EFFICACY OF CHLOROPHYLLIN ON TOBACCO INDUCED CHROMOSOMAL ABERRATIONS IN RATS

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ABSTRACT

The present study has investigated the cytoprotective effects of chlorophyllin in tobacco induced chromosomal aberrations in bone marrow of rats. Chromosomal aberrations per cell and percent aberrant cells were observed to calculate genotoxic action of tobacco. Oral treatment with different doses of chln was able to reduce chromosomal aberrations significantly at the pre-treatment level and concurrent treatment level in tobacco treated animals. The present study thus demonstrates the cytoprotective role of Chlorophyllin in tobacco induced chromosomal aberrations in rats (Rattus rattus).

Keywords - Chromosomal Aberrations, Chlorophyllin, Chln, Rattus Rattus

I. INTRODUCTION

Tobacco was selected as there is growing public health concern because the habits associated with the use of tobacco are found globally and the genetic risk to the individuals for non-smoking tobacco was well established. Number of studies has characterized smokeless tobacco as an etiological factor in the development of cancer of the oral cavity [1] and the oesophagus [2]. Several studies have shown that prevalence of cancer is due to genetic damage in somatic cells. Agents that can alter organism‘s genome by causing toxic effect on cellular genetic materials are referred to as mutagenic. Such substances damage organisms DNA by induced mutations [3 and 4]. An abnormality in chromosome structure and chromosome number due to exposure of chemical or physical mutagens are referred to as chromosomal aberrations. Such chromosomal aberrations were seen in the cell populations of human and experimental cancer [4]. The rodent bone marrow has been used as a tool to evaluate genotoxicity of environmental mutagens and carcinogens in the present study because of low frequency of chromosomal aberrations in the control, a high proliferative activity of the cells and simple and rapid preparation of slides[3 and 5].

Smokeless tobacco includes both chewing tobacco and snuff [6]. The effect of water soluble tobacco smoke extract from filter and non-filter cigarette on seed germination of onion and tomato have been observed [7,8 and 9] and with increasing concentrations percent of mitotic changes increased and abnormalities like stickiness, lagging, breaks of chromosomes during metaphase and bridges were noticed. The effect of aqueous extract of a processed tobacco leaf on the roof meristems of *Allium cepa* showed that with higher concentrations of extract was highly mitostatic, lower concentration produced various type of chromosomal damage [10] .

Ethanolic extract of chewing tobacco induces mutations in *Salmonella typhimurium* and Chinese hamster V79 cells and also induces micronuclei in bone marrow cells of Swiss mice. Chromosomal and mitotic abnormalities resembled those prevalent in tumor cells, irradiated cells and chemically treated cells [11, 12 and 13].

Many antimutagenic or anticarcinogenic agents, which reduces genetic instability in somatic cells have been identified in fruits and vegetables, the most potent being the chlorophyllin, a derivative of the natural chlorophyll found in green vegetables, Chln is more stable form of chlorophyll [14] being widely used as food additive. Anticlastogenic activity of chlorophyllin correlates with the form and route of treatment, its mode of action indicates that when administered simultaneously with a mutagenic or carcinogenic agent, it is capable of reducing DNA-drug binding, thus acting as a desmutagenic compound or interceptor [15]. The protective effects of chlorophyllin have been described against chemical-benzopyrene, aflatoxin [16 and 17] and physical (e.g. γ-rays) [18 and 19]. The best results in animals at suppressing carcinogenesis were in the 2-4mg per kg range [20], the same as the dosage used in the human intervention trials [21]. No reports are available for cytoprotective role of Chlorophyllin on tobacco induced Chromosomal Aberrations in *Rattus rattus.* The present study was thus designed to evaluate the efficacy of chlorophyllin on tobacco induced Chromosomal aberrations in Rats.

II. MATERIALS AND METHODS

Animals: For the present investigations Albino rats *(Rattus rattus)* in the age group of 10-15 weeks and weighing 75-100 gm were used as test animals. The animals were housed as 6 animals per cage, in the animal house of Biosciences Department, H.P. University, Shimla, at the optimum temperature of 25±5°C. Special care was taken to use sanitary cages and bedding. They were given standard pellet diet (Hindustan Lever Ltd.) and water was given *ad libitum.*The local instituitional animal ethics committee, Himachal Pradesh University, Summer Hill,Shimla, India approved the experimental design. The material used for cytological study was bone-marrow in case of somatic tissue analysis. Chemicals:Tobacco(Panchi Brand Tobacco-Shiva tobacco Company, Novelty Road, Ambala city)

Preparation of various concentrations of Chemicals:For the chemical(tobacco) undertaken for the study stock solutions were prepared, from the stock solution, working solutions from time to time were prepared according to the requirements.

For the chemical (tobacco) undertaken for the study: a) Stock solutions**:** For tobacco 4 gms of Zarda was boiled in distilled water for 15 minutes, which was then filtered. The filtrate was made upto 100 ml to make it 4% solution. From this stock solution different concentrations of working solutions were made. They were kept in stoppered airtight, dark bottle and were marked S.S-1 of the respective chemical.

b) Working solutions**:** Frequently, a solution of desired volume and concentration of the test chemical was prepared from the respective stock solution by using the following mathematical equation:

$$N_1 V_1 = N_2 V_2 \quad (1)$$

Where, N_1 = concentration of the solution to be prepared.

V_1 = Volume of the solution to be prepared.

N_2 = concentration of the stock solution.

V_2 = Volume of the stock solution

c)Various concentrations of test chemicals taken for study:

For tobacco extract, working solutions of different concentrations as 0.5%, 1%, 1.5% and 2% were made from stock solution.

(b)Chlorophyllin:

The chemical was purchased from Sigma Chemical Company USA. Stock solution of chlorophyllin was prepared and was stored in refrigerator but the solution was prepared fresh after every 3-4 days to avoid any contamination and to ensure their best quality (there being no preservative used). Further dilution for making working solution or concentration (1, 2 and 4 mg/kg b.wt.) according to test animals were done with distilled water.

2.1 Experimental Design

For Animals as Control**:** For Group 1 – 1 ml i.p. distilled water for 5 days having three animals each, for Group 1a – 0.5 ml orally chlorophyllin for 5 days (1 mg/kg b. wt.), Group 1b – 0.5 ml orally chlorophyllin for 5 days (2 mg/kg b. wt.) and Group 1c – 0.5 ml orally chlorophyllin for 5 days (4 mg/kg b. wt.) were given.

For Chemicals**:** The animals were divided into four groups A, B, C and D for each test chemicals separately. Twelve animals were kept in each group. For Crude tobacco extract doses were given in the order: Group A – 1 ml of 0.5% of the crude tobacco extract, Group B – 1 ml of 1% of the crude tobacco extract, Group C – 1 ml of 1.5% of the crude tobacco extract and Group D – 1 ml of 2% of the crude tobacco extract

For Chlorophyllin**:** The animals were divided into 4 groups for each test chemical—A, B, C and D. 27 animals were kept in each group and 9 animals in each sub division. i.e.:Group 1B – for chlorophyllin (1 mg/kg. b. wt.), Group 2B – for chlorophyllin (2 mg/kg. b. wt.) and Group 3B – for chlorophyllin (4 mg/kg. b. wt.) The subdivisions i.e. 1, 2, 3 were again divided into 3 sub-categories having 3 animals each:Pre-treatment-Plant.Ext./Der.Comp →Test chemical (Ext/Der. Comp→ C), Concurrent treatment- PlantExt./Der.Comp.+Test Chemical (Ext/Der. Comp + C) and Post-treatment- Test Chemical → Plant Ext./Der. Comp.) C → Plt. Ext./Der. Comp.

2.2 For Cytological Studies

All the animals were weighed, and an overall mean weight was determined. According to that mean weight, volume of the test chemicals or extracts was administered per animal.

Procedures for cytological studies were as follows: All the animals were killed by cervical dislocation with the help of others. For Control animals: Control animals were sacrificed after 5 days of the last treatment for somatic cell preparation and sperm slide preparation. Animals treatment with Chemicals: Three animals each were sacrificed on 3^{rd}, 5^{th}, 7^{th} and 10^{th} day of the chemical treatment for all the groups, in case of somatic cell preparations. Animals treated Plant Derived Compounds: In case of somatic cell preparations five animals each were sacrificed on 5^{th} day after the last treatment (pre, concurrent and post)

All the animals were given i.p. (intraperitoneally) 2.5 mg/kg body weight of colchicine prepared in HBSS (concentration 0.7 mg/ml), 2½ hours before sacrificing them. Then each animal was dissected out for femurs (bone marrow) and testis. Chromosomal preparations from bone narrow cells were made [22] with some modifications. Finally, observations from well spread metaphase plates with the help of Research Binocular Microscope under different magnifications. Photomicrographs were taken under (100x10) x magnifications for chromosome slides and (45x10) x and for sperm slides with Leica DMLS-2 photomicroscope(camera-DFC 320)

2.2.1 Chromosomal Aberration Study:

50 well spread intact metaphases were scored from each animal (bone narrow).Chromosomal aberrations per cell (CA/Cell) were calculated as total aberrations observed per total number of cell studied. Percent aberrant cells (% AC) were calculated as total number of aberrant cells per total number of cells studied multiplied by hundred. Statistical analysis was carried out using student's t- test.

III. RESULTS

TABLE-1: Chromosomal Aberrations caused by Tobacco in the Bone marrow cells of Rats (n=3).

Chemical/Group	Post-treatment (days)	Dose (mg/kg b.wt.)	Total Chromosomal Aberrations										M.I.	Mean ± SE	
			Structural Aberrations							Numerical Aberrations					
			Br	Ga	F	Pulv	RC	DC	Clump	EEA	Poly	Hyper	Hypo		CA/Cell	%AC
Control /1	5 dy		0	1	0	0	0	0	1	0	0	0	0	20.00	0.01333±0.01333	0.6667±0.6667
Tob (A)	3 dy	0.5%	0	2	1	0	1	2	1	1	0	0	0	14.45	0.0533±0.006667	4.6667±0.6667*
	5 dy		0	1	1	1	3	2	2	1	0	0	0	13.19	0.0733±0.01764	6.0000±1.1547*
	7 dy		0	2	1	0	1	1	2	0	0	0	0	12.08	0.0466±0.006667	4.0000±0.0000**
	10 dy		0	2	0	0	2	0	1	0	0	0	0	11.36	0.0333±0.00667	2.6667±0.6667
Tob (B)	3 dy	1%	0	2	1	0	4	3	1	1	0	0	0	11.11	0.0800±0.01155*	7.3333±0.6667**
	5 dy		1	2	1	1	3	2	1	0	0	0	0	9.852	0.06667±0.006667*	6.0000±1.1547*
	7 dy		1	1	1	1	1	1	2	0	0	0	1	8.970	0.0600±0.01155	5.3333±0.6667**
	10 dy		0	2	1	0	1	1	0	1	0	0	0	11.88	0.0400±0.01155	4.0000±1.1547
Tob (C)	3 dy	1.5%	0	1	1	1	4	3	2	0	0	1	0	7.352	0.08667±0.006667**	7.3333±1.3333*
	5 dy		1	3	2	0	3	2	2	0	0	1	0	8.429	0.09333±0.01764*	8.6667±1.3333**
	7 dy		1	3	1	0	3	2	1	1	0	0	0	8.090	0.08000±0.01155*	6.6667±0.6667**
	10 dy		1	4	2	0	5	1	3	0	0	1	0	10.21	0.01133±0.01764*	10.0000±1.1547**
Tob (D)	3 dy	2%	2	3	3	0	3	2	2	1	0	0	0	7.522	0.1067±0.006667**	9.3333±0.6667**
	5 dy		1	3	2	1	2	3	0	2	0	0	0	9.125	0.1267±0.01764**	12.0000±2.0000**
	7 dy		1	2	2	1	1	4	0	1	0	0	0	7.818	0.10000±0.01155**	8.0000±1.1547**
	10 dy		1	2	3	0	2	3	1	0	0	1	0	9.183	0.08667±0.01333*	7.3333±0.6667**

Student's t-test; *and ** superscripts indicate level of significance. * - $p < 0.05$, ** - $p < 0.01$.

Tob- Tobacco, MI-Mitotic Index, SE-Standard Error, CA-Chromosomal Aberrations, AC-Aberrant Cells, SE-Standard Error, b.wt.-Body Weight, Br-Chromosomal Break, Ga-Chromosomal Gap, F-Chromosomal Fragmentation, Pulv-Pulverization, RC-Ring Chromosomes, DC-Dicentric Chromosomes, Clump-Clumping, EEA-End to end association, Poly-Polyploidy, Hyper-Hyperploidy, Hypo-Hypoploidy.

The material used for cytological study was bone marrow. 50 metaphase plates per animal from the bone marrow tissue were observed. The frequency of chromosomal aberrations in control and experimental animals are given in Table 1. In rats treated with different doses of Tobacco metaphase plates showed different kinds of chromosomal aberrations viz. chromosomal break (Br.), chromosomal gap (G), chromosomal fragmentation (F), pulverization (Pulv), ring chromosomes (RC), dicentric chromosomes (DC), clumping (clump), end to end association (EEA), polyploidy (Poly), hyperploidy (Hyper) and hypopolidy (Hypo). Significant differences were found in the M.I., %AC and CA/Cell of the animals with different doses of tobacco extract as compared to those of the control group Thus, overall there was observed increase in %AC and CA/Cell in relation to the dose increase of crude tobacco extract (Zarda). (Table-,1).

Treatment with different doses of Chln was able to reduce chromosomal aberrations to a significant level in tobacco treated animals. This proved Chln as an efficient anti-clastogen and significant reduction for the different doses of Chln 1, 2 and 4 mg/kg b.wt. was observed in case of dose 2 mg/kg b.wt. of Chln (Table-2) at the pre-treatment level and concurrent level. Post-treatment with different doses of Chln reduces the chromosomal aberrations but not to a significant level in the tobacco treated animals.

TABLE-2: Minimization of Chromosomal Aberrations caused by Tobacco in Bone marrow cells of Rats, primed with Chlorophyllin extract (n=3)

Chemical/ Group	Post-treatment (days)	Dose (mg/kg b.wt.)	Total Chromosomal Aberrations											M.I.	Mean ± SE	
			Structural Aberrations								Numerical Aberrations					
			Br	Ga	F	Pulv	RC	DC	Clump	EEA	Poly	Hyper	Hypo		CA/Cell	%AC
Group																
Chln (1a)	5 dy	1	0	0	0	0	1	0	1	0	0	0	0	18.07	0.01333±0.00667	1.3333±0.6671
Chln (1b)	5 dy	2	0	0	0	0	2	0	0	0	0	0	0	18.25	0.01333±0.01333	1.3334±1.3335
Chln (1c)	5 dy	4	0	1	0	0	0	0	1	0	0	0	0	17.88	0.01333±0.01333	1.3334±1.3335
Tob	5 dy	2%	1	3	2	1	2	3	0	2	0	0	0	9.125	0.1267±0.01764	12.0000±2.0000
Chln→ Tob (1B)	5 dy → 5 dy	1 → 2%	0	1	1	2	2	2	2	0	0	0	0	11.82	0.06667±0.006667*	5.3333±0.6667*
Chln→ Tob (2B)	5 dy → 5 dy	2 → 2%	0	0	1	1	1	1	2	1	0	0	0	12.81	0.04667±0.006667*	3.3333±0.6667*
Chln→ Tob (3B)	5 dy → 5 dy	4 → 2%	0	2	2	0	1	1	2	0	0	0	0	12.24	0.05333±0.006667*	4.6667±0.6667*
Tob+Chln (1B)	5 dy	2%+1	1	2	1	1	1	2	1	2	0	0	0	11.23	0.07333±0.01764	4.6667±1.3333*
Tob+Chln (2B)	5 dy	2%+2	0	0	2	0	1	1	2	1	0	0	0	11.02	0.04667±0.006667*	4.0000±0.0000*
Tob+Chln (3B)	5 dy	2%+4	1	2	2	0	1	2	2	2	0	0	0	10.87	0.08000±0.01155	6.0000±1.1547
Tob→Chln (1B)	5 dy → 5dy	2%+1	1	2	1	2	4	2	2	0	1	0	0	10.95	0.1000±0.02000	10.6667±0.6667
Tob→Chln (2B)	5 dy →5 dy	2%+2	1	2	2	1	1	2	3	0	0	0	0	10.92	0.08000±0.01155	9.3333±0.6667
Tob→Chln (3B)	5 dy → 5 dy	2%+4	2	3	2	1	3	2	3	0	0	0	0	10.60	0.1067±0.01764	8.000±1.1547

Student's t-test; *& ** superscripts indicate level of significance. * - p <0.05, ** - p < 0.01.

Chln-Chlorophyllin, Tob-Tobacco, **Chln→ Tob-Pre-treatment**, **Tob+Spi-Concurrent treatment**, **Tob→Chln-Post-treatment** b.wt.-Body Weight, SE-Standard Error, MI-Mitotic Index, CA-Chromosomal Aberrations, AC-Aberrant Cells, Br-Chromosomal Break, Ga-Chromosomal Gap, F-Chromosomal Fragmentation, Pulv-Pulverization, RC-Ring Chromosomes, DC-Dicentric Chromosomes, Clump-Clumping, EEA-End to end association, Poly-Polyploidy, Hyper-Hyperploidy, Hypo-Hypoploidy.

IV. DISCUSSION

The aim of the present study was to demonstrate the protective effects of Chlorophyllin in tobacco treated bone marrow chromosomal abnormalities in Rattus rattus.. Chromosomal and mitotic abnormalities resembled those prevalent in tumor cells, irradiated cells and chemically treated cells [11, 12 and 13]. The aqueous extracts from four types of tobacco led to abnormalties of spindle disturbances like stickiness, somatic bridge formation, diplochromatic clumping, non-disjunction and abnormal configuration [23]. Saliva collected from chewing Indian tobacco induces chromosomal

aberrations in Chinese hamster cells [24]. Similarly the effect of water soluble extracts of different forms of tobacco preparations in plant systems was observed and chromosomal abnormalities were noticed [25]. The effect of higher concentrations of aqueous extract of zarda on the root meristem of Allium cepa showed higher mitostatic effect and produces chromosomal damage including clumped metaphase, pulverization, polyploidy, stretching fracture in centromeric regions in metaphase leading to nuclear disintegration [26]. Similar affect on the chromosomes was observed in the present study (Table-1). Present study showed dose dependent increase in chromosomal abnormality and percentage achromatic cells (Table-1) The genotoxicity of bidi tobacco was evaluated by aqueous, ethanolic and chloroform extracts of processed tobacco used in India and the Ames test was used to detect the mutagenticity and was found that the workers in bidi industry were exposed to potentially mutagenic and genotoxic chemicals [27]. The workers in cigarette manufacturing industry were exposed to tobacco dust which induced chromosome damage including chromosomal aberrations [28]. The frequency of chromosomal aberration in peripheral blood lymphocyte increased with increase in environmental tobacco smoke in case of passive smoking was also observed [29]. Similarly increase in the frequency of chromosomal aberrations was observed in rats when exposed to tobacco (Table-1). The affect of the aqueous tobacco extract led to abnormalities of spindle disturbances like stickiness, somatic bridge formation, diplochromatid, clumping and fragmentation which were a consequence of the known action of nicotine and its related substances on the disulfide and sulfhydryl linkages which enter into spindle formation [23]. Other action leads to breaks, gaps and translocation indicating effects on DNA or DNA protein complex.

The protective role of Chln on chromosomal aberrations induced by tobacco extract was investigated in rats (*Rattus rattus*). Chlorophyllin administration to the test animals was observed to significantly reduced the number of chromosomal aberrations per cell, as well as percentage of aberrated cells on the tobacco treated animals (Table2). The antimutagenic agents were identified in fruits and vegetables and the most potent being the chlorophyllin was also observed [30]. Chlorophyllin protects against cigarette smoke condensate in the *Salmonella* microsome assay [31]. Chln modes of action includes the formation of complexes with genotoxic agents, acting as a desmutagenic compound or interceptor when administered simultaneously with a mutagenic or carcinogenic agent, is capable of reducing DNA-drug binding interceptor[32, 33 and 34]. Inhibition of mutagenicity *in vivo* system by chln may be due to phase to phase complexing with planar compound and mainly as a scavenger [35].The present study thus demonstrates the cytoprotective role of Chlorophyllin in tobacco treated chromosomal aberrations in rats*(Rattus rattus*) (Table 2).

V. CONCLUSION

Tobacco was proved to be an efficient genotoxic chemical, causing significant chromosomal alterations which increased with the increase in dose of tobacco. Plant derived compounds (chlorophyllin) proved to be efficient antigenotoxic against tobacco. Genotoxicity of tobacco was reduce with chlorophyllin and 2 mg/kg b.wt. dose of chlorophyllin was observed to be more effective in minimizing the genotoxicity of the test chemical at the pre-treatment followed next by concurrent level of administration. It was concluded that chemical carcinogens were highly genotoxic and daily intake of plant and plant derived compounds proved to be efficient in minimizing their genotoxic effect. Therefore, a balanced vegetable diet can be suggested which is more effective

in protecting against genotoxicity or dietary supplements may be used to prevent the harmful effects of genotoxicants.

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