermatogenesis in Drosophila (21–23), the crop of gametes belonging to the spermatozoa stage at the time of treatment are used during the first 4 days of mating, those to the meiotically dividing spermatocyte stage during the 5th to the 8th day, and those of the premeiotic stages of the spermatogenic cycle during the 9th and beyond days. It can therefore be inferred that the pesticide could not affect the chromosomes present in spermatozoa head where the genetic material remains in very compact state due to its association with
protamines. In primary spermatocytes, the chromosomes, being in an active state of division, are much more prone to clastogenic and other genotoxic effects. Among the pre-meiotic cells, some improvement was noted. As the gonial cells keep on dividing (gonial mitosis), more damages ought to have occurred in them. But it has also been found, specially in a heterogeneous cell population like this (spermatogenic), that many of the sensitive cells which get damaged and destroyed ("apoptosis" of Kondo (24)) are replaced by the compensatory division in the remaining resistant cells ("germinal selection" of Auerbach (25). The net effect is an improvement in the total number of healthy spermatozoa, giving more opportunities for fertilization and embryonic development. Vitamin C alone did not affect (in comparison to the control group) significantly the pattern of egg hatchibility, but it certainly improved it (egg hatchibility) among the meiotic (spermatocytes) and the pre-meiotic (gonial) cells of the pesticide-fed groups. The cumulative result (of all the five broods) also indicate the protective effect of vitamin C against the genotoxic nature of the pesticide (Fig. 2).

The vitamin also decreased the induction rate of pesticide-induced X-chromosome-linked recessive lethal mutations (Table 6). Note that this is a highly objective test and highly reliable.

**DISCUSSION**

The results suggest that the mitotic and meiotic inhibitions as well as the clastogeny and mutations induced by the pesticide can be appreciably minimized by vitamin C. The concurrent treatment with the vitamin appears to be more effective than pretreatment of organisms with it. Pesticide-mediated alkylation of not only guanine but also of thymine, cytosine, and uracil (26) and subsequent base-replacements is perhaps the most common mechanism of mutagenesis in microorganisms, cells in culture, or in experiments carried out in vitro, where virtually unmodified pesticide molecules are available for causing the damage. But among higher organisms, like *Drosophila* or laboratory mammals, the pesticides undergo a series of enzyme-mediated biotransformations in the gastrointestinal tract, liver/
hepatic caecae, etc. Therefore, for a mutation to take place, it is these biotransformed forms that have to be mutagenic. According to Hutson (27), the fats and lipids present in the body/cells combine with pesticide metabolites to produce a conjugate type of chemical, and it is believed that these conjugates have the real mutagenic nature. Vitamin C is known to stimulate 7-α-hydroxylation of lipid and cholesterol nuclei thus enhancing their degradation to bile acids for their being excreted out of the body (28–31). Hence the antimutagenic, anticlastogenic, and anticytotoxic effects of vitamin C, as observed in the present case, may be due to this vitamin C-mediated hydroxylation of the lipids/cholesterol, thus concomitantly minimizing the chances of the formation of mutagenic conjugates of pesticide with them.

According to Tazima (32), scavenging, metabolic modification, and detoxification of the mutagen are three more efficient factors in altering the biological response of higher organisms to chemical mutagens. The hydroxylation of fats/lipids/cholesterol by vitamin C come under such general category of metabolic modification and detoxification. Selective cell-killing and elimination of damaged/dead cells followed by compensatory division may also play some role, but they may or may not be vitamin C-dependent.

Whatever the mechanism(s) of vitamin C-mediated antigenotoxicity be, it is sufficiently clear that vitamin C does produce some minimizing effect, and it can therefore be suggested that this vitamin be almost necessarily added to the diet of the populations which are unknowingly being chronically exposed to pesticides.

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