Modulatory Effect of Vitamin C on Genotoxic Effect of Endosulfan in Developing Albino Rats

SHIMOGA DURGOJIRAO MANJULA, SUSAN BENJAMIN and KURADY LAXMINARAYANA BAIRY

ABSTRACT

The genotoxic effect of endosulfan and the modulatory effect of vitamin C in growing albino rats were studied by bone marrow micronucleus assay. Seven days old male Wistar rats were treated with 3, 6, 9, 12 mg/kg Endosulfan orally (10 pups/group), for up to 60 days, at intervals of 24 h. For 2 more groups (n=10/group), Endosulfan 9 mg/kg and 12 mg/kg was administered along with vitamin C (20 mg/kg). One more group of rats were treated with cyclophosphamide as positive control. The genotoxic effect was studied by bone marrow micronuclei assay. There was an increase in micronuclei in polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) and a decrease in PCE and P/N ratio in endosulfan treated rats. The effect was similar to the standard mutagen cyclophosphamide. In rats treated with vitamin C and endosulfan, there was an increase in PCE and P/N ratio and decrease in micronuclei in PCE and NCE. This could be due to the antimitogenic effect of vitamin C.

Keywords: Endosulfan, Cyclophosphamide, Vitamin C, Bone marrow micronuclei, PCE, NCE

Pesticides are economically important chemicals and their use in agriculture has increased crop yields leading to a decrease in food costs. The exposure to these toxic chemicals may alter the genetic material of animals, which may affect them and their descendants. Endosulfan is a widely used insecticide belonging to the cyclodiene group of organochlorine pesticides. Endosulfan is highly toxic, regardless of route of its exposure [1], causing incoordination, imbalance, difficulty in breathing, gagging, vomiting, diarrhea, agitation, convulsions, loss of consciousness, and central nervous system disorders [2]. Chronic exposure results in liver enlargement, seizures, reduced growth and survival, changes in kidney structure and blood parameters. Endosulfan is known to get excreted from the mammary gland via the human breast milk in a comparatively higher rate than other pesticides [3].

Long-term exposure of endosulfan adversely affects the kidney, liver, blood cells and respiration. It also affects biochemical parameters by increasing glucose-6-phosphate dehydrogenase activity and increased blood glucose level, phospholipid contents of the microsomal and surfactant system, and profoundly increases the activity of alcohol dehydrogenase and cytosolic glutathione-s-transferase. It also causes immunologic toxicity by significant decrease in IgG, IgM and γ-globulin [3].

Vitamin C (L-ascorbic acid) is a bio-antimutagen [5,6]. Vitamin C prevents genetic damage caused by toxicants by several mechanisms and is known as an antimutagen which acts mainly by interfering with free radical generation and the formation of toxic metabolites [7].

Children are the least protected population from exposure to pesticides. Children, due to their small size, greater intake of air and food relative to body weight, developing organ systems and other unique characteristics are at a higher risk than adults to pesticides [8]. There are reports that endosulfan is genotoxic in adult rats [9]. But there are no reports of endosulfan in developing rats.

Hence, the present work was undertaken to study the genotoxic effect of endosulfan in prepubertal rats. The possible protective effects of vitamin C on toxicity of endosulfan are also evaluated. These findings will pave the way to its rational use in future. This study is expected to furnish further data on how endosulfan acts on genetic material on germ cell line.
**MATERIALS AND METHODS**

**Animals**

In the present study, growing male Wistar rats (one week old, weighing between 8-12 g) were divided into 10 groups.

Animals were housed in polypolyethylene cages with paddy husk bedding at 28±10°C temperature and 50±5% humidity. In each cage, a maximum of three animals were housed to avoid stress due to overcrowding. Animals were fed on laboratory rat feed (Gold Mohur; Lipton India Ltd.) and water ad libitum. The number of animals/group was eight and the treatment was given orally for 60 days. Animals were sacrificed by giving overdose of pentobarbitone. The study protocol was approved by the Institutional Ethical Committee.

**Chemicals and Treatment**

Endosulfan (Meerut Agro Chemicals Industries Ltd., Meerut, India), was used as the test drug. It was dissolved in groundnut oil and was given at four different doses - 3, 6, 9, and 12 mg/kg body weight orally. Endosulfan dose was selected based on earlier studies [6]. The maximum dose used in this study was half of the LD50 of endosulfan in rats (24-355 mg/kg body weight) [10]. Cyclophosphamide (Khandelwal Laboratories, Mumbai, India) was used as a positive control. Commercial grade of cyclophosphamide, which is available in powdered form was dissolved in water and given at a dose of 10 mg/kg. Vitamin C (Extra pure from Loba Chemicals (P) Ltd., Mumbai, India) was used as a test antimutagen and was dissolved in distilled water and given at a dose of 20 mg/kg.

- Group I, served as control and received saline.
- Group II, received vehicle (groundnut oil).
- Group III, was administered with Vitamin C (20 mg/kg body weight).
- Group IV, received 10 mg/kg dose of cyclophosphamide.
- Groups V-VIII, received 3, 6, 9 and 12 mg /kg doses of endosulfan respectively.
- Group IX and X, received endosulfan in doses of 9 and 12 mg /kg, respectively along with 20 mg/kg dose of Vitamin C.

All the agents were administered orally daily for 60 days. On the 70th day, the animals were sacrificed and bone marrow from all the animals was removed and processed for micronucleus analysis.

**Bone Marrow Micronucleus Assay**

This assay evaluates the genotoxicity of any mutagen. It has been accepted as a standard and a reliable in vivo assay to screen chemicals including drugs for their genotoxicity [10]. Schmid’s standard procedure [11] was followed with slight modification.

The animals were killed, lower abdomen and limbs were incised and the femora were cleaned and separated from the hip joint. The ends of the femur were trimmed and a blunt needle was pushed to pierce the marrow cavity. Bone marrow was flushed out into 0.9% saline. The suspension was made up to 5 mL in a centrifuge tube and centrifuged at 1000 rpm for 10 minutes. The clear supernatant was discarded, 2-3 drops of fetal calf serum were added and the pellet was mixed thoroughly. Smears were drawn on to precleaned coded slides using a drop of the suspension. The slides were air dried and fixed in absolute methanol. The slides were then stained with 0.125% acridine orange (BDH, UK, Gurr Cat. No 340019704644440E) in Sorensen’s buffer (pH 6.8). Then it was washed in Sorensen’s buffer and observed under fluorescent microscope. 2000 PCE and NCE each were counted per animal. Micro-nucleated PCE’s and NCE’s (MNPCE and MNCE) were counted for each animal.

**Statistical Analysis of Data**

Data obtained from all the above experiments were analysed by one way Analysis of Variance (ANOVA) followed by Bonferroni’s post test wherever applicable using statistical software package, InStat (Graph Pad) 1990: version 1.13. Values of p<0.05 were considered statistically significant.

**RESULT**

The present study showed that the vehicle and vitamin C did not significantly alter the PCE, NCE, MPCE, MNCE and P/N ratio when compared to control. Cyclophosphamide, the standard mutagen used in this study produced significant decrease in PCE and P/N ration and significant increase in NCE, MPCE and MNCE (p<0.05). Endosulfan (6-12 mg/kg) produced significant decrease in PCE and P/N ration compared to 9 mg endosulfan alone; *p<0.05, compared to control. *p<0.05, compared to 9 mg endosulfan alone; †p<0.05, compared to 12 mg endosulfan alone; All values are expressed as mean±SE (n=8).

<table>
<thead>
<tr>
<th>Groups</th>
<th>PCE (%)</th>
<th>NCE (%)</th>
<th>MPCE (%)</th>
<th>MNCE (%)</th>
<th>P/N Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.5 mL saline)</td>
<td>52.67±0.42</td>
<td>47.79±1.05</td>
<td>2.66±0.08</td>
<td>0.74±0.01</td>
<td>1.10±0.02</td>
</tr>
<tr>
<td>GN Oil</td>
<td>51.54±0.35</td>
<td>47.96±0.48</td>
<td>2.61±0.06</td>
<td>0.66±0.07</td>
<td>1.07±0.02</td>
</tr>
<tr>
<td>Vit C (20 mg/kg)</td>
<td>52.53±0.64</td>
<td>47.76±0.71</td>
<td>2.54±0.06</td>
<td>0.73±0.01</td>
<td>1.14±0.06</td>
</tr>
<tr>
<td>CP (10 mg/kg)</td>
<td>33.68±0.94*</td>
<td>66.31±0.94*</td>
<td>19.37±0.50*</td>
<td>4.26±0.18*</td>
<td>0.50±0.02*</td>
</tr>
<tr>
<td>END (3mg/kg)</td>
<td>61.60±0.79*</td>
<td>48.39±0.79*</td>
<td>2.53±0.15*</td>
<td>0.76±0.01</td>
<td>1.06±0.03</td>
</tr>
<tr>
<td>END (6 mg/kg)</td>
<td>45.57±0.40*</td>
<td>54.41±0.41*</td>
<td>4.33±0.11*</td>
<td>0.79±0.01*</td>
<td>0.83±0.01*</td>
</tr>
<tr>
<td>END (9 mg/kg)</td>
<td>41.58±0.87*</td>
<td>58.41±0.87*</td>
<td>8.31±0.26*</td>
<td>2.01±0.09*</td>
<td>0.70±0.02*</td>
</tr>
<tr>
<td>END (12 mg/kg)</td>
<td>38.51±0.81*</td>
<td>61.48±0.82*</td>
<td>15.68±0.81*</td>
<td>3.21±0.07*</td>
<td>0.61±0.02*</td>
</tr>
<tr>
<td>END+Vit C (9 mg/kg+20 mg/kg)</td>
<td>46.68±0.75*</td>
<td>53.32±0.75*</td>
<td>5.12±0.10*</td>
<td>1.21±0.07*</td>
<td>0.88±0.02*</td>
</tr>
<tr>
<td>END+Vit C (12 mg/kg+20 mg/kg)</td>
<td>42.31±0.03*</td>
<td>57.71±0.01*</td>
<td>11.37±0.53*</td>
<td>2.30±0.05*</td>
<td>0.73±0.03*</td>
</tr>
</tbody>
</table>

PCE=Polychromatic erythrocytes; NCE=normochromatic erythrocytes; MPCE= Micronucleated polychromatic erythrocytes MNCE= Micronucleated normochromatic erythrocytes; GN Oil= Groundnut oil; Vit C= Vitamin C; CP=Cyclophosphamide; End 3 to E 12=Endosulfan in different dosages; *p<0.05, compared to control. †p<0.05, compared to 9 mg endosulfan alone; ††p<0.05, compared to 12 mg endosulfan alone; All values are expressed as mean±SE (n=8).
increase in MPCE and MNCE in rat bone marrow and decrease in PCE and P/N ratio. Endosulfan in the entire dose range tested increased NCE when compared to control. On administration of vitamin C along with endosulfan, significant increase in the levels of PCE and P/N ratio and decrease in NCE, MPCE and MNCE were observed compared to rats treated with endosulfan alone (Table 1).

**DISCUSSION**

Micronuclei assay is an important tool to represent the consequence of chromosomal aberrations induced during the preceding mitotic divisions in erythroblasts [12]. In the present study, the result obtained revealed the genotoxic effect of endosulfan in developing rats. The maximum frequency of micronuclei was observed at 12 mg/kg dose of endosulfan. Significant micronuclei were observed in PCE for all doses and in NCE at higher doses. This confirms the clastogenic potential of endosulfan [13]. In an earlier study, the genotoxicity of endosulfan in adult rats has been reported where an increase in frequency of micronuclei was observed [14]. The observed effect may be due to interaction of endosulfan with chromosomes by attacking nucleic sites on their DNA [14]. This indicates that endosulfan induces cytogenetic effects in growing rats. There is a difference between frequency of chromosomal abnormalities in bone marrow cells of adult and prepubertal rats. The maximum tolerated dose in adult is was 3 mg/kg, whereas maximum tolerated dose in prepubertal rats was 12 mg/kg. The frequency of chromosomal abnormalities in adult rats treated with 3 mg/kg of endosulfan was similar to the one produced by 9 mg/kg of endosulfan in prepubertal rats [9]. This shows that developing rats can tolerate endosulfan better than adult rats. Kiran and Verma reported that the toxic effects of endosulfan are age-dependent. They observed that maximum hyperglycemia, maximum depletion of liver glycogen and maximum inhibition of brain acetylcholine esterase activity were observed in 365-day-old (adult) animals whereas these changes were found to be negligible in 15-day-old animals when endosulfan (12.5 mg/kg body weight), was administered daily, for 4 days in female rats of 4 different age groups [15].

The frequency of PCEs observed in the bone marrow provides an index of mitotic activity [13]. The PCE % declined with increasing dose, suggesting the suppression of proliferative activity of the bone marrow (or suppression of erythrocyte production), which in turn indicates the toxic effect of endosulfan. The decreased PCE percentage also indicates the extent of cell death. The formation of micronuclei indicates that a considerable amount of genetic information is no longer available to the cell, which would result in the cell death [16].

An increased frequency of micronucleated cells reflects the damage by agents which, cause chromosome breakage or non-disjunction. Since both these effects have been correlated with tumor initiation, the micronucleus test is appropriate for the screening of potentially genotoxic environmental agents [13]. The P/N ratio showed a significant and dose dependant decline upon endosulfan administration suggesting the cytotoxic effect of endosulfan. The exact mechanism of action of endosulfan in inducing genotoxicity is not known. Attention is increasingly being focused upon electrophilic reactivity as the fundamental cause of the toxicity of many cytoxic compounds.

In endosulfan treated animals, the magnitude of response was similar to cyclophosphamide, a positive mutagen used in the present study, which is a well known genotoxic agent. Cyclophosphamide induces micronucleus formation and chromosomal aberrations both *in vivo* and *in vitro* [17, 18, 19].

In the present study, vitamin C along with endosulfan produced significant increase in the levels of PCE and P/N ratio and decrease in NCE, MPCE and MNCE compared to endosulfan 9 and 12 mg/kg alone (*p*<0.05). This shows that administration of vitamin C with endosulfan can significantly decrease the extent of cytogenetic damage induced by endosulfan.

The observed protective effect may be due to the antioxidant property of vitamin C. It has been reported to modify response to oxygen radicals and known to be an excellent antioxidant [20, 21] along with its nucleophilic character [22]. Its anti mutagenic property therefore might be due to its antagonistic action at all probable levels of pesticide genotoxicity [23]. It might also be due to detoxification or incapacitation of the toxin molecules via chemical processes mediated by various detoxification enzymes [24] and elimination of abnormal cells by apoptosis [25]. The antimutagenic effect of vitamin C observed in the present study is in full conformity with clinical observations where individuals with a high vitamin C status when exposed to environmental toxins recovered faster [26].

It is clear from the present study that endosulfan at different doses produced significant increase in MPCE (%) and MNCE (%) in rat bone marrow and the magnitude of the response was similar to cyclophosphamide, a positive mutagen used in the present study, which is a well-known genotoxic agent. Vitamin C along with endosulfan produced significant increase in the levels of PCE and P/N ratio and decrease in NCE, MPCE and MNCE. This shows that administration of vitamin C with endosulfan can significantly decrease the extent of cytogenetic damage induced by endosulfan. Vitamin C can modify response to oxygen radicals and is known to be an excellent antioxidant and its observed protective effect is probably due to its antioxidant property.

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