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ANTIGENOTOXIC EFFECT OF FERULIC ACID IN 7,12-DIMETHYL BENZ(A)-
ANTHRACENE (DMBA) INDUCED GENOTOXICITY

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Abstract

The antigenotoxic effect of ferulic acid was carried out by evaluating the cytogenetic markers, the micronuclei frequency and chromosomal aberrations, in the bone marrow of hamsters in 7,12-dimethylbenz(a)anthracene (DMBA) induced genotoxicity. Genotoxicity was induced in experimental hamsters by single intraperitoneal injection of DMBA (30mg kg⁻¹ b.w). Pretreatment of ferulic acid orally at a dose of 40mg kg⁻¹ b.w for five days significantly reduced the frequency of micronucleated polychromatic erythrocytes (MnPCEs) and the percentage of chromosomal aberrations in hamster's bone marrow. Our results thus suggest that ferulic acid has potent antigenotoxic effect in DMBA induced genotoxicity in golden Syrian hamsters.

Key words: DMBA, ferulic acid, genotoxicity, chromosomal aberrations, lipid peroxidation, antioxidants, hamster.

Abbreviations: CAT - Catalase; DMBA - 7,12-dimethylbenz(a)anthracene; GPx - Glutathione peroxidase; MnPCEs - Micronucleated polychromatic erythrocytes; ROS - Reactive oxygen species; SOD - Superoxide dismutase; TBARS - Thiobarbituric acid reactive substances

Introduction

Cytogenetics, the study of the structure and function of chromosomes, is useful in the study and treatment of patients with malignancy and haematologic disorders. Cytogeneticists could easily identify chromosome defects, subtle deletions, inversions, insertions, translocations, fragile sites and other more complex rearrangements and refine break points. Cells used for chromosome analysis are taken mostly from amniotic fluid, blood sample and bone marrow. Gene mutation and chromosomal damage are essential in the multistep process of carcinogenesis. Genotoxic agents such as carcinogens can enhance the error rate in the genome reduplication and cause mutation in the DNA of an organism (Britto and Ravindran, 2007; Poppe *et al.*, 2007).

Genotoxicity tests are commonly used to identify genotoxic chemicals with carcinogenic potential. Cytogenic markers such as chromosome aberrations (abnormality in chromosomal structure and number), micronuclei frequency and sister chromatid exchanges are relatively rapid, facile and sensitive indicators of genetic damage (Ohba *et al.*, 2007; Wu *et al.*, 2004). A hallmark of neoplastic transformation is the accumulation of genetic lesions and is characterized by having aberrant chromosomes. The degree of chromosomal aberrations is correlated with tumor progression (Cheung and Loeb, 1997). Exposure of the human populations to genotoxic agents can be monitored by measuring DNA and protein adducts and by

assessing chromosomal aberrations and gene mutations (Gaizev *et al.*, 1996). Cancer patients are often exposed to high level of DNA damaging agents. Somatic cell mutation induced by genotoxic agents has been implicated in the pathogenesis of cancer (Nersesian *et al.*, 1993).

DMBA, a potent organ specific carcinogen, is proficient in oxidizing both DNA bases and deoxyribose sugars through its metabolites diol epoxide. It has been demonstrated that DMBA exposure results in pronounced mutagenic response in several *in vivo* and *in vitro* mutation assay system. Polyploidy and sister chromatic exchanges have been shown in DMBA induced genotoxicity (Han *et al.*, 2007; Bhuvaneshwari *et al.*, 2004; Chandramohan *et al.*, 2003).

Reactive oxygen species (ROS) can induce chromosomal aberrations through oxidative base damage and strand breaks in DNA contributing to mutagenesis and carcinogenesis. ROS mediated oxidative DNA damage may consist of strand break, abasic sites, alkali-labile sites and oxidized bases (Nagy *et al.*, 2007). The genome is particularly vulnerable to oxidative damage when antioxidants defenses are inefficient.

Measurement of MnPCEs frequency and chromosomal aberrations in bone marrow has been widely used as tool to assess the antigenotoxic effect of chemopreventive agents. Ferulic acid, a well-established antioxidant, arises from the metabolism of phenylalanine and tyrosine by Shikimate pathway in plants. This phenolic acid is present in fruits, vegetables, barley, rice and wheat. Ferulic acid possesses diverse pharmacological functions including anti-inflammatory, antiageing, anticancer, antidiabetic, antiapoptotic, hepatoprotective and neuroprotective (Tanaka *et al.*, 1993; Srinivasan *et al.*, 2005; Rukkumani *et al.*, 2004). The anticarcinogenic potential of ferulic acid in skin, colon, liver, tongue and mammary carcinogenesis has been reported (Huang *et al.*, 1988; Kawabata *et al.*, 2000; Tanaka *et al.*, 1993). Ferulic acid has an important role in the prevention of LDL oxidation and scavenging superoxide anion radicals (Castelluccio *et al.*, 1996). Ferulic acid acts as a strong membrane antioxidant in humans and is known to be effective against various disorders including cancer. The health benefits of ferulic acid is gaining a lot of attention currently in the research world but its influence against genotoxicity has not yet been entirely proven. To the best of our knowledge, no scientific research has been carried out on antigenotoxic effect of ferulic acid in DMBA induced genotoxicity. Thus, the present study was designed to evaluate the antigenotoxic effect of ferulic acid in DMBA induced genotoxicity. The mechanistic pathway for antigenotoxic potential of ferulic acid was monitored by measuring lipid peroxidation byproducts and endogenous antioxidant status in plasma and erythrocytes of DMBA treated hamsters.

Materials and Methods

Animals

Male golden Syrian hamsters 8-10 weeks old weighing 80-120g were purchased from National Institute of Nutrition, Hyderabad, India, and maintained in Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The hamsters were housed four or five in a polypropylene cage and provided standard pellet diet (Agro Corporation Private Limited, Bangalore, India) and water *ad libitum*. The standard pellet diet is composed of 21% protein, 5% lipids, 4% crude fiber, 8% ash, 1% calcium, 0.6% phosphorus, 3.4% glucose, 2% vitamin, and 55% nitrogen-free extract (carbohydrates). The hamsters were maintained under controlled conditions of temperature and humidity with a 12h light/dark cycle.

Chemicals

7,12-dimethylbenz(a)anthracene (DMBA), ferulic acid, colchicines, May-Grunwald stain, and Giemsa stain were purchased from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore, India. All other chemicals used were of analytical grade, purchased from Hi-media Laboratories, Mumbai, India.

Experimental protocol

The experimental design was approved by the Annamalai University animal ethical committee [Reg. No: 160/1999/CPCSEA], Annamalai University, Annamalainagar. A total number of 24 hamsters were divided into 4 groups and each group contained 6 hamsters. Group 1 served as untreated control. Group 3 hamsters were pretreated with ferulic acid (40mg kg⁻¹ b.w) for five days. At the end of the 5th day,

groups 2 and 3 hamsters were intraperitoneally injected with DMBA (30mg kg⁻¹ b.w) after two hrs of administration of ferulic acid. Group 4 hamsters were received ferulic acid alone and were not received DMBA. All hamsters were provided standard pellets and water *ad libitum*. All the hamsters were anaesthetized and sacrificed at the 6th day by cervical dislocation for the assessment of micronucleus frequency and chromosomal aberrations.

Assessment of chromosomal aberrations in bone marrow was carried out according to the procedure of Kilian *et al.*, (1977). The femur bones were removed from hamsters injected intraperitoneally with 0.1% colchicines (1ml/100gm b.w) 90 minutes before sacrificing the hamsters. The bone marrow contents were flushed into 5 ml of physiological saline and centrifuged at 500 g for five minutes. The sediment obtained were resuspended in 6 ml of hypotonic KCl (0.075 M) and incubated at 37^o C for 25 min. The pellets were then fixed using methanol:acetic acid (3:1) fixative and stained with Giemsa stain. One hundred well spread metaphase cells were scored for each hamster and structural chromosomal aberrations were observed and recorded.

Bone marrow micronucleus test was carried out according to the method of Schmid (1975). The femur bones removed from the hamsters were cleaned and the content was flushed into tube containing 1 ml of calf serum and was centrifuged at 500 g for 10 min. The obtained pellet was suspended with few drops of fresh serum and slides were prepared and air-dried for 18 h. After drying, the slides were stained with May-Grunwald stain followed by Giemsa stain. The frequency of MnPCEs in each group was calculated by scoring 2500 polychromatic erythrocytes (PCEs) per hamster.

Lipid peroxidation as evidenced by formation of TBARS was assayed in plasma by the method of Yagi (1978). Enzymatic antioxidants activities were measured in plasma according to the methods of Kakkar *et al* (1984) [superoxide dismutase], Rotruck *et al* (1973) [glutathione peroxidase] and Sinha (1972) [catalase]. The above mentioned biochemical parameters were analyzed by using specific colorimetric methods.

Statistical analysis

The data are expressed as mean \pm SD. Statistical comparisons were performed by one way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). The results were considered statistically significant if the p values were 0.05 or less.

Results

The frequency of MnPCEs and chromosomal aberrations in control and experimental hamsters in each group are given in Tables 1 and 2 respectively. Hamsters treated with DMBA (Groups 2 and 3) showed a high frequency of MnPCEs and chromosomal aberrations (chromatic gap, chromosomal gap, chromatic break, chromosomal break, fragment, minute) as compared to control hamsters. However, hamsters treated with DMBA alone (group 2) showed highest frequency of MnPCEs and chromosomal aberrations as compared to control hamsters. The frequency of MnPCEs and chromosomal abnormalities were significantly reduced in DMBA treated hamsters pretreated with administration of ferulic acid orally. Oral pretreatment of ferulic acid alone displayed no significant differences in MnPCEs and chromosomal aberrations as compared to control hamsters.

Table 3 shows the status of TBARS and enzymatic antioxidants status in plasma of control and experimental hamsters in each group. TBARS and enzymatic antioxidants were increased in DMBA treated hamsters as compared to control hamsters. Oral pretreatment of ferulic acid brought back the status of TBARS and antioxidants in DMBA treated hamsters. No significant difference was observed between control hamsters and ferulic acid alone treated hamsters.

Discussion

The field of cancer genetics deals with the transformation of somatic cell to mutant cell through intrinsic DNA alterations, epigenetic changes, structural chromosomal aberrations and numerical chromosomal changes (Stitich *et al.*, 1983). Chromosomal instability is a common phenomenon of malignant tumours. Cytogenetic markers (micronuclei frequency and chromosomal aberrations) have been

widely used to assess the mutagenicity of genotoxic chemicals, mainly carcinogens, which can induce various DNA lesions in different proportions (Miyamoto *et al.*, 2007). Micronucleus assay is a valuable complement to the chromosome aberration measurement and is comparatively easy to perform and inexpensive. Scientific studies have suggested that the presence of one or more micronuclei in peripheral erythrocytes in rodent is an accepted marker of chromosomal breakage or loss that occurred prior to the extrusion of the nucleus during erythrocyte differentiation (Venkatesh *et al.*, 2007; Gaizev *et al.*, 1996).

Table 1.: Effect of ferulic acid on DMBA-induced bone marrow micronuclei formation

Group	Parameters	MnPCEs / 2500 PCEs	PCEs/NCEs	PCEs* (%)
1.	Control	5.43 ± 0.21 ^a	1.02 ± 0.06 ^a	50.4
2.	DMBA	60.2 ± 5.3 ^b	0.73 ± 0.08 ^b	42.3
3.	DMBA + Ferulic acid	22.8 ± 3.1 ^c	0.90 ± 0.07 ^c	47.9
4.	Ferulic acid alone	6.09 ± 0.73 ^a	1.04 ± 0.08 ^a	50.2

Values are expressed as mean ± SD; n = 6. Values not sharing a common superscript significantly differ at P < 0.05. (DMRT). * Percentage of polychromatic erythrocytes was calculated as follows: [PCEs / (PCEs+NCEs) x 100].

Table 2.: Mitotic index and frequencies of chromosomal abnormalities in experimental and control animals

Group	Parameters	Mitotic index (%) [*]	Chromosomal aberrations ^{**} hamster ⁻¹					Total aberrations rat ⁻¹	Abnormal metaphase rat ⁻¹
			G [#]	B [']	B ^{''}	F	M		
1.	Control	4.51±0.73 ^a	0.45±0.06 ^a	1.18±0.22 ^a	0.00 ^a	1.37±0.11 ^a	0.29±0.03 ^a	2.9±0.32 ^a	1.52±0.13 ^a
2.	DMBA	1.83±0.09 ^b	9.73±0.6 ^b	6.91±0.79 ^b	2.32±0.31 ^b	6.93±0.57 ^b	2.16±0.28 ^b	18.3±2.1 ^b	14.2±1.64 ^b
3.	DMBA + Ferulic acid	2.97±0.58 ^c	4.79±0.51 ^c	3.21±0.52 ^c	1.2±0.17 ^c	3.69±0.27 ^c	1.34±0.16 ^c	9.5±0.96 ^c	5.8±0.98 ^c
4.	Ferulic acid alone	4.33±0.84 ^a	0.47±0.07 ^a	1.36±0.26 ^a	0.00 ^a	1.58±0.15 ^a	0.33±0.04 ^a	3.2±0.34 ^a	1.54±0.14 ^a

Values are expressed as mean ± SD; n = 6. Values not sharing a common superscript significantly differ at P < 0.05. (DMRT). G[#] - Gap, B['] - Chromatic Break, B^{''} - Isochromatid Break, F - Fragment, M - Minute, * - Mitotic index has been calculated by analyzing 1000 cells/animal (for a total of 6000 cells/treatment) and percentage of the mitotic cells calculated for each treatment group. ** - Frequency per 100 cells. Each chromosomal aberration has been counted by analyzing 100 cells/animal (6 animals/group, for a total of 600 cells/treatment) and mean ±SD were calculated per treatment group. # - Gaps were not included in total chromosomal aberrations.

Table 3: Plasma TBARS and antioxidants in control and experimental animals.

Group	Parameters	TBARS (nmol/ml)	GPx (U ^A /L)	SOD (U ^B /ml)	CAT (U ^C /ml)
1.	Control	1.78±0.21 ^a	103.7±8.9 ^a	3.01±0.27 ^a	1.12±0.07 ^a
2.	DMBA	3.03±0.46 ^b	139.7±12.6 ^b	4.72±0.51 ^b	1.59±0.12 ^b
3.	DMBA + Ferulic acid	2.39±0.34 ^c	118.5±11.8 ^c	3.59±0.42 ^c	1.38±0.17 ^c
4.	Ferulic acid alone	1.73±0.16 ^a	105.2±7.4 ^a	3.13±0.21 ^a	1.15±0.08 ^a

Values are expressed as mean ± SD (n=6), Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT). B = The amount of enzyme required to inhibit 50% NBT reduction, C = Micromoles of H₂O₂ utilized/min, A = Micromoles of glutathione utilized/min

Several studies have been carried out which showed that chromosomal aberrations are the consequences of DNA damage, poor DNA repair and misreplication (Sang and Li, 2005; Stitich *et al.*, 1983).

DMBA can cause chromosomal damage by binding with adenine residues of DNA through its active metabolite diol epoxide and other free radicals formed during metabolic activation (Manjanatha *et al.*, 1998). Husain *et al.* (1989) have shown H-ras mutations and over expression of C-erb N and its product in DMBA induced hamster buccal pouch carcinogenesis. A to T transversions in Ha-ras codon 61 has been demonstrated in DMBA induced mouse skin and hamster buccal pouch carcinogenesis (Chang *et al.*, 1996). An increase in micronucleus frequency and chromosomal aberrations in the bone marrow of DMBA painted or injected rodents have been reported earlier (Bhuvaneswari *et al.*, 2004). Osaka *et al.*, (1996) have suggested that N-ras mutation is an earliest event in DMBA induced leukemogenesis. Oral pretreatment of ferulic acid significantly decreased the frequency of MnPCEs and percentage of chromosomal aberrations in DMBA treated hamsters, which clearly indicate its potent antigenotoxic effect in DMBA induced genotoxicity.

Reactive oxygen species (ROS) are formed in human body as an unavoidable byproducts of cellular oxygen metabolism. However, human body has an array of antioxidant defense mechanism to combat the harmful effects of reactive oxygen species. Reactive oxygen species that are overproduced during oxidative stress are among the most intracellular modifiers of DNA. In the present study, we observed an increase in TBARS and activities of antioxidant enzymes in DMBA treated hamsters. Elevated activities of antioxidant enzymes in the plasma confirm the oxidative stress in DMBA treated hamsters. Oxidative stress, an imbalance in oxidant and antioxidant status that has been repeatedly addressed as an indicator of indirect genotoxicity, is considerably increased in certain pathological conditions including cancer. Carcinogenic chemicals generate excess ROS and induce lipid peroxidation process during its metabolic activation can lead to different types of toxicity including genotoxicity and cell death (Bhuvaneswari *et al.*, 2004; Manoharan *et al.*, 2006). Chandramohan *et al.*, (2003) have demonstrated the DNA damaging and mutagenic effects of DMBA in DMBA induced genotoxicity and oxidative stress in mice. Our results thus suggest that the observed increase in the frequency of MnPCEs and chromosomal aberrations in DMBA treated hamsters are due to excessive generation of reactive oxygen species in the system.

Profound studies have reported that substances that can reduce the genetic instability in somatic cells exert their protective role in carcinogenesis by interfering with covalent interaction of carcinogens with DNA, modifying DNA repair process and antioxidant functions (Kussmaul *et al.*, 2006; Bhuvaneswari *et al.*, 2004; Manoharan *et al.*, 2006; Chandramohan *et al.*, 2003). *In vivo* and *in vitro* studies have shown the free radical scavenging properties of ferulic acid and its dihydrodimers (Castelluccio *et al.*, 1996; Garcia-Conesa *et al.*, 1997). Ferulic acid is known to protect oxidative DNA damage and prevents lipid peroxidation process (Kanski *et al.*, 2002). In the present study, oral pretreatment of ferulic acid restored the status of plasma TBARS and antioxidant enzymes, which indicates its free radical scavenging property during DMBA induced genotoxicity.

Conclusion

The present study has demonstrated the antigenotoxic effect of ferulic acid in DMBA induced genotoxicity in golden Syrian hamsters. The antigenotoxic mechanism of ferulic acid can be related to its antioxidant potential to neutralize the toxic effects of ROS generated by DMBA during its metabolic activation.

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