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PHYLLANTHUS EMBLICA PREVENTS CYCLOPHOSPHAMIDE INDUCED CYTOGENETIC DAMAGE IN GERM CELLS OF MICE

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ABSTRACT

In the present investigation the antimutagenic effects of *Phyllanthus fruit extract* (PFE) has been evaluated against cyclophosphamide induced genotoxicity in germ cells of mice. The administration of Phyllanthus fruit extract at various doses i.e. 170, 340 and 680 mg /kg.When treated individually did not induce chromosomal aberrations in germ cells of mice A single Intra peritoneal injection of 50mg/kg of cyclophosphamide induced significant increase in the percentage of chromosomal aberrations in germ cells of mice. However after co administration three doses of PFE extract there was a dose dependent decrease in the % of chromosomal abberations was observed. When animals were administered with *Phyllanthus fruit extract* PFE 170, 340 & 680 mg/kg/bw orally for two months. CP (50 mg/kg/bw) was given intraperitonially as a single dose. For each experimental group control, animals were maintained. Two days after the administration of the last dose, the animals were sacrificed and air dried metaphase preparations were made and processed for identification of chromosomal aberrations in germ cells of mice. In animals treated with single dose of CP, an increase was observed when compared with the values of control group. But when animals primed with PFE + CP group, there was a decrease in the frequency of chromosomal aberrations. Thus the results clearly indicated the protective role of PFE on cyclophosphamide induced genotoxic damage in germ cells of mice.

KEYWORDS: Cyclophosphamide, Phyllanthus, germ cells.

INTRODUCTION

A number of antineoplastic drugs are in common use to combat various types of cancers. These are shown to be mutagenic in different test systems and these antineoplastic drugs such as Cyclophosphamide, Cisplatin, Tamoxifen, Gemcitabine and Paclitxel etc., have shown clastogenic effects in various test systems. Potential genetic damage due to drugs and other chemicals is well recognized. Extensive studies have been carried out on mutagenicity of various drugs in microorganisms, insects, mammals and in exposed population.[1-4]

Cyclophosphamide (CPM) is a well-known bifunctional alkylating agent, widely used in cancer chemotherapy and expresses its genotoxicity when metabolically activated.[5] It is extensively used for the treatment of various cancers as well as an immunosuppressant in organ transplantation, rheumatoid arthritis, systemic lupus erythromatosis, multiple sclerosis, and other benign diseases.[6,7] According to the International Agency for Research on Cancer (IARC), CPM is widely used as reference mutagen and has been classified as carcinogenic for animals and humans.

According to believe in ancient Indian mythology, *Phyllanthus emblica* is the first tree to be created in the universe. It belongs to family Euphorbiaceae. It is also named as Amla, *phyllanthus Emblica* or Indian gooseberry. The species is native to India and also grows in tropical and subtropical regions including Pakistan, Uzbekistan, Srilanka, South East Asia, China and Malaysia. The fruits of PF are widely used in the Aryuveda and are believed to increase defense against diseases. It has its beneficial role in cancer, diabetes, liver, heart trouble, ulcer, anemia and various other diseases.

Diet can modify the pathological processes, because certain naturally occurring substances known as antioxidants are present in plants and other sources have shown to be protective against mutagens or carcinogens or endogenous mutagens.[9] Among the various phytonutrients, phyllanthus emblica posses good antioxidants. It was described in Indian Ayurvedic literature more than 200 years ago. It has been widely used by traditional medical practitioners for the treatment of various diseases. It exhibits many properties like antiviral, antimutagenic, hepato protective activity, hypoglycemic activity etc.[10-13]. In the present investigation, the studies were carried out on protective role of PFE on cyclophosphamide induced genetic damage in Germ cells of mice.

MATERIALS AND METHOD

Chemicals

Trisodium citrate (Merck), NaCl (Loba Chemie), Methanol (s.d fine chemie), acetic acid (Qualingens), and 2% Giemsa stain solution in phosphate buffer (pH 6.8) were all purchased from E. Merck, India. Cisplatin, *Phyllanthus fruit extract*, ethanol, mitomycin-C and eosin were purchased from Cipla. However, all other chemicals used in the experiments were of analytical grade.

PFE Extract preparation

Cameron and Puling.[14] suggested the daily intake of vitamin C is 1-10g/day for human being. Data based on maximum ascorbate concentrations in human body suggest a maximum body pool of around 5000mg, which is approximately 70mg/kg body weight in man.[15] In the present study, a corresponding amount of an aqueous extract of PFE containing the same amount of vitamin C was used for mice, as calculated from daily 1 g intake for a 60kg person. The fruits were procured in bulk, cut into pieces and dried in sunlight. Known quantities weighed and kept in distilled water for 24hr. The AA content of the decoction was estimated b the 2, 6-dichlorophenol indophenol method.[16] and it amounted to 685mg/kg body weight.

Experimental animals

Eight weeks old random bred male Swiss albino mice (Mus musculus) average body weight of 25 ± 2 gms were purchased from National Institute of Nutrition, Hyderabad, were maintained in the departmental animal house under an absolute hygienic conditions as per the recommended procedures by fulfilling all the necessary ethical standards. They were housed in polypropylene shoe box type cages dimensions were 13.5" L x 7.0" W x 6.5" to 8.5"H cages, bedded with rice husk (rice husk procured locally and autoclaved to free from microorganisms) and kept in AC room at the temperature 25°C (± 2°C) and RH 65 ± 5% and a photo-cycle of 12:12 h light and dark periods, were fed with pelleted diet (from National Institute ofNutrition, Hyderabad)composed of 20.0% crude protein, 4.0% crude fiber, 1.0% calcium, 0.6% phosphorus, 8% fish meal, 20% ground nut cake and enriched with stabilized vitamins A, B, C, D3, K, thiamine, riboflavin, pantothenic acid, niacin, folic acid, minerals & trace elements and water.

Dosage schedule: In the present study two experiments were conducted. The animals were feed orally with cyclophosphamide and PFE extract and categorized in to following groups

Group I : controls

Group II: PFE extract 170 mg/kg

Group III: PFE extract 340 mg/kg

Group IV: PFE extract 680 mg/kg

In the second experiment for modulation studies all the three groups as follows:

Group I: controls

Group II: Cyclophosphamide 50 mg/kg

Group III: PFE extract 170 mg/kg + Cyclophosphamide 50 mg/kg

Group IV: PFE extract 340 mg/kg + Cyclophosphamide 50 mg/kg

Group V: PFE extract 680 mg/kg + Cyclophosphamide 50 mg/kg

Analysis of chromosomal aberrations in germ cells of mice

The mice were killed on 28th day, 24 h after administration of last dose of the drug. Seminiferous tubules from testis were collected in 5ml of isotonic 1.2% trisodium citrate solution and incubated at the temperature 37ºC for 45 min. The cell suspension was centrifuged in 120x17 mm conical centrifuge tubes for 10 min at 1000 rpm. To the pellet 5 ml of freshly prepared pre-chilled fixative (3:1 methanol and acetic acid) added and centrifuged. This process repeated for 4 to 5 times. The Chromosomal preparations were made by the air drying technique6 and stained with 2 ml of 2% Giemsa (2 ml of 2% Giemsa in 46 ml of double distilled water plus 2 ml of phosphate buffer* pH 6.8) for 7-8 min. Approximately 500 meiotic metaphases screened for numerical (Autosomal Univalents, Sex- Autosomal Univalents, euploids and aneuploids) and structural (translocations) Aberrations.acording to the method of evans et al.[17]

STATISTICAL ANALYSIS

The significance for the differences between control and treated groups was statistically analyzed by using x 2 test. Data are expressed as mean ± SES in the Tables 1 and2

RESULTS

The data on the frequencies of chromosomal aberrations in control and treated groups are depicted in table 1 & 2. The frequencies (%) in the controls recorded were 3.00% of abnormalities and the percentages of chromosomal aberrations were 3.20, 3.40 & 3.60 after administration of 170,340., 680 mg/kg phyllunthus fruit extract respectively The differences

in the frequencies in the chromosomal aberrations between controls and treated mice were analyzed using X2 test and the results were found to be insignificant (P>0.05, Table-1

Table 1: Frequency of chromosomal aberrations in germ cells of mice treated with various doses of phyllanthus Fruit Extract

Treatment (hrs) Dose (mg/kg)	Normal Metaphases Scored (%)	Abnormal Metaphases Scored (%)
Control	242(96.8)	8(3.2)
170 mg/kg	240(96)	10(4.0)*
340 mg/kg	239(95.6)	11(4.4)*
680 mg/kg	238(95.2)	12(4.8)*

*P<0.01

Table 2: Frequency of chromosomal aberrations in germ cells of mice treated with Cyclophosphamide and primed with phyllanthus emblica.

Time Dose	Non-primed		Primed with phyllanthus emblica					
			170 mg/kg		340 mg/kg		680 mg/kg	
	Normal Metaphases Scored (%)	Abnormal Metaphases Scored (%)	Normal Metaphases Scored (%)	Abnormal Metaphases Scored (%)	Normal Metaphases Scored (%)	Abnormal Metaphases Scored (%)	Normal Metaphases Scored (%)	Abnormal Metaphases Scored (%)
Control	235 (94)	15 (6.0)						
Mitomycin	202 (80.8)	48 (19.2)*						
50 mg/kg	204 (81.6)	46 (18.4)	235 (94)	15 (6.0)*	238 (95.2)	12 (4.8)*	243 (97.3)	7 (2.7)*

*P<0.05

DISCUSSION

Cytogenetic evaluation of chromosomal damage in germ cells induced by a toxicant or mutagen is of specific significance. Since the gametes transmit the effects from one generation to another generation. The studies on behavior of sex-chromosomes and autosomes are of cardinal importance since the inherited anomalies like congenital malformations, still births, neonatal deaths, repeated abortions and other genetic disorders may arise due to the mutations. A study revealed marked protective role of both vitamin E and ginger oil on cyclophosphamide induced male gonadal dysfunction. The later represented by altered male gonadal weight, disturbed sperm quality parameters, decreased testosterone level, disturbed oxidative stress and lipid peroxidation markers in addition to altered

spermatogenesis, testicular histology and increased incidence of apoptosis among germ cells,. The findings of the present study pass in accordance with similar studies which previously reported that cyclophosphamide induced testicular androgenic and gametogenic dysfunction.[18,19]

Cyclophosphamide is widely used drug among alkylating agents in cancer chemotherapy. Acrolein and phosphoramide are active compounds of CP and further these compounds reduce the growth of cancerous cells by acting at DNA level.[20] There are many studies showing chemotherapeutic agents and CP cause gene mutations, CA and aneuploidy and rearrangements in somatic and germ cells of mice in vivo and in vitro test systems,[21] and an elevated frequency of secondary treatment related tumors in human cancer survivors.[22,23] Earlier studies have shown that post meiotic germ cells are specifically sensitive to cyclophosphamide treatment.[24,25] The administration of low doses of cyclophosphamide to male rats for 6 week produced greater than 95% post implantation loss among their progeny.[25] This loss caused to male rat with cyclophosphamide was characterized by early pre implantation embryonic death[26]. Some abnormalities in progeny outcome caused by cyclophosphamide treatment persisted to a subsequent generation,[27] Thus the effects of cyclophosphamide exposure were both specific and heritable. Further chronic low dose exposure to cyclophosphamide produced adverse effects on progeny by altering sperm nuclear components.[28] The results of present study showed that there is a significant increase in the frequency of chromosomal aberrations when compared with control values ($p<0.05$). The mutagenicity of CP is clearly related to the formation of ultimate cytotoxic metabolite phosphoramide mustard through intermediate agent is hydroxyl cyclophosphamide and deschloroethyl cyclophosphamide which is responsible DNA cross links and strand.

There are several reports on such effects of plant extracts,bioactive constituents and natural compounds. Flavonoids are the phenolic compounds which are naturally found in fruits, vegeta-bles and other plant parts. They have many favourable biological effects due to their antioxidant and free radical scavenging abili-ties. Sharma and Agrawal [29] studied the antigenotoxic effects of Glycyrrhiza glabra root extract against CP induced chro-mosomal aberration in Swiss albino mice. According to them the protective effect is due to the reduced immunosuppressant effects of CP by Glycyrrhiza glabra and presence of the phytotherapeutic molecules such as flavonoids, tannins, saponins, triterpinoids inthis plant. Cevallos et al.[30] observed thechemo protective effects of spirulina against CP induced muta-genicity in mouse

test system. The positive response obtained inthis study is hypothesised as due to the synergistic action of widespectrum of antioxidants present in the algal extract Cyclophosphamide (CP) is one of the anti-neoplastic drugs. Despite its numerous clinical applications, it has devastating effects on the testicles and declines the sperm quality in treated patients in another study the protective effect of crocin in improving the toxicity induced by CP in reproductive system was investigated. In this study, 24 male adult mice (6 to 8 weeks) were randomly divided into three groups, control group received normal saline (0.1 mL, IP, daily), the CP group received CP (15 mg kg-1, IP, weekly) and the CP + crocin group received CP along with crocin (200 mg kg-1, IP, daily). After 35 days of treatment, animals were sacrificed. The samples of epididymis in human tubal fluid medium incubated for 30 min in 5% CO2 for flotation of sperm. Sperm were obtained from caudal epididymis using dissecting method. Then, the parameters of sperm quality including sperm count, motility, viability, DNA damage, nuclear maturation, and sperm morphology were evaluated. In CP group, the sperm count, motility, viability, nuclear maturation and sperm morphology were significantly decreased compared to control group ($p < 0.05$) and in the CP + crocin group all of these parameters significantly increased compared to CP group ($p < 0.05$). The percentage of sperm with DNA damage in the CP group significantly increased compared to other groups ($p < 0.05$). The results of this study indicated that the crocin was able to suppress free radicals and enhance the quality of sperm in CP treated animal.[31]

Phyllanthus emblica enjoyed a hallow position in Ayurveda an Indian system o medicine. It is a first tree to be created in the universe. Its fruit juice contains highest vitamin C contains as 478.56mg/100ml. It is used in the preparation of Indian pickles. The fruit when blended with other fruits boosted their nutritional quality in terms of vitamin C content. It is often used as Triphala which is a herbal formulation containing fruit of Terminalia chebula and Terminlia belerica in equal proportions. It has important medicinal value against various diseases. *In vitro* and *in vivo* animal studies suggested wide range of potential therapeutic or preventive effects has been reported. Such effects in humans have not conformed so far. PFE when prepare in the Triphala delayed the development of fore stomach Papillomagness, breast cancer, skin tumors, liver fibrosis, diabetic cataract, Alzheimer's diseases.[32-34]

The present results are comparable with the reports of other investigators. When cadmium chloride administered orally 3mg/kg in a single dose, co-treatment with phyllanthus fruit extract at dose of 500mg/kg showed decreased mortality in rats. Further there are

histopathological changes reduced peroxidation in liver, kidney and testis after acute cadmium exposure.[35] The protective effects of *phyllanthus* fruit extract aganist adriamycin and chromium induced genotoxicity in bone marrow cells of mice has been reported.[36,37] The crude extract of *phyllanthus emblica* decreased the percentage of chromosomal aberration induced by Cesium chloride and aluminium etc.,.[38-39] In the present study pretreatment of phyllanthus fruit extract was shown to be more effective in reducing the genotoxicity of cyclophosphamide. The protective nature of *phyllanthus emblica* is because of presence of Vitamin C, tannins, polyphenolic compounds and elligic acid.[40] Ascorbic acid (vitamin c) polyphenolic compounds such as elligic and tannic acids are inhibitors and blocking agents aganist carcinogens on direct acting N- Nitroso compounds. Elligic acid protects DNA attack of electrophyilic species of free radicals by binding to nitcleophyilic sites.[41]

CONCLUSIONS

Animals when treated with various doses of *phyllanthus fruit* extract showed non mutagenic nature and the percentage of chromosomal aberration in germ cells of mice were equivalent with that of control values. There was an increase in the incidence of chromosomal aberrations in cyclophosphamide treated group when compared to the control Group. There was a significant decrease in the percentage of chromosomal aberration in germ cells of mice when cyclophosphamidewas primed with various doses of *phyllanthus fruit* extract. Thus amla fruit extract showed protective effects against the cyclophosphamide induced genotoxicity in germ cells of mice. Hence *phyllanthus emblica* fruit extract supplementation is a safer dietary component in chemotherapeutic strategy. Our results have a practical decline of genotoxic effects of cyclophosphamide in drug exposed population and pharmaceutical lead plant workers handle this drug which may alternate the higher risks for development of secondary malignancy and for abnormal reproductive outcome.

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