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N-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)-induced *in vivo* clastogenicity: protective effects of aqueous neem leaf extract

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We evaluated the modifying effects of aqueous neem leaf extract on the *in vivo* clastogenicity of N-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), a potent gastric carcinogen by quantitation of micronuclei and chromosomal aberrations in metaphase cells from the bone marrow of male Wistar rats. Intraperitoneal injection of MNNG (40 mg/kg body weight) induced a significant increase in the frequency of micronuclei and chromosomal aberrations. Pretreatment with aqueous neem leaf extract (100 mg/kg body weight) significantly reduced MNNG-induced increase in micronuclei and chromosomal aberrations. These results reveal the chemoprotective potential of aqueous neem leaf extract against the clastogenic effects of MNNG.

1. Introduction

Chemoprevention has evolved as an effective approach to control the incidence of stomach cancer, the second most common malignancy worldwide and a major cause of mortality in Chennai, India [1, 2]. Accumulating evidence supports the hypothesis that medicinal plants and phytochemicals may offer chemoprotection during experimental gastric carcinogenesis induced by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) [3–5]. In previous reports we documented the tumour inhibitory effects of garlic, lycopene and neem leaf in the MNNG model [6–8].

Azadirachta indica A Juss, commonly known as neem has attained worldwide prominence because of its medicinal properties [9]. Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex. All parts of the neem tree, leaves, flowers, oil and bark have been used in Ayurveda as well as in other systems of traditional medicine for the treatment of infections, skin diseases and dental disorders. Neem preparations have been reported to exhibit a wide range of pharmacological properties including anti-inflammatory, antihyperglycemic and immunomodulatory effects [9–12]. Studies from this laboratory have demonstrated the hepatoprotective, antioxidant and the anticarcinogenic activities of aqueous neem leaf extract during hamster buccal pouch carcinogenesis [13, 14]. Most notably, extracts of neem leaf have been reported to protect against gastric ulcer, a premalignant lesion and inhibit infection by *Helicobacter pylori*, an important etiological agent in gastric carcinogenesis [15, 16]. Previously, we reported a positive correlation between the chemopreventive efficacy of aqueous extract of neem leaf against MNNG-induced gastric carcinogenesis and its modulatory effects on lipid peroxidation, antioxidant and detoxification systems [17, 18].

The current focus of chemoprevention is on biomarkers capable of detecting early changes that can be correlated with tumour progression as well as regression. Cytogenetic biomarkers have assumed significance as reliable, early indicators of the biological effects of carcinogen-induced DNA damage due to the strong association between chromosomal alterations and carcinogenesis [19]. We therefore evaluated the effect of pretreatment with aqueous neem leaf extract on MNNG-induced genetic damage by quantitation of micronuclei and chromosomal aberrations as endpoints of chemoprevention.

2. Investigations and results

The micronucleus test was carried out according to the method described by Schmid [20]. The bone marrow from the femur was flushed in the form of a fine cell suspension into a centrifuge tube containing fetal calf serum. The cell suspension was centrifuged at 500 × g for 10 min and the supernatant was discarded. The pellet was resuspended in a drop of serum and used for preparing slides. The air-dried slides were stained with May-Grünwald and Giemsa. A total of 1000 polychromatic erythrocytes were scored per animal to determine the frequency of micronucleated polychromatic erythrocytes (MnPCE).

Bone marrow cells from control and experimental animals were processed for analysis of chromosomal aberrations by the method of Sharma and Sharma [21]. The bone marrow from the femurs was flushed into a centrifuge tube containing 0.9% saline and centrifuged at 500 × g for 5 min. The supernatant was removed and hypotonic KCl was added to the sediment. After incubation for 20 min at 37 °C, the contents were centrifuged for 5 min and the sediment was fixed in methanol-acetic acid (3 : 1 v/v). Three changes of fixative were given prior to slide

Table 1: Frequencies of micronucleated polychromatic erythrocytes in control and experimental animals

Group	Treatment	MnPCEs/1000 PCEs Mean ± SEM
1	MNNG	40.3 ± 5.6[a]
2	MNNG + Neem leaf	21.3 ± 3.2[ab]
3	Neem leaf	15.4 ± 3.0[ab]
4	Control	16.1 ± 2.5

Transformed values are presented as mean ± SEM for groups of ten rats
A total of 1000 cells were counted/animal and averaged over the number of animals
Values are statistically significant at $p < 0.05$
[a] Significantly different from group 4
[b] Significantly different from group 1

preparation. The slides were air-dried, stained in Giemsa solution and scored blindly. One hundred well-scattered metaphase plates were scored for each animal, giving a total of 1000 metaphases per group.

The data were transformed using Arcsine table and analysed by two way analysis of variance (ANOVA) followed by Neuman-Keuls' multiple range test to compare the significance of differences among the different experimental sets.

The data presented in Table 1 show the influence of pretreatment with aqueous neem leaf extract on the frequency of MnPCE induced by MNNG. A significant increase in the frequency of MnPCE was observed in Group 1 compared with Group 4. Pretreatment with neem leaf extract significantly reduced the frequency of MNNG-induced MnPCE.

Table 2 shows the effect of pretreatment with neem leaf extract on MNNG-induced chromosomal aberrations. The frequency of chromosomal aberrations was significantly increased in group 1 compared with group 4. Pretreatment with neem leaf extract significantly reduced the frequency of MNNG-induced chromosomal aberrations.

3. Discussion

N-Nitroso compounds, the predominant aetiological agents in gastric carcinogenesis are genotoxins that induce gastric mucosal mutagenesis leading to intestinal metaplasia, dysplasia and finally carcinoma [22]. MNNG, a nitroso compound and a potent gastric carcinogen is widely known as a mutagen [23, 24]. MNNG induces single and double strands in DNA [25]. In an earlier study, we showed that oxygen free radical (OFR)-induced lipid peroxidation plays a key role in the mutagenic and carcinogenic effects of MNNG [26]. OFR, primarily hydroxyl radicals are unstable and highly reactive. They produce a wide range of DNA lesions and activate oncogenes [27, 28]. The enhanced frequencies of micronuclei and chromosomal aberrations in MNNG-treated animals in the present study confirm reports by other workers on the clastogenic effects of MNNG [29, 30].

Pretreatment with neem leaf extract significantly ameliorated the clastogenic effects of MNNG. In an earlier report, we demonstrated the modulatory effects of pretreatment with aqueous neem leaf extract on MNNG-induced oxidative stress [26]. The protective effects of neem leaf on MNNG-induced genotoxicity may therefore be related to its free radical scavenging properties. Aqueous neem leaf contains a number of potent antioxidants and anticarcinogens that lower lipid peroxidation levels. These include ascorbic acid and the flavonoids, quercetin and kaempferol. Ascorbic acid present in neem leaf is known to trap hydroxyl radicals, inhibit the formation of N-nitroso compounds and attenuate the mutagenic potency of MNNG [31–33]. Ascorbic acid and quercetin are recognised to modulate the DNA-damaging effect of MNNG [33]. Quercetin and kaempferol have been reported to retard both initiation and promotion stages of carcinogenesis [34].

The results of the present study indicate that pretreatment with aqueous extract of neem leaf exerts significant protection against MNNG-induced chromosomal damage. These findings strengthen the observation that medicinal plants have potential inhibitory effects on chemical mutagenesis and carcinogenesis. Due to lack of toxicity and ubiquitous distribution in nature, neem leaf may be regarded as a valuable plant source for use in traditional medicine and modern drug development.

4. Experimental

4.1. Animals

All the experiments were carried out with male Wistar rats aged 6–8 weeks obtained from the Central Animal House, Annamalai University, India. The animals housed in polypropylene cages were provided food and water *ad libitum* and maintained under standard conditions of temperature and humidity with an alternating light/dark cycle. All animals were fed standard pellet diet (Mysore Snack Feed, Mysore, India). The animals used in the present study were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad and approved by the ethical committee, Annamalai University.

4.2. Chemicals

MNNG was obtained from Fluka-Chemika-Biochemika, Buchs, Switzerland. All other reagents used were of analytical grade.

4.3. Collection of plant material

Fresh matured leaves of *A. indica* collected locally during March–April were identified by a pharmacognosy expert. These leaves were dried in the shade, powdered and the powders were used for extraction. Voucher specimens were deposited at the herbarium of the Botany Department, Annamalai University.

Table 2: Frequencies of chromosomal aberrations in control and experimental animals

Group	Treatment	Types of aberrations							Chromosomal aberrations Mean ± SEM*
		G′	G″	B′	B″	F	M	Total	
1	MNNG	4	7	12	7	116	5	151	16.8 ± 1.2[a]
2	MNNG + neem leaf	5	6	10	6	36	2	65	7.2 ± 0.80[b]
3	Neem leaf	6	5	7	4	28	2	52	5.8 ± 0.83[b]
4	Control	7	6	4	3	24	3	47	5.2 ± 0.76

G′ – chromatid gap; G″ – isochromatid gap; B′ – chromatid break; B″ – chromosome break; F – fragment; M – minute
* Transformed values are presented as mean ± SEM for groups of 10 rats. 1000 metaphases from ten animals per treatment were analysed. Values are statistically significant at $p < 0.05$
[a] Significantly different from group 4
[b] Significantly different from group 1

4.4. Preparation of aqueous neem extract

An aqueous extract of neem leaf was prepared by homogenising the required amount of fresh leaves from the neem tree in an appropriate volume of distilled water to give a concentration of 25 mg/mL [12]. The homogenate was centrifuged at 3120 × g for 10 min to remove the particulate matter and the supernatant fraction was used for the experiment. At this stage of preparation, 96% of the extract was remaining.

4.5. Treatment schedule

The animals were randomised into control and experimental groups and divided into four groups of 10 animals each. Animals in group 1 were injected MNNG (40 mg/kg body weight) intraperitoneally [23]. Group 2 animals received 100 mg/kg body weight neem leaf extract by intragastric intubation for 5 days followed by intraperitoneal injection of MNNG (40 mg/kg body weight) 90 min after the final feeding. Rats in group 3 were given neem leaf extract alone for 5 days. Group 4 received the same amount of distilled water and served as control. All animals were injected colchicine 90 min prior to sacrifice. The animals were killed by cervical dislocation 27 h after MNNG exposure.

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