Original Paper

Protective Role of *Ocimum sanctum* Infusion against Norethynodrel-induced Genotoxic Damage in Cultured Human Peripheral Blood Lymphocytes

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ABSTRACT

Synthetic progestins have wide spread use in medicine, but their side effects are often debatable. Norethynodrel is a synthetic progestin used either as single entity drug, or in combination with an estrogen such as ethinylestradiol in oral contraceptives. It induces chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), and inhibits lymphocyte proliferation in the presence of metabolic activation (S9 mix) in cultured human peripheral blood lymphocytes. The genotoxic effects of steroids can be reduced by the use of various antioxidants and natural plant products. Aqueous extract of *Ocimum sanctum* L. (Sacred Basil) leaves have been used for the treatment of a variety of conditions since ancient times. Pharmacological evidence shows that Sacred Basil possesses immunomodulating, hepatoprotective, chemopreventive, anticancer, antioxidant, antimutagenic and antigenotoxic properties. Infusion concentrations of 1.075×10^{-4}, 2.127×10^{-4} and 3.15×10^{-4} g/ml of culture medium were tested against 60 mg/ml of norethynodrel, separately in the presence of S9 mix. Aqueous plant infusion resulted in the reduction of the genotoxic damage by norethynodrel. Our study on other synthetic progestins such as ethynodioldiacetate, lynestrenol, and medroxy-progesterone acetate showed genotoxic effects only in the presence of S9 mix. Estrogens such as estradiol-17b and ethinylestradiol undergoes aromatic hydroxylation by cytochrome P450 and generates various forms of quinones. Quinones, via redox cycling in the presence of NADP generates reactive oxygen species (ROS). Pharmacologically active compounds of *O. sanctum* L. like eugenol, rosmarinic acid and epigenin are excellent antioxidants. Flavonoids, orientin and vicenin have shown a protective effect against radiation induced genotoxic damage in cultured human lymphocytes by scavenging free radicals. Infusion of medicinal plants can modulate DNA damage when combined with other substances.

Key Words: *Ocimum sanctum*, Norethynodrel, Genotoxicity

Introduction

Synthetic progestins have widespread use in medicine, but their side effects are often troublesome. They are used as oral contraceptives and in hormonal therapy. Norethynodrel is a synthetic progestin used either as single entity drug, or in combination with estrogens such as ethinylestradiol or mestranol in oral contraceptives.[1] It tested negative in bacterial tests.[2] It was found to be UDS positive in male rat hepatocytes in vitro,[3] but was found UDS negative in another study[4]. It induces chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), and inhibits lymphocyte proliferation in the presence of metabolic activation in vitro.[5] Norethynodrel induces pituitary, mammary, vaginal, and cervical tumours in mice when given alone, or with mestranol.[6] Chronic users of oral contraceptives are reported to have a relatively high frequency of sister chromatid exchanges (SCEs), significant increase in the number of lymphocytes with DNA migration,[7] and different types of cancer[8]. The genotoxic effects of steroids can be reduced by the use of various antioxidants,[9-10] and natural plant products.[11-12]

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Aqueous extract of *Ocimum sanctum* L. leaves have been used for the treatment of a variety of conditions including vomiting, fever, bronchitis, earache, diseases of the heart and blood, diabetes, arthritis, and asthma since ancient times.[13-14] Pharmacological evidence shows that Sacred Basil possesses immunomodulating,[15] chemopreventive,[16] anti-cancer,[17] antioxidant,[18-20] anti-mutagenic,[18,21-22] and antigenotoxic[23] properties. In the present study, the effect of *Ocimum sanctum* infusion at different dosages has been studied against the genotoxic damage induced by norethynodrel in human lymphocytes.

Materials and Methods

Chemicals: Norethynodrel (CAS No: 68-23-5) (17a)-17-Hydroxy-19-norpregn-5(10)-en-20yn-3-one (Sigma); RPMI 1640 (Gibco); Fetal calf serum (Gibco); Phyto-haemagglutinin-M (Gibco); Hoechst 33258 stain (0.05% w/v; Sigma); Dimethyl sulphoxide (5 ml); E. Merck, India); Colchicine 0.20 mg/ml (Microlab); Antibiotic-antimycotic mixture (Gibco); 3% Giemsa solution in phosphate buffer (pH 6.8) (E. Merck, India); Cyclophosphamide (SRI, India); 5-bromo-2-deoxyuridine (SRL, India).

Infusion preparation: The infusion was prepared with *in nature* leaves chopped with scissors into small pieces using medicinal plant *Ocimum sanctum* L. One gram of plant leaves was placed in 100 ml of boiling distilled water and covered for 5 min without heating. The material was then shaken for 5 min and filtered for sterilization. The infusion concentrations of 1.075×10^{-4}, 2.127×10^{-4} and 3.15×10^{-4} g/ml of culture medium were established.

Human lymphocyte culture: Duplicate peripheral blood cultures were prepared according to Carballo et al.[24] Briefly, 0.5 ml of heparinized blood samples were obtained from a healthy female donor, and were placed in a sterile flask containing 7 ml of RPMI-1640 medium, supplemented with 1.5 ml of fetal calf serum, 0.1 ml of phytohaemagglutinin, and 0.1 ml of antimycotic-antibiotic mixture and kept in an incubator for 72 hr at 37°C.

Preparation of S9 mix: S9 mix was prepared from the liver of healthy rats (Wistar strain) as per standard procedure of Maron and Ames.[25] The S9 fraction was enhanced by addition of 5 mM NADP and 10 mM of glucose-6-phosphate just before use. 0.5 ml of S9 mix was given along with each treatment.

Chromosomal aberrations (CAs) analysis: After 24 hr, 60 mg/ml of norethynodrel (dissolved in DMSO) was treated along with 1.075×10^{-4}, 2.127×10^{-4} and 3.15×10^{-4} g/ml of plant infusion separately, and kept for another 48 hr at 37°C in the incubator. After 47 hr, 0.2 ml of colchicine (0.2 mg/ml) was added to the culture flask. Cells were centrifuged at 1000 rpm for 10 min. The supernatant was removed and 5 ml of pre-warmed (37°C) KCl hypotonic solution (0.075 *M*) was added. Cells were re-suspended and incubated at 37°C for 15 min. The supernatant was removed by centrifugation at 1000 rpm for 10 min, and 5 ml of chilled fixative (methanol: glacial acetic acid; 3:1) was added. The fixative was removed by centrifugation and the procedure was repeated twice. The slides were stained in 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min. A total of 300 metaphases were examined for the occurrence of different types of abnormalities. Criteria to classify the different types of aberrations were in accordance with recommendations of EHC 46 for Environmental Monitoring of Human Population.[26]

Sister chromatid exchange (SCE) analysis: For sister chromatid exchange (SCE) analysis, bromodeoxyuridine (10 mg/ml) was added at the beginning of the culture. After 24 hr, 60 mg/ml of norethynodrel (dissolved in DMSO) was given along with 1.075×10^{-4}, 2.127×10^{-4} and 3.15×10^{-4} g/ml of plant infusion separately, and kept for another 48 hr of 37°C in the incubator. Mitotic arrest was done 1 hr prior to harvesting by adding 0.2 ml of colchicine (0.2 mg/ml). Hypotonic treatment and fixation were performed in the same way as described in the CAs analysis. The slides were processed according to Perry and Wolff.[27] The sister chromatid exchange average was taken from an analysis of fifty metaphases during second cycle of division.

Statistical analysis: Student's '*t*' test was used for the analysis of CAs and SCEs. The level of significance was tested from standard statistical table of Fisher and Yates.[28]

Results

In chromosomal aberration (CA) analysis, all the tested dosages of *Ocimum sanctum* L. infusions resulted in significant reduction of the genotoxic damage induced by 60 mg/ml of norethynodrel **(Table 1)**. Chromatid exchanges were completely eliminated by the very first tested dosages of *OS*I i.e. 1.075×10^{-4} g/ml. The tested *OS*I dosages *per se* did not induce any significant chromosomal aberrations.

In sister chromatid exchange (SCE) analysis, a significant decrease in SCEs/cell was observed at all the tested dosages of *OS*I **(Table 2)**. The *OS*I dosages *per se* did not induce significant SCEs/cell.

Discussion

The results of the present study show that the aqueous plant infusion from the leaves of *O. sanctum* reduced the genotoxic damage induced by norethynodrel in the

Table 1: Effect of *Ocimum sanctum* L. infusion on chromosomal aberrations (CAs) induced by norethynodrel in the presence of S9 mix

Treatments	Abnormal metaphases without gaps		Chromosomal aberrations				
	Number	Mean%±SE	Gaps	CTB	CSB	CTE	DIC
Norethynodrel (mg/ml)							
60	30	10.0±1.73[a]	18	15	12	3	-
Norethynodrel (mg/ml) + *OS* I (g/ml)							
60 + 1.075x10^{-4}	15	5.0±1.25[b]	9	9	6	-	-
60 + 2.125x10^{-4}	10	3.33±1.03[b]	4	7	3	-	-
60 + 3.15x10^{-4}	8	2.67±0.93[b]	3	6	2	-	-
Untreated	3	1.00±0.57	2	2	1	-	-
OS I (g/ml)							
1.075x10^{-4}	2	0.66±0.46	1	2	-	-	-
2.125x10^{-4}	2	0.66±0.46	1	2	-	-	-
3.15x10^{-4}	4	1.33±0.66	2	2	2	-	-
CP (mg/ml)							
0.16	42	14.0±2.00[a]	20	19	17	3	3

*OS*I : *Ocimum sanctum* infusion; SE: Standard error; CTB: Chromatid break; CSB: Chromosome break
CTE: Chromatid exchange; DIC: Dicentric; CP: Cyclophosphamide
[a]Significant difference with respect to untreated (P<0.01)
[b]Significant difference with respect to Norethynodrel (P<0.05)

Table 2: Effect of *Ocimum sanctum* L. infusion on sister chromatid exchanges (SCEs) induced by norethynodrel in the presence of S9 mix

Treatments	SCEs/Cell (mean±SE)	Range
Norethynodrel (mg/ml)		
60	13.4±0.43[a]	3 - 14
Norethynodrel (mg/ml) + *OS* I (g/ml)		
60 + 1.075x10^{-4}	9.12±0.29[b]	2 - 10
60 + 2.125x10^{-4}	7.10±0.26[b]	2 - 8
60 + 3.15x10^{-4}	6.16±0.23[b]	2 - 7
Untreated	1.14±0.10	0 - 5
OS I (g/ml)		
1.075x10^{-4}	1.64±0.13	0 - 5‘
2.125x10^{-4}	2.62±0.18	0 - 5
3.15x10^{-4}	2.84±0.20	0 - 5
CP (mg/ml)		
0.16	19.4±0.65[a]	3 - 20

*OS*I: *Ocimum sanctum* infusion; SE: Standard error; CP: Cyclophosphamide
[a]Significant difference with respect to untreated (P<0.01)
[b]Significant difference with respect to Norethynodrel (P<0.05).

presence of metabolic activation (S9 mix). Some synthetic progestins generate free radicals that are responsible for the genotoxic damage. Our study on other synthetic progestins such as ethynodiol diacetate, lynestrenol and norethynodrel showed genotoxic effects only in the presence of metabolic activation (S9 mix). Considering our study on synthetic progestin, we conclude that progestins having double bond between carbon-6 and carbon-7 undergo nucleophilic reaction and generate free radicals in the system,[10,29-30] while progestins in which double bond between carbon-6 and carbon-7 is absent undergo metabolic activation, and like estrogens, generate various forms of quinones which are responsible for the genotoxic damage.[31-32] Progestins like estrogens such as estradiol-17b and ethinylestradiol, undergo aromatic hydroxylation by cytochrome P450, and generate various forms of quinines.[33-34] Quinones by undergoing redox cycling in the presence of NADP, generate reactive oxygen species (ROS), which attack DNA and thus are responsible for the genotoxic damage.[35-36]

The leaf extract of *O. sanctum* reduces the radiation-induced damage in mouse bone marrow cells by scavenging free radicals.[18,22] Pharmacologically active compounds of *O. sanctum* like eugenol, rosmarinic acid and epigenin are excellent antioxidants.[21] Two water soluble flavonoids isolated from the Holy Basil, i.e., orientin and vicenin showed a protective effect against radiation induced genotoxic damage in cultured human lymphocytes by scavenging free radicals in the system.

In our present study, an infusion of *O. sanctum* significantly reduces the genotoxic damage induced by norethynodrel in the presence of metabolic activation system. Infusions of medicinal plants can modulate DNA damage when combined with other substances. Therefore, they should be used with utmost care, exactly following the traditional methods of preparation, especially with regard to the concentration of the infusions and the duration of treatments, so that infusions have the desired pharmacological effects without toxicity.

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