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THE PROTECTIVE EFFECT OF PHYLLANTHUS EMBLICA AGAINST CYCLOPHOSPHAMIDE INDUCED ABERRANT SPERMS IN MICE

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ABSTRACT

A number of antineoplatic drugs are used to combat with different types of cancer, and had been shown to be cytotoxic. Cyclophosphamide is one of most commonly used in malignant lymphomas. The antioxidants such as *phyllanthus emblica* inhibits the toxicity of mutagens/carcinogens in animals and Humans. The protective effects of *phyllanthus emblica* in cyclophosphamide induced toxicity in swiss male mice was evaluated using sperm morphology assay. Two experiments were conducted in germ cells of Swiss male mice. The cauda epidymis was collected from control and exposed group of animals after five weeks of treatment schedule and screened for presence of various types of sperm head abnormalities such as amorphous, banana hammer head etc., The animals were treated with 170, 380 and 640 mg/kg of *phyllanthus emblica* showed as non mutagenic where as the cyclophosphamide induced significant increase in the percentage sperm head abnormalities but when primed with AA there is a significant decrease in the percentages of abnormal sperms. *Phyllanthus emblica* protects the cyclophosphamide induced genotoxicity in germ cells of mice. Hence *phyllanthus emblica* supplementation is safer in chemotherapeutic strategy.

KEYWORDS: *phyllanthus emblica*, genotoxicity, cyclophosphamide, sperm morphology.

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.[1-3]

Cyclophosphamide (CPM) is a well-known bifunctional alkylating agent, widely used in cancer chemotherapy and expresses its genotoxicity when metabolically activated.[4] 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.[5,6] 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.[7] 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.[8]

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] The mouse spermmorphology test is commonly used for measurement of spermato-genic damage induced by test agents. Studies have shown thatinduced changes in sperm morphology reflect the genetic dam-age in male germ cells. There are several reports on chemically induced abnormal sperms.[14,15] reported the sperm abnormalities in mouse germ cells after short term exposure to pesticides acetamiprid, propineband their mixture. From various studies it has been concluded that chemicals yielding positive response in mouse sperm mor-phology test should be regarded as suspected germ cell mutagens in mammals and agent's positive responses in these sperm tests should be considered with high priority against human applica-tions[16,17] Sperm abnormality assay is extensively being used for the evaluation of

genotoxicity of chemicals and also for the study of antigenotoxic protective effects of natural compounds.[18-23]

MATERIALS AND METHODS

PFE Extract preparation

Cameron and Puling[24] 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 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[25] and it amounted to 680mg/kg body weight.

Animal treatment

The study was conducted after taking the approval of Institutional Ethical Committee on twenty adult male swiss albino mice 30 to 50 days old and weighing around to 30 to 40 g were maintained in plastic cages under controlled lighting conditions (12:12 light and dark cycle) relative humidity (50±5%) and temperature (37±2^{o}C) fed with mice feed and were given ad libitium access to water. A group of 5 mice per experiment were taken and treated with CP and PEF. The doses were prepared daily in distilled water and were administered by gastric gavage method for PEF and 26G needle intraperitoneal injection for CP treatmen

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

Sperm morphology assay

All the control and treated animals were sacrificed on 35th day. This is because spermatogenesis takes about 34.5 days to complete in mice. Sperms were sampled from the caudal epididymis after the animals had been sacrificed by cervical dislocation. Sperm suspension was prepared from the caudal of each testis by mincing the caudal in physiological saline. To the suspension 2- 3 drops of 1% aqueous eosin was added and kept for about 20 min undisturbed. Smears were made on clean slides and allowed to dry in air. 1000 sperm cells/mouse were assessed for morphological abnormalities according to the criteria of Wyrobek and Bruce.[16] Sperm collection was done from Cauda epididymidis in physiological saline and stained in .aqueous Eosin-Y. 500 perms were scored per animal and sperm head abnormalities were categorized, following Wryobek and Bruce[16] method. The various types were observed as banana, Amorphous, hammer head shaped were observed in control and treated group of animals and sperm head abnormalities were categorized and data was analysed using Chi-Square test.

RESULTS

The data on the incidence of aberrant sperms in control and treated groups are depicted in table 1-4. Various doses of *Phyllanthus emblica* of 170, 340 and 680 mg/ kg body weight have been selected. The frequencies of abnormal sperm heads in controls showed (3.0%) of when compared to the treated groups were 3.6, 3.8 and 4.0%. There was a gradual increase in the frequency of the sperm abnormalities with different doses of *Phyllanthus emblica* ***(Table 2)*** The differences in the frequency of aberrent sperms between control and PFE treated groups were found to be insignificant (Table 2, $P>0.05$).

The frequency of aberrant sperms were 12.6% in cyclophosphamide treated mice and significant decrease was observed when animals were primed with *Phyllanthus emblica* at all dose levels (Table 3). The differences in the frequency of aberrant sperms between control and primed groups were found to be statistically significant. (Table 4, $P<0.05$).

Table 1: Frequency of sperm head abnormalities in mice treated with various doses of phyllanthus fruit extract (PFE).

Treatment	Normal sperms (%)	Abnormal sperms (%)
Control	485(97.0)	15(3.0)
170 mg/kg	482(96.4)	18(3.6)*
340 mg/kg	481(96.2)	19(3.8)*
680 mg/kg	480(96.0)	20(4.0)*

*P<0.05.

Table 2: X^2 values for the differences in the frequencies of sperm head abnormalities between control and phyllanthus fruit extract treated group.

Treatment	X^2 values
Control Vs 170 mg/kg	0.28
Control Vs 340 mg/kg	0.48
Control Vs 680 mg/kg	0.74
170 mg/kg Vs 340 mg/kg	0.02
170 mg/kg Vs 680 mg/kg	0.10
340 mg/kg Vs680 mg/kg	0.02

*P<0.05.

Table 3: Frequency of sperm head abnormalities in cyclophosphamide treated mice primed with Phyllanthus fruit extract.

Time Dose	Non-primed		Primed with phyllanthus fruit extract					
			170 mg/kg		340 mg/kg		680 mg/kg	
	Normal sperms (%)	Aberrant sperms (%)	Normal sperms (%)	Aberrant sperms (%)	Normal sperms (%)	Aberrant sperms (%)	Normal sperms (%)	Aberrant sperms (%)
B. Control II	480 (96.0)	20 (4.0)						
Mitomycin	440 (88.0)	60 (12.0)*						
50 mg/kg	452 (90.40)	48 (9.6)*	468 (93.6)	32 (6.4)*	472 (94.4)	28 (5.6)*	484 (96.8)	16 (3.2)*
*P<0.05								

Table 4: X2 values for the differences in the frequencies of sperm head abnormalities between control and cyclophosphamide treated animals and primed with phyllanthus fruit extract (PFE)

Dose	X^2 values
Control Vs 50 mg/kg	12.37
50 mg/kg Vs 170 mg/kg	3.47
50 mg/kg Vs 340 mg/kg	5.69
50 mg/kg Vs 680 mg/kg	17.09

DISCUSSION

Sperm head abnormality test is one of the quickest, simplest and least expensive methods for identifying mutagens and carcinogens. The abnormalities may be as a result of the mistakes made in packaging the genetic material in the sperm head or perhaps as a result of an abnormal chromosome complement. However, it is probable that sperm with abnormal shapes would contain abnormal genetic material. Wyrobek[17] reported that large reductions in sperm number or motility or large increases in sperm with abnormal shapes are associated with reduced fertility. Sperm tests provide a direct measure of the quality of sperm produced in chemically treated animals such as in the use of formaldehyde.

The sperm morphology assay, is one of the most widely used genetic toxicology assays, has potential in identifying chemi-cals that induce spermatogenic dysfunction and perhaps heritable mutations. Furthermore, the development of sperm head abnormality has been used as a reliable short-term biolog-ical indicator in the evaluation of chemical genotoxicity.[25,26] Vilar et al.[27] studied the antimutagenecity protection of Ginkgo biloba extract against mitomycin C and CP in mouse bone marrow. According to them there are some common mechanism of action of plant extracts such as antioxidant activity, free radical scavenging and even the gene regulation which contributes its direct or indirect anti mutagenic effects. CP is an alkylating anticancer drug which is activated invivo by passing through various metabolic steps producing reac-tive molecules. Scavenging of these reactive molecules is one of hypothesis has techniques in the armoury of antimutagenesis. This may be one reason for the protective effect of septilin. Similar hypothesishas been given by Hosseinimehr and Karami[28] while reporting the chemoprotective effects of captopril against CP induced geno-toxicity. They reported the reduction of CP induced genotoxicity in bone marrow cells and they opine that this effect is due to the antioxidant activity of thiols in captopril. There are several reports on such effects of plant extracts, bioactive constituents and natural compounds. 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 effectsof CP by Glycyrrhiza glabra and presence of the Phytotherapeutic molecules such as flavonoids, tannins, saponins, triterpinoids in this plant.

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.[30]

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.[31-35]

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.[36] The protective effects of *phyllanthus* fruit extract aganist adriamycin and chromium induced genotoxicity in bone marrow cells of mice has been reported.[37,38] The crude extract of *phyllanthus emblica* decreased the percentage of chromosomal aberration induced by Cesium chloride and aluminium etc.[39-40] 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.[41] 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[42] Earlier the protective effects of *phyllanthus emblica* against Chromium & adraimycin induced genotoxicity in mice has been reported from our laboratory.[43-44]

These observations are in accord with our results where PFE has been showed to render protection against CP induced toxicity. Such observations indicate that when organisms. administered with fruit extract for a longer period of time than perhaps toxic agents such as anticancer drugs might not be effective in inducing the sperm cell damage.

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