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IN-VITRO CARBOFURAN INDUCED MICRONUCLEUS FORMATION IN HUMAN BLOOD LYMPHOCYTES

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Abstract

The farmers in general get exposed to different chemicals including pesticides. Many of these compounds are capable of inducing mutations in DNA and lead to several diseases including cancer. Carbofuran is a broad spectrum pesticide and frequently used in agricultural practices in India. In this study we intended to evaluate DNA damage inflicted by pesticide exposure in human blood lymphocytes under *in vitro* condition. The lymphocytes were exposed to varying concentrations of carbofuran (0-50μM) and analyzed by means of the micronucleus (MN) test. The results obtained showed significant increase in MN frequency after exposure to 5, 10, 25 and 50μM of carbofuran as compared to the control group. The frequencies of MN were observed to be in concentration dependent manner. As we further increase the concentration of carbofuran, we observed significant decrease in the mean percentage of binucleated cells (70-49%) and increase in the number of micronuclei formed per 1000 binucleated cells. Simultaneously, we also observed reduction in Cytokinesis-Block Proliferation index (CBPI) with increase in the carbofuran concentrations. The results indicate that this pesticide may exhibit genotoxic effect at higher concentrations. This study emphasizes the need to reinforce the good practices campaigns in order to enlighten those who work with pesticides and also to make them aware about the importance of using protective measures.

Key words: Carbofuran, DNA damage, Micronucleus assay, Human blood lymphocytes, cytokinesis block proliferation index (CBPI).

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INTRODUCTION

Carbofuran is a pesticide often used in agriculture practices in the country with an unintended human exposure (1). Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol methyl carbamate), a broad-spectrum pesticide, is commonly used in agricultural activities. The chemical structure of this molecule is shown in Figure 1. It is known as a systemic insecticide, acaricide and nematicide with a broad spectrum of activity. It is extensively used for the control of all types of stem borers in rice, sugarcane, fruit, and vegetables. Carbofuran is lipophilic in nature and its chronic exposure is reported to be responsible for oxidative injury leading to perturbations in membrane structure and functions (10, 13, 15).

Micronuclei (MN) are acentric chromosome fragments or whole chromosomes that are left behind during mitotic cellular division and appear in the cytoplasm of interphasic cells as small additional nuclei. The cytokinesis-block micronucleus (CBMN) assay (4), employing cytochalasin-B (Cyt-B) to block cytokinesis and identify cells that have divided once in vitro by their binucleated appearance, has made the identification of MN a useful tool for assessing genetic damage (3).

The toxicity of pesticides (organocarbamate) has been an important research line in our laboratory for many years. The evidence that commonly used pesticides represent a potential hazard to humans and to nature, together with an increasing environmental occurrence of these chemicals, has made the study of their genotoxicity a field of immediate concern. In this sense we have recently published genotoxic properties of carbofuran and their mitigation by vit C and E using comet assay (14).

MN provides a measure of both chromosome breakage and chromosome loss and it has been shown to be as sensitive an indicator of chromosome damage as classical metaphase chromosome analysis. The key advantage of the MN assay is the relative ease of scoring and the statistical power obtained from scoring larger numbers of cells than are typically used for metaphase analysis (6). Keeping these facts in perspective, the present study has been undertaken to evaluate pesticide induced cytogenetic alterations by different concentrations of carbofuran in human lymphocytes using the cytokinesis-block micronucleus (CBMN) assay.

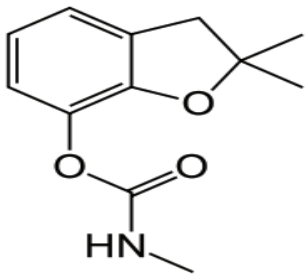

Figure 1. Chemical structure of carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol methyl carbamate).

MATERIALS AND METHODS

Chemicals

Carbofuran (99.5% pure) was kindly gifted by Rallis India Ltd. Dimethyl sulphoxide (DMSO) and cytochalesin B were purchased from Sigma. Phytohaemagglutinin (PHA), RPMI 1640 growth medium and foetal calf serum were purchased from Gibco BRL. Other chemicals required in

this experiment were purchased locally.

Subject and sample collection

Venous blood was taken from each of 4 healthy, male donors using sterile heparinized tubes from 24-28 year old non-smoking healthy four male donors, not exposed to radiation, drugs or any antioxidant supplementation in the recent past.

Whole blood lymphocytes culture and pesticide treatment

Carbofuran was dissolved in DMSO, to give final concentrations of 1.25, 2.5, 5, 10, 25 and 50µM, were added 41 h after the initiation of the cultures. The doses of carbofuran were chosen by taking into account the cell survival and the cytotoxicity found in human blood lymphocytes and previous experiments conducted with this pesticide in our laboratory (data not shown).

Whole blood cultures were set up by inoculating 1 ml of whole blood in 9 ml of culture medium [RPMI 1640 medium supplemented with 16% heat-inactivated fetal calf serum, antibiotics (penicillin and streptomycin) and glutamine (all obtained from Gibco)]. Immediately after this, 0.3 ml of phytohemagglutinin (PHA) was added to stimulate each culture (0h). After treatment, cells were incubated at 37^0C in a 5% CO_2 incubator for 72h and, at 41h from the start, various concentrations of carbofuran dissolved in DMSO, were diluted in culture medium such that addition of 100µl to cultures would achieve the desired pesticide concentration. The final solvent concentration is 0.1% for all treatments. Carbofuran was used at final concentrations of 1.25, 2.5, 5, 10, 25 and 50µM. For each donor 4 negative controls (two untreated and two vehicle treated cultures) were performed and run simultaneously with 12 carbofuran treated cultures. None of the treatments produced significant pH change in the culture medium, even at highest concentration of carbofuran tested i.e. 50µM and at 44h from the start, cytochalasin-B (Cyt-B) at a final concentration of 6mg/ml was added to arrest cytokinesis. This concentration of Cyt-B was selected because it gives a higher percentage of binucleated cells and a lower baseline MN frequency (16). Harvesting was performed at 72h from the start. Each culture was transferred to a correspondingly labeled Universal tube and centrifuged at 1500 rpm for 10 min. The supernatant was discarded and the pellets resuspended in RPMI and then, a mild hypotonic treatment (2–3 min in 0.075M KCl at room temperature) was carried out. Thereafter, the cells were centrifuged, and acetic acid/methanol (1:5, v/v) solution was gently added. This fixation step was repeated twice and the resulting cells were resuspended in a small volume of fixative solution (16). Air-dried preparations were made and the slides were stained with 10% Giemsa in Sorenson's buffer for 20 min (12).

The slides were stained with 10% Giemsa in Sorenson's buffer. The buffer-mounted slides were examined for the presence of micronuclei in the binucleated cells using a light microscope (Leica, Germany) at 1000X magnification. Four thousand binucleated cells from each concentration (1000 binucleated cells from each concentration per Individual) were scored. In the MN study, a minimum of 1000 binucleated cells from each concentration per individual were scored to evaluate the percentage of cells with 1, 2, 3, and 4 or more nuclei. A nuclear division index or Cytokinesis-Block Proliferation index (CBPI) was calculated as recommended in the OECD Guideline No. 487 (OECD, 2007). The cytokinesis block proliferation index (CBPI) was calculated as follows:

Cytokinesis Block Proliferation Index (CBPI) = (Number of mononucleated cells + 2 ×Number of binucleated cells + 3 ×Number of multinucleate cells)/Total Number of cells

Statistical Analysis

The statistical analysis of the MN data has been done using software PRISM 5.01. All values were expressed as mean ± SD .The results of each experiment were compared using One-way ANOVA followed by Dunnett's test for multiple pair wise comparisons between the various carbofuran concentration treated groups with control.

RESULTS

The results of treatment of human lymphocytes from four different donors with varying concentrations of carbofuran (0, 1.25, 2.5, 5, 10, 25 and 50 µM) are shown in Tables 1, 2, 3 and 4. Each table represents the effect of carbofuran on the lymphocytes from one individual. A human blood lymphocyte undergoing mitotic division *in vitro* is shown in Figure 1. The binucleated normal lymphocyte is shown without any presence of micronucleus. On the other hand, the binucleated and quadrinucleated human blood lymphocyte treated with carbofuran *in vitro* indicating appearance of single and double micronuclei, respectively, are shown in Figures 3 and 4. As shown in Tables 1, 2, 3 and 4, a dose-dependent increase in the mean frequency of binucleated cells containing micronuclei was observed in the lymphocytes. This increase was 12 (Table 1), 8 (Table 2), 11 (Table 3) and 9 (Table 4) fold higher in comparison with the negative control and was highly statistically significant.

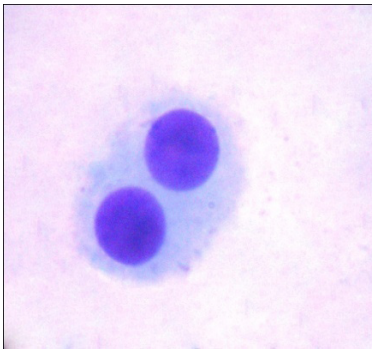

Figure 2. The human blood lymphocyte undergoing mitotic division in vitro. The binucleated normal lymphocyte is shown without any presence of micronucleus.

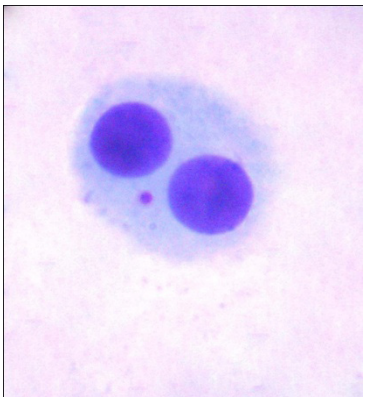

Figure 3. Binucleated human blood lymphocyte treated with carbofuran *in vitro* indicating appearance of a single micronucleus.

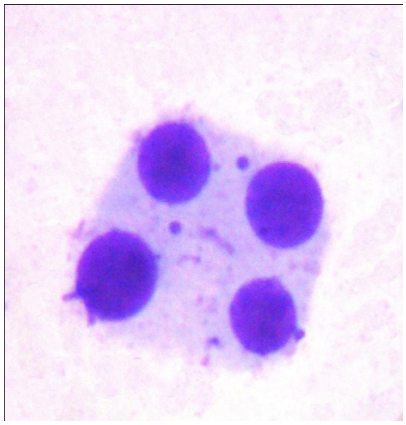

Figure 4. Quadrinucleated human blood lymphocyte treated with carbofuran *in vitro* indicating appearance of two micronuclei.

The results indicated that the formation of more than two nuclei (shown as B^+) in the lymphocytes from donors displayed biphasic response with respect to carbofuran concentration. Excepting the lymphocytes obtained from donor-1 (Table 1), the cells from other three donors (Tables 2 to 4) exhibited increase in the formation of more than two nuclei (B^+) with increase in the pesticide concentration up to 5µM; the values being 47, 47 and 51, respectively. The donor 1 (Table 1) registered formation of maximum number of nuclei (B^+) at 10µM.

However, further increase in the carbofuran concentration (10, 25 and 50 µM for donors 2, 3 and 4 and for donor 1, the concentrations were 25 and 50 µM) caused gradual decrease in the number of formation of more than two nuclei per cell (Tables 1-4). Since there was an increasing trend in the number of micronuclei formed in a cell in response to increasing concentration of carbofuran (0, 1.25, 2.5, 5.0, 10.0, 25.0 and 50µM) with concomitant decrease in the mean percentage of binucleated cells (Bi) for all the donors (70-49%), the percent values of MNCs / Bi also showed rising pattern (Tables 1-4). Because of the increase in the micronuclei formation with increase in the carbofuran concentration, the values as calculated from the data for Cytokinesis-Block Proliferation Index (CBPI) indicated a declining trend; the values being in the range of 1.7 -1.5 as against the carbofuran concentrations 0-50µM (Tables 1-4).

The statistical analysis of the data obtained from the four blood donors as displayed in Tables 1-4 is presented in Table 5. It is clear from this analysis that the number of micronuclei formed per 1000 binucleated cells increased from about 8 to 75 in response to the increasing concentrations of carbofuran i.e. 0 to 50 µM. The CBPI value was maximum (1.72±0.02) in absence of carbofuran,

Table 1. Effect of different concentrations of carbofuran on the extent of micronucleus (MN) formation in human blood lymphocytes from blood donor-1.

Carbofuran Dose (µM)	Mono	Bi	Bi+	Total	Bi (%)	Micronuclei	MNC/ Bi (%)	CBPI
0µM	479	1000	20	1499	66.71	5	0.07	1.69
1.25µM	505	1000	17	1522	65.70	11	0.16	1.67
2.5µM	543	1000	20	1563	63.97	17	0.26	1.66
5.0µM	651	1000	42	1693	59.06	31	0.52	1.64
10.0µM	725	1000	47	1772	56.43	49	0.86	1.61
25.0µM	885	1000	39	1924	51.97	55	1.05	1.56
50.0µM	985	1000	19	2004	49.90	62	1.24	1.51

Mono: mononucleated cells; Bi: binucleated cells; Bi+: cells with more than two nuclei; Bi (%): Percent frequency of binucleated cells; MNC/Bi(%):Percent frequency of binucleated cells containing micronuclei; CBPI: cytokinesis block Proliferation Index, the values calculated as shown in Materials and Methods.

Table 2. Effect of different concentrations of carbofuran on the extent of micronucleus (MN) formation in human blood lymphocytes from blood donor-2.

Carbofuran Dose (µM)	Mono	Bi	Bi+	Total	Bi (%)	Micronuclei	MNC/ Bi (%)	CBPI
0µM	407	1000	29	1436	69.63	9	0.12	1.73
1.25µM	527	1000	38	1565	63.89	12	0.18	1.68
2.5µM	556	1000	31	1587	63.01	19	0.30	1.66
5.0µM	620	1000	47	1667	59.98	30	0.50	1.65
10.0µM	810	1000	32	1842	54.28	37	0.68	1.57
25.0µM	735	1000	25	1760	56.81	59	1.03	1.59
50.0µM	923	1000	23	1946	51.38	73	1.42	1.53

Mono: mononucleated cells; Bi: binucleated cells; Bi+: cells with more than two nuclei; Bi (%): Percent frequency of binucleated cells; MNC/Bi (%):Percent frequency of binucleated cells containing micronuclei; CBPI: cytokinesis block Proliferation Index, the values calculated as shown in Materials and Methods.

Table 3. Effect of different concentrations of carbofuran on the extent of micronucleus (MN) formation in human blood lymphocytes from blood donor-3.

Carbofuran Dose (µM)	Mono	Bi	Bi+	Total	Bi (%)	Micronuclei	MNC/ Bi (%)	CBPI
0µM	405	1000	17	1422	70.32	7	0.09	1.72
1.25µM	507	1000	26	1533	65.23	10	0.15	1.68
2.5µM	545	1000	35	1580	63.29	21	0.33	1.67
5.0µM	660	1000	47	1707	58.58	36	0.61	1.64
10.0µM	723	1000	36	1759	56.85	56	0.98	1.60
25.0µM	853	1000	29	1882	53.13	71	1.33	1.56
50.0µM	905	1000	31	1936	51.65	78	1.51	1.54

Mono: mononucleated cells; Bi: binucleated cells; Bi+: cells with more than two nuclei; Bi (%): frequency of binucleated cells; MNC/Bi (%): frequency of binucleated cells containing micronuclei; CBPI: cytokinesis block Proliferation Index the values calculated as shown in Materials and Methods.

Table 4. Effect of different concentrations of carbofuran on the extent of micronucleus (MN) formation in human blood lymphocytes from blood donor-4.

Carbofuran Dose (µM)	Mono	Bi	Bi+	Total	Bi (%)	Micronuclei	MNC/ Bi (%)	CBPI
0µM	391	1000	27	1418	70.52	10	0.14	1.74
1.25µM	532	1000	33	1565	63.89	14	0.21	1.68
2.5µM	560	1000	39	1599	62.53	23	0.36	1.67
5.0µM	672	1000	51	1723	58.03	38	0.65	1.63
10.0µM	819	1000	34	1853	53.96	47	0.87	1.57
25.0µM	642	1000	19	1661	60.20	63	1.04	1.62
50.0µM	875	1000	21	1896	52.74	86	1.63	1.54

Mono: mononucleated cells; Bi: binucleated cells; Bi+: cells with more than two nuclei; Bi (%): Percent frequency of binucleated cells; MNC/Bi(%):Percent frequency of binucleated cells containing micronuclei; CBPI: cytokinesis block Proliferation Index, the values calculated as shown in Materials and Methods.

Table 5. Statistical analysis of the data obtained for the four blood donors as shown in the Tables 1, 2, 3 and 4.

Carbofuran (µM)	No. of micronuclei / 1000 binucleated cells	Cytokinesis block proliferation index (CBPI)
Control	7.7 ±1.1	1.72±0.02
1.25 µM	11.75±1.7ns	1.68±0.003 ns
2.50 µM	20.00±1.2*	1.67±0.005*
5.00 µM	33.75±1.9***	1.64±0.008 **
10.0 µM	47.25±3.9***	1.59±0.02***
25.0 µM	62.0±6.8***	1.58±0.03***
50.0 µM	74.75±5.2***	1.53±0.014***

All values were expressed as mean±SD of four independent experiments in duplicate for each carbofuran concentration per subject. The results of each experiment were compared using One-way ANOVA followed by Dunnett's test for multiple pair wise comparisons between the various carbofuran concentration treated groups with control. *, ** and *** represent the values significant at $p < 0.05$, $p < 0.01$and $p < 0.001$, respectively.

which gradually decreased to 1.68±0.003, 1.67±0.005, 1.64±0.008, 1.59±0.02, 1.58±0.03 and 1.53±0.014, respectively, at 1.25, 2.50, 5.0, 10.0, 25.0 and 50.0 µM carbofuran concentrations (Table 5).

DISCUSSION

There are some recently published reports showing carbofuran's genotoxicity (17, 18). It is one of the most toxic carbamate pesticides and known to exert high toxicity to mammalian systems (9). A case report shows genotoxic effect of acute carbofuran intoxication in humans as evidenced by Comet assay and micronucleus assay in the human blood samples showing significant DNA damage (17). The results from the present work have shown that carbofuran may produce a dose dependent increase in micronucleus formation in human lymphocytes when tested *in vitro*. The micronucleus index reduction at the 2.5,5,10,25 and 50µM doses compared with that of control could be due to the fact that higher necrosis was observed in this dose range. It may also be due to the response of the cells to the genotoxin causing necrosis as discussed by

Kirsch-Volders and Fenech (11). The necrosis observed at higher doses of carbofuran suggests that while apoptosis has an important role in the elimination of cells with DNA damage, the majority of cells were eliminated by necrosis in lymphocyte cultures. This has also been reported in an earlier study where hydrogen peroxide was used as the DNA-damaging agent (6, 7). This is an important parameter in understanding the toxicity of a compound and has been extensively reviewed (5, 11). This study has demonstrated that carbofuran may exert cytogenetic effects in human lymphocytes under *in vitro* conditions.

The mechanism of genotoxicity of this compound is not fully known and the results of some studies remain often inconclusive. Carbofuran treatment has also been reported to induce mitotic inhibition, chromosomal aberrations, micronucleus formation and sperm abnormality in a dose dependent manner in rats (2, 8).

Another possible mechanism responsible for the observed cytogenetic effects of carbofuran may involve the metabolites of carbofuran, which have been commonly detected in plasma.3-hydroxycarbofuran and 3-ketocarbofuran have been reported to be potentially genotoxic (18), which may greatly increase the cytotoxic and mutagenic activities of N-methyl carbamate pesticides during carbofuran metabolism in human lymphocytes. However, while studying the carbofuran induced genotoxic effects in epithelial cells across cryptvillus axis in rat intestine, some workers have suggested another possible mechanism responsible for the observed genotoxic effects of carbofuran (8). It may involve conversion of this compound to nitrosoamides in the stomach, because of the presence of nitrite in human gastric juice. N-nitrosation greatly increases the cytotoxic and mutagenic activities of N-methyl carbamate pesticides (8). The results obtained by incubating the human lymphocytes with carbofuran resulted into micronucleus formation, which is considered as a potential marker of DNA damage. The results of this study support the notion that carbofuran may cause cytogenetic effect on the human lymphocytes and hence may act as a potential causative agent of cancer at higher concentrations.

In conclusion, the present results indicate that carbofuran is capable of inducing DNA damage. The effect was biphasic and dose dependent. Carbofuran may cause cytogenetic effect on the human lymphocytes and possibly at higher concentrations may act as a potential factor of carcinogenesis. The lack of protective measures during activities with pesticides can be considered as risk factors due to exposure. This information may be used to guide public health laws and policies in the work place and residential communities in order to provide better health to occupationally exposed individuals to different pesticides.

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Other articles in this theme issue include references (19-46).

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