

## Protective role of nordihydroguaiaretic acid (NDGA) against the genotoxic damage induced by ethynodiol diacetate in human lymphocytes *in vitro*

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**Abstract:** Antioxidants and plant products are reported to reduce the genotoxic damage of steroids. In our present study, we have tested different dosages of nordihydroguaiaretic acid (NDGA) against the genotoxic damage induced by ethynodiol diacetate in the presence of S9 mix. Treatments with nordihydroguaiaretic acid (NDGA) results in the reduction of the genotoxic damage. A significant decrease was observed at all the tested doses of NDGA in sister chromatic exchanges of number of abnormal cells. The results suggest a protective role of NDGA against the genotoxic damage.

**Key words:** Nordihydroguaiaretic acid, Ethynodiol acetate, Chromosomal aberrations, Sister chromatid exchanges

### Introduction

In our earlier study, ethynodiol diacetate was found to be genotoxic in cultured human lymphocytes in the presence of metabolic activation system (Siddique and Afzal, 2004a). Steroids are used in the treatment of sexual and metabolic disorders and also in oral contraceptives (IARC, 1987). Earlier reports suggest that synthetic progestins have genotoxic potential (Singh *et al.*, 1994; Dhillon *et al.*, 1994; Brambilla and Martelli, 2002; Biri *et al.*, 2002; Martelli *et al.*, 2003; Siddique and Afzal, 2004bc; Siddique and Afzal, 2005a; Siddique *et al.*, 2005a). The prolonged use of the steroidal drugs can generate a risk of developing different types of cancer (IARC, 1999). The genotoxic effect of the steroids can be reduced by the use of antioxidants (Ahmad *et al.*, 2002; Siddique and Afzal, 2005b; Siddique *et al.*, 2005b) and natural plant products (Ahmad *et al.*, 2004; Siddique and Afzal, 2004d). In this context, natural plant products having antioxidant or free radical scavenging property can form a good base. Nordihydroguaiaretic acid (NDGA) is a phenolic lignan, present in the ever green shrubs *Larrea divaricata* and *Guaiacum officinale* (Agarwal *et al.*, 1991). NDGA possesses a number of interesting biological properties, which are potentially useful for humans, such as enzyme inhibitor (Capdevilla *et al.*, 1988), having antimicrobial properties (Hurtado *et al.*, 1979), protector from neurotoxicity and bladder toxicity (Nakayama *et al.*, 1991; Frasier and Kehrer, 1993), stimulator of corpus luteum for the secretion of progesterone (Carlson *et al.*, 1995), potential vaso and branchiodilator (Nagano *et al.*, 1996), antitumorigenic and antimutagenic properties (Wang *et al.*, 1991). NDGA has also been reported to possess genotoxic and antigenotoxic potential (Bujaidar *et al.*, 1998a, 1998b) both *in vivo* and *in vitro*. In the present study we have tested NDGA for its protective effects against ethynodiol diacetate induced genotoxic damage in human lymphocytes *in vitro*.

### Materials and Methods

**Chemicals:** RPMI 1640, Fetal calf serum, phytohaemagglutinin-M, antibiotic-antimycotic mixture (Gibco); Ethynodiol diacetate (CAS No.: 297-76-7, Wyeth Lab); Dimethylsulphoxide, Giemsa stain (Merck, India); 5-Bromo-2-deoxyuridine (SRL, India); Hoechst-33258 (Sigma), Nordihydroguaiaretic acid (Fluka).

### Human lymphocyte culture and sister chromatid exchanges (SCEs) analysis:

Duplicate peripheral blood cultures were prepared according to Carballo *et al.* (1993). 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 rpm 1640, supplemented with 1.5 ml of fetal calf serum, 0.1 ml of phytohaemagglutinin-M and 0.1 ml of antibiotic-antimycotic mixture. An amount of, 150 µg/ml of ethynodiol diacetate dissolved in dimethylsulphoxide (DMSO, 5 µl/ml) was added along with 1 ml of S9 mix, at the beginning of the culture and then incubated for 72 hr at 37°C. Simultaneously, 0.5, 1.0 and 1.5 µM of NDGA was given along with 150 µg/ml of ethynodiol diacetate separately in the presence of S9 mix. Normal, negative and positive control cultures were grown under identical conditions. Rat liver S9 fraction (S9 mix) was prepared from Swiss albino healthy rats (Wistar strain) as per standard procedures of Maron and Ames (1983). The S9 fraction was enhanced by addition of 5 µM of NADP and 10 µM of glucose-6-phosphate just before use.

For sister chromatid exchange (SCE) analysis, bromodeoxyuridine (10 µg/ml) was added at the beginning of the culture and the cells were harvested after 72 hr of incubation at 37°C. Two hours before harvesting, 0.2 ml of colchicine (0.2 µg/ml) was added to the culture flask. Cells were centrifuged at 1000 rpm for 10 min. The supernatant was removed and 5 ml prewarmed (37°C) 0.075M KCl hypotonic solution was added. Cells were

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resuspended and incubated at 37°C for 15 min. The supernatant was removed by centrifugation and fixation of cells was done by slowly adding 5 ml of fixative (methanol: glacial acetic acid; 3:1 ratio). The fixative was removed and the procedure was repeated twice. The slides were processed according to Perry and Wolff (1974). Slides were stained for 20 min in a 0.05% (w/v) Hoechst 33258 solution, rinsed with tap water and placed under a UV lamp for 90 min, covered with Sorensen's buffer and stained with a 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min. The SCE average was taken from an analysis of the metaphases during second cycle of cell division.

**Chromosomal aberrations (CAs) analysis:** For the analysis of CAs, parallel cultures were carried out without bromodeoxyuridine for 72 hr. Mitotic arrest was initiated 2 hr prior to harvesting by adding 0.2 ml of colchicine (0.2 µg/ml). Hypotonic treatment and fixation were performed in the same way as in SCE, analysis. To prepare slides, 3-5 drops of the fixed cell suspension were dropped on a clean slides and air dried. The slides were stained in 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min. 300 well-spread metaphases were examined for the occurrence of different types of structural CAs. The criteria to classify the different types of aberrations were in accordance with the recommendation of EHC 46 for Environmental Monitoring of Human Populations (IPCS, 1985).

**Statistical analysis:** Student's 't' test was used for the analysis of SCEs and CAs. The level of significance was tested from standard statistical tables of Fisher and Yates (1963).

### Results and Discussion

In SCEs analysis, a significant decrease was observed at all the tested doses of NDGA with ethynodiol diacetate (Table 1).

In CAs analysis NDGA treatment results in the reduction of abnormal cells. A significant decrease in the number of abnormal cells was observed at all the tested doses of NDGA with 150 µg/ml of ethynodiol diacetate (Table 2). Chromatid exchanges and dicentric chromosomes were completely eliminated even at lowest tested dose of NDGA.

The results of the present investigations reveal that treatment with NDGA results in the reduction of the genotoxic

**Table - 1:** Effect of nordihydroguaiaretic acid (NDGA) on sister chromatid exchanges (SCEs) induced by ethynodiol diacetate in the presence of S9 mix

Treatment	Cells scored	SCEs/Cell (mean±SE)
Ethynodiol diacetate 150 µg/ml	100	14.43±1.02 <sup>a</sup>
NDGA 0.5 µM	100	5.01±0.13
1.0 µM	100	5.33±0.16
1.5 µM	100	6.01±0.33
Ethynodiol diacetate + NDGA 150 µg/ml + 0.5 µM	100	9.34±0.73 <sup>b</sup>
150 µg/ml + 1.0 µM	100	8.01±0.63 <sup>b</sup>
150 µg/ml + 1.5 µM	100	6.69±0.54 <sup>b</sup>
Untreated	100	5.34±0.18 <sup>a</sup>
Negative control (DMSO, 5 µl/ml)	100	5.67±0.27
Positive control (Cyclophosphamide, 0.16 µg/ml)	100	29.63±1.33 <sup>a</sup>

<sup>a</sup>Significant with respect to untreated ( $p < 0.01$ ), <sup>b</sup>Significant with respect to ethynodiol diacetate ( $p < 0.05$ ), DMSO: Dimethylsulphoxide; SE: Standard error; NDGA: Nordihydroguaiaretic acid

**Table - 2:** Effect of nordihydroguaiaretic acid (NDGA) on chromosomal aberrations (CAs) induced by ethynodiol diacetate in the presence of S mix

Treatment	Cells scored	Abnormal cells (%)	Total structural CA					Total breaks without gaps (%)
			Gaps	CTB	CSB	CTE	DIC	
Ethynodiol diacetate 150 µg/ml	300	28 (9.33) <sup>a</sup>	24	30	11	3	1	49 (16.33)
NDGA 0.5 µM	300	6 (2)	5	4	2	-	-	6 (2.00)
1.0 µM	300	7 (2.33)	6	5	2	-	-	7 (2.33)
1.5 µM	300	9 (3.00)	7	6	3	-	-	9 (3.00)
Ethynodiol diacetate + NDGA 150 µg/ml + 0.5 µM	300	18 (6) <sup>b</sup>	11	17	8	-	-	25 (8.33)
150 µg/ml + 1.0 µM	300	13 (4.33) <sup>b</sup>	9	10	5	-	-	15 (5.00)
150 µg/ml + 1.5 µM	300	9 (3.00) <sup>b</sup>	7	7	2	-	-	9 (3.00)
Untreated	300	5 (1.67)	3	4	1	-	-	5 (1.67)
Negative control (DMSO, 5 µl/ml)	300	6 (2.00)	4	4	2	-	-	6 (2.00)
Positive control (Cyclophosphamide, 0.16 µg/ml)	300	82 (27.33) <sup>a</sup>	40	52	24	3	2	86 (28.67)

<sup>a</sup>Significant with respect to untreated ( $p < 0.01$ )

<sup>b</sup>Significant with respect to ethynodiol diacetate ( $p < 0.05$ )

CTB: Chromatid break; CSB: Chromosome break; CTE: Chromatid exchange; DIC: Dicentric; NDGA: Nordihydroguaiaretic acid

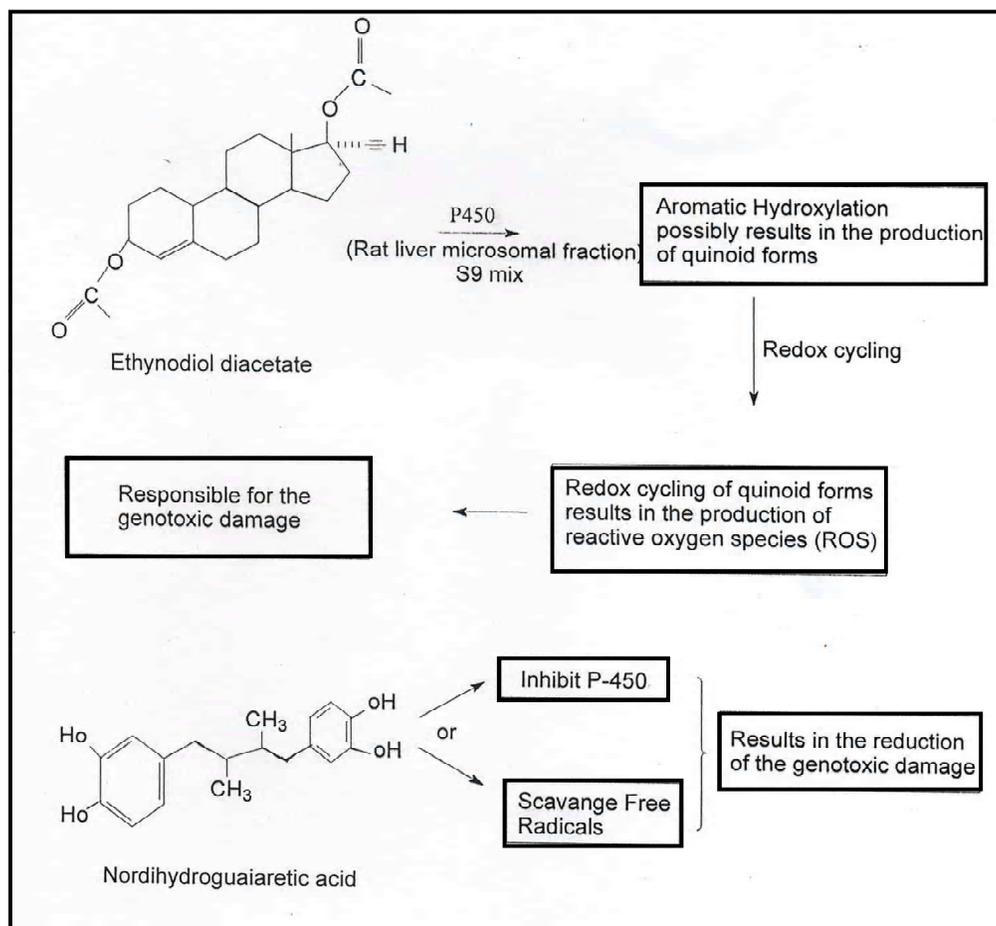


Fig. 1: Possible metabolism of ethynodiol diacetate and protective mechanism of nordihydroguaiaretic acid (NDGA)

damage caused by 150 µg/ml of ethynodiol diacetate in the presence of S9 mix. Our earlier study shows that metabolic activation of ethynodiol diacetate and possible conversion of it to reactive species is responsible for its genotoxicity (Siddique and Afzal, 2004a). Metabolic activation of estrogens, such as estradiol-17β and ethinylestradiol, results in the production of reactive oxygen species via redox cycling between quinone and their semiquinone derivatives (Bolton, 2002; Siddique *et al.*, 2005a). Cytochrome P450s in liver S9 fractions plays an important role in activating promutagens to proximate and/or ultimate mutagens. Rat and human liver P450s is involved in the activation of some chemical carcinogens having different isoforms (Maron and Ames, 1983; Guengerich and Shimada, 1991). In our earlier studies synthetic progestins have been shown to be genotoxic by generating reactive oxygen species either by nucleophilic reaction (Siddique and Afzal, 2005a; Siddique and Afzal, 2004b) or possibly by redox cycling between various forms of quinones in the presence of S9 mix (Siddique *et al.*, 2005a).

Since the NDGA treatment, in the presence of S9 mix, reduced the genotoxic damage by ethynodiol diacetate, the reduction may be due to the inhibition of cytochrome P450s (Capdevilla *et al.*, 1988) which prevents the metabolic activation

of ethynodiol diacetate or NDGA may act as a free radical scavenger (Nakadate, 1989; Marthy *et al.*, 1990) (Fig. 1). The identification of specific mechanism is under investigation. In the light of the results obtained in our present study, we can say that NDGA can reduce the genotoxic damage induced by ethynodiol diacetate in the presence of metabolic activation (S9 mix).

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