RESEARCH COMMUNICATION

Antioxidative and Modifying Effects of a Tropical Plant *Azadirachta indica* (Neem) on Azoxymethane-induced Preneoplastic Lesions in the Rat Colon

Junya Arakaki[1,2], Masumi Suzui[1], Takamitsu Morioka[1], Tatsuya Kinjo[1, 2], Tatsuya Kaneshiro[1], Morihiko Inamine[1], Nao Sunagawa[1], Tadashi Nishimaki[2], Naoki Yoshimi[1]

Abstract

The purpose of the present study was to examine whether Neem leaf (*Azadirachta indica*) has short-term chemopreventive effects on endpoint preneoplastic lesions involved in rat colon carcinogenesis and might also exert antioxidative activity. Forty- two male F344 rats were randomly divided into 6 experimental groups. Groups 1 to 4 were given a subcutaneous injection of azoxymethane (AOM, 20 mg/kg body weight) once a week for 2 weeks. Starting one week before the first injection of AOM, rats in groups 2 to 4 received an aqueous extract of Neem leaf (20, 100, and 250 mg/kg, respectively) by gavage 3 times per week, for 5 weeks. Rats in group 5 also were given the Neem extract by gavage feeding 3 times per week for 5 weeks, while group 6 served as untreated controls. The experiment was terminated 5 weeks after the start. Dietary feeding of the Neem extract at all dose levels significantly inhibited the induction of aberrant crypt foci (ACF) ($P<0.0002$), when compared to the AOM-treated group (group 1). In groups 2 to 4, treatment of rats with the Neem extract also significantly decreased the proliferating cell nuclear antigen (PCNA) labeling indices ($P<0.0006$) of colon epithelium and ACF. Moreover, the Neem extract also showed antioxidative activity. The finding that dietary Neem has possible chemopreventive effects in the present short-term colon carcinogenesis bioassay suggests that longer-term exposure may cause suppression of tumor development.

Key Words: Neem leaf - chemoprevention - colon carcinogenesis - preneoplastic lesions - cell proliferation

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Introduction

Medicinal plants have received considerable attention in the drug discovery process for various human disorders including cancer. Herbal products have been used for the prevention and treatment of several chronic diseases in ethnic societies worldwide. *Azadirachta indica*, termed Neem, is a tropical evergreen tree in South and Middle Asian countries (Conrick, 2001) and the origin of this plant is in the northwest region of India. Neem has in fact been known by people in India to be a valuable medicinal plant for more than 2000 years. Its leaves are commonly consumed in ordinary life as a dietary supplement in tea (Conrick, 2001). Many parts of Neem, including leaves, flowers, seeds, fruits, roots and bark have been shown to have a wide range of biological activities, such as anti-bacteria, anti-inflammation, antioxidation, antimutagenesis and anticarcinogesis properties (Bhargava et al., 1970; Dasgupta et al., 2004; Kusamran et al., 1998; Pillai et al., 1984; Sadekar et al., 1998; Sithisarn et al., 2005). Furthermore, several studies have demonstrated the chemopreventive effects of the water extract of the Neem leaf on *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG)-induced rat gastric carcinogenesis (Arivazhagan et al., 2000; Arivazhagan et al., 2004; Subapriya et al., 2003).

Colorectal cancer is a common malignancy ranking third in frequency on a world wide basis and causes about 500,000 deaths annually (Parkin, 2001), and mortality rate of this disease is still rising (Parkin, 2001). Aberrant crypt foci (ACF) are putative precursor lesions of colon carcinogenesis in human and rodents (Bird, 1987; Pretlow et al., 1991). Mucin depleted foci (MDF) have also been described as preneoplastic lesions of rat colon carcinogenesis (Caderni et al., 2003; Kinjo et al., 2006; Yoshimi et al., 2004). MDF can be detected by staining with high-iron diamine Alcian blue (HID-AB) (Caderni et al., 2003; Yoshimi et al., 2004).

[1]*Tumor Pathology,* [2]*Division of Digestive and General Surgery, University of the Ryukyus Faculty of Medicine, 207 Uehara Nishihara-cho, Okinawa 903-0215, Japan*

**For correspondence Tel.: +81-98-895-1120; FAX: +81-98-895-1406 E-mail: junya-arakaki@ryukyu-surg1.org*

The occurrence of MDF is likely to be correlated with the development of rat colon tumors induced by azoxymethane (AOM) (Caderni et al., 2003). Therefore, MDF are considered as novel biomarkers in colon carcinogenesis and are also useful to evaluate possible chemopreventive effects of a wide variety of candidate agents (Caderni et al., 2003; Yoshimi et al., 2004).

In the present study, we used a short-term rat colon carcinogenesis bioassay system to investigate the cancer preventive effect of Neem leaf. To obtain insights into its mechanism of action, we examined the proliferating cell nuclear antigen (PCNA) labeling index of carcinogen-induced preneoplastic lesions and antioxidative activity of the aqueous extract of the Neem leaf.

Materials and Methods

2.1. Preparation of the aqueous extract of Neem leaves

Neem leaves were provided by Ryu-celo Co., Ltd. (Okinawa, Japan) and ground to powder. The aqueous extract was made on a weekly basis and stored in a cold room (<4˚C) until use. In brief, three to sixty g of powdered leaves was boiled with 1000 ml distilled water at 95˚C for 10 min and filtered through filter paper. By this procedure, we obtained aqueous extracts at concentrations of 3 to 60 mg Neem/ml distilled water.

2.2. Animal experimentation

A total of 42, four-week-old male, F344 rats were purchased from Japan SLC Inc. (Hamamatsu, Japan). All animals were housed in wire cages (3 rats/cage) with a CE-2 control diet (CLEA Japan Inc., Tokyo) under controlled conditions of humidity (50 ± 10 %), lighting (12 h light/dark cycle) and temperature (23 ± 2 °C). After arrival, the animals were quarantined for 7 days and then assigned into 6 experimental groups. The animal experiment was approved by the animal welfare committee of the University of the Ryukyus Faculty of Medicine and the animals were maintained according to the Animal Cure Guidelines. The experimental protocol of the present study is shown in Fig. 1. When treating rats with the indicated dose levels of the Neem extract, the volume of the extract solution was determined according to their body weight. Each rat was fed by gavage at dose levels of 20, 100, and 250 mg Neem/kg body weight. Rats in groups 1-4 were given subcutaneous (s.c.) injections of AOM (20 mg/kg body weight) (Sigma Chemical Co., St Louis, MO) once a week for 2 weeks. A week before the first injection of AOM, rats in groups 1 and 6 were given distilled water by gavage-feeding 3 times per week for 5 weeks. Rats in groups 2 and 3 were given the aqueous extract of the Neem leaf (20 and 100 mg Neem/kg body weight, respectively) by gavage-feeding 3 times per week for 5 weeks. Rats in groups 4 and 5 were given the aqueous extract of the Neem leaf (250 mg/kg body weight) by gavage-feeding 3 times per week for 5 weeks. The rats in group 6 were served as untreated controls. All animals were sacrificed at 5 weeks after the start of the experiment. Colon tissues were removed and carefully cut open along the longitudinal axis, flushed with saline and then fixed with 10% buffered formalin.

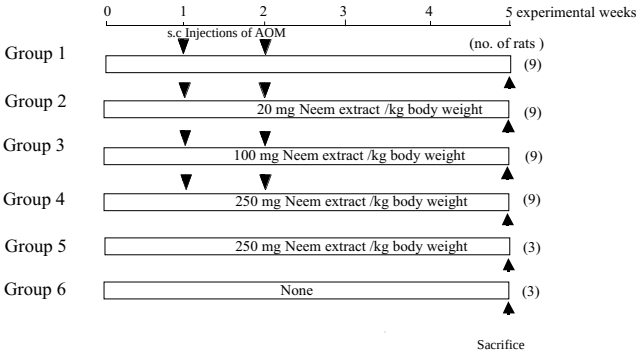

Figure 1. Experimental Protocol

2.3.Detection of preneoplastic lesions and immunohistochemical staining of PCNA

Colon tissues were dipped into 3% acetic acid for 2 min and then stained with a 3% alcian blue (AB) solution (pH 2.5, Sigma) for 5 min. After AB staining, each tissue was again dipped into 3% acetic acid for 5 min and rinsed with tap water. MDF were identified according to the criteria described elsewhere (Caderni et al., 2003; Yoshimi et al., 2004) under a light microscope at a magnification of x 40. After MDF counting, colon tissues were stained in a 0.5% methylene blue solution for 30 s and immediately washed with distilled water. Using the same magnification, ACF were counted with a light microscope according to the criteria described earlier (Bird, 1987). After ACF counting, colon tissues were longitudinally cut into two exact halves. One piece of the colon tissue was rolled up and embedded in a Swiss Roll form. Three μm-thick serial sections were prepared with the rolled colonic mucosa. After staining with an anti-PCNA antibody (DAKO Co. Ltd., Kyoto, Japan, 1:100 dilution), PCNA labeling index was measured in the colonic epithelial cells. The labeling index was determined by calculating the ratio of PCNA-positive nuclei/total number of nuclei counted as described earlier (Morioka et al., 2004). The second piece of the colon tissue was cut at 2-cm intervals from the anal side, divided into 9 compartments, and embedded in paraffin for histological and immunohistochemical analyses. In the middle and distal colon, we chose compartments where ACF and MDF frequently occurred. Dysplastic foci (DF) were detected in hematoxylin and eosin (HE)-stained sections. According to the criteria described earlier (Yoshimi et al., 2004), DF were identified as focal lesions displaying nuclear stratification, loss of nuclear polarity, structural abnormality of the crypts, Paneth cell metaplasia, decrease or loss of goblet cells, and presence of mitosis. PCNA labeling indices were then determined in the epithelial cells in the crypts of ACF and DF.

2.4.Measurement of antioxidative activity of Neem leaves

In these assays, we examined the increasing dose levels

Table 1. Body, Liver, and Relative Liver Weights

Group/Treatment	No. of rats	Body weight (g)	Liver weight (g)	Liver weight (g/100g bw)
1 AOM	8	205.8 ± 11.2*	8.4 ± 1.1	4.1 ± 0.18
2 AOM + 20 mg#	9	209.4 ± 11.1	8.8 ± 0.2	4.2 ± 0.13
3 AOM + 100 mg#	9	205.4 ± 10.9	8.4 ± 0.1	4.2 ± 0.14
4 AOM + 250 mg#	9	202.1 ± 15.1	8.5 ± 0.3	4.2 ± 0.06
5 Saline + 250 mg#	3	209.0 ± 13.3	8.7 ± 0.6	4.2 ± 0.08
6 None	3	213.0 ± 5.0	8.7 ± 0.2	4.1 ± 0.02

*Mean ± SD, #Neem extract /kg body weight

(2, 10, and 25 mg Neem/ml distilled water) of the aqueous extract of Neem leaf. The measurement of the antioxidative activity of the extract was performed as described in a previous paper (Morioka et al., 2004). In brief, the reaction mixture consisted of 0.5 ml of 0.1 mM 1,1-diphenyl-2-picryl hydrazyl (DPPH, Wako Pure Chemicals, Osaka, Japan radical) in 100 % ethanol, 0.475 ml of 0.05 M Tris-HCl (pH 7.4), 0.5 ml of 100 % ethanol, and 0.025 ml of the aqueous extract of the Neem leaf or distilled water (control). After incubation for 30s, the absorbance was measured at 517 nm by SmartSpec 3000 (Bio-Rad Laboratories, Hercules, CA). As a positive control, the antioxidative activity of vitamin C (ascorbic acid, Wako Pure Chemicals, Osaka, Japan) was also measured. The concentration of the sample required to scavenge 50% (IC_{50}) of the DPPH free radical was determined. Each assay was carried out in triplicate.

2.5. Statistical analysis

Statistical analyses by Dunnett's test were performed to determine the significance of the differences in mean body, liver, the relative liver weights, the mean number of ACF, MDF and PCNA labeling index, between experimental groups. Significance was concluded at $P < 0.05$.

Table 2. Inhibitory Effects of the Aqueous Extract of Neem Leaves on ACF and MDF Formation

Group	ACF/colon	ACF of 1-3 crypts/colon	ACF of ≥4 crypts/colon	MDF/ colon
1	121.6 ± 26.3*	114 ± 23.0	7.9 ± 4.6	6.1 ± 4.3
2	83.3 ± 12.3**	75.0 ± 11.1**	5.3 ± 2.2	4.1 ± 0.9
3	76.0 ± 14.0**	70.0 ± 13.6**	6.0 ± 3.5	3.9 ± 2.2
4	80.2 ± 17.2**	75.4 ± 16.5**	4.8 ± 2.7	4.7 ± 1.9
5	0	0	0	0
6	0	0	0	0

ACF : aberrant crypt foci, MDF: mucin depleted foci
* Mean ± SD ** Significantly different from group 1 by the Dunnett's t-test (P<0.0002)

Table 3. Inhibitory Effects of the Aqueous Extract of Neem Leaves on PCNA Labeling

Group	PCNA labeling index (%)		
	Colonic epithelium	ACF	DF
1	13.1 ± 3.5	17.4 ± 4.5	38.7 ± 8.0
2	7.9 ± 3.1*	10.9 ± 4.4**	35.3 ± 7.6
3	5.4 ± 1.2*	8.1 ± 2.7**	30.7 ± 10.9
4	4.0 ± 1.3*	8.9 ± 3.0**	32.7 ± 7.7
5	2.5 ± 1.7*		
6	3.4 ± 2.7*		

*,** Significantly different from group1 by Dunnett's t test at P<0.0006 and P<0.002

Results

3.1 General observations of the animal experiment

A total of 41 F344 rats survived to the end of the experiment and none of them developed colon tumors. One rat in group 1 died of an unknown cause. Mean body, liver and relative liver weights in all groups are shown in Table 1. There were no statistical differences in these values between experimental groups. No symptomatic adverse side effects were seen in any of the rats.

3.2 Inhibition of the occurrence of ACF and MDF by the aqueous extract of Neem leaves

The mean number of ACF and MDF per colon is shown in Table 2. All rats in groups 1-4 developed ACF in their colon. No ACF were seen in rats of groups 4 and 5. The mean number of ACF/colon in groups 2 (83.3 ± 12.3), 3 (76.0 ± 14.0) and 4 (80.2 ± 17.2) was significantly lower than that of group 1 (121.6 ± 26.3) (P<0.0002). The mean number of ACF containing 1-3 crypts/colon of groups 2 (75.0 ± 11.1), 3 (70.0 ± 13.6) and 4 (75.4 ± 16.5) was also significantly lower than that of group 1 (113.8 ± 23.0) (P<0.0002). The number of MDF/colon in groups 2-4 was decreased compared to that of the group 1 but the difference was not statistically significant. In groups 2-4, the number of ACF containing more than 4 crypts/colon decreased compared to the group 1 but this decrease was not statistically significant.

3.3. Inhibition of PCNA labeling index by the aqueous extract of Neem leaves

The PCNA labeling index in each experimental group is shown in Table 3. In groups 2-4, treatment of rats with the aqueous extract of Neem leaf caused a significant and dose-dependent decrease in PCNA labeling index of the colonic epithelium (P<0.0006), when compared to the control group (group 1). The PCNA labeling index in ACF was significantly lower than the control (P<0.002). The PCNA labeling index in DF was also decreased in the Neem extract-treated groups compared to the control group but the decrease was not statistically significant.

3.4. Measurement of antioxidative activity of Neem leaf extract

DPPH radicals were scavenged by the aqueous extract of Neem leaf and Vitamin C, with IC_{50} values of 7321.1 ± 1543.9, and 100.8 ± 15.1 mg/ml, respectively. This result indicates that the Neem extract has antioxidative activity.

Discussion

The present studies provide the first detailed examination

of the effects of the Neem leaf extracts in carcinogen-induced short-term rat colon carcinogenesis. In these studies, we found that dietary administration of the aqueous extracts of Neem leaf inhibited the occurrence of the earliest morphological changes of colonic epithelium such as ACF and MDF induced by a carcinogen AOM. We also found that the PCNA labeling index in the colonic epithelial cells in ACF and DF was inhibited by the treatment of rats with the Neem extracts. Therefore, these results suggest that the aqueous extract of Neem leaf has potent chemopreventive effects in a short-term colon carcinogenesis bioassay system. ACF are visible preneoplastic lesions in the colonic mucosa of rats treated with a carcinogen (Rodrigues et al., 2002; Tanaka et al., 2000; Yoshimi et al., 2004). These lesions are classified into two distinct fractions including histological ACF and DF (Yoshimi et al., 2004). MDF have recently been identified as focal lesions characterized by the absence or very small production of mucins under light microscope examination (Yoshimi et al., 2004).

Carcinogens such as AOM, 1,2-dimethylhydrazine (DMH) and methylazoxymethanol (MAM) enhance cell proliferation and increase the vertical length of crypts and the number of cells constructing crypts in the rat colon (Chewonarin et al., 1999; Mori et al., 1991; Rodrigues et al., 2002; Tanaka et al., 2000). In general, cell proliferation in ACF and colon tumors is higher than that in normal colonic mucosa (Tanaka et al., 2000). Thus, cell proliferation may contribute to the development of ACF and colon tumors. In the present study, we found that the Neem extract inhibited the ACF formation and that PCNA labeling index of ACF was significantly decreased by treatment with the Neem extract, and this decrease occurred in a dose-dependent fashion (Table 3). Thus, inhibitory effects of the colonic preneoplastic lesions by the Neem extract may be due to the inhibition of cell proliferation in these lesions. We earlier demonstrated that the β-catenin accumulated crypts and MDF both play a critical role in short-term rat colon carcinogenesis models induced by a specific carcinogen (Kinjo et al., 2006; Yamada et al., 2000; Yoshimi et al., 2004). Therefore, more detailed studies are required to further characterize the possible effects of the Neem extract on these lesions.

More than 140 active substances have been isolated from the different parts of Neem leaves (Subapriya et al., 2005). The Neem leaf has been demonstrated to include possible chemopreventive phytochemicals such as limonoids and quercetin (Conrick, 2001; Lamson et al., 2000; Subapriya et al., 2005). Of these, both limonin and limonin 17β-D-glucopyranoside inhibit DMBA-induced buccal pouch tumors in hamsters (Miller et al., 1992) and DMBA-induced skin tumors in mice (Miyagi et al., 2000). Quercetin, a flavonoid present in Neem leaf, has been demonstrated to inhibit buccal pouch carcinogenesis by its radical scavenging property (Balasubramanian et al., 1996). Quercetin has also been shown to inhibit the growth of several types of human cancer cell lines (Castillo et al., 1989; Lamson et al., 2000). In the present study, we found that the Neem extract displays antiproliferative activity in the colonic epithelial cells, suggesting that the Neem leaf contains more than one possible compound(s) that have an antiproliferative effect in colon carcinogenesis. As mentioned above, the Neem leaf contains biologically active compounds such as limonoids and quercetin. Thus, these compounds may be effective in the present short-term bioassay system. To address these issues, further studies are in progress to identify the active and distinct component of Neem leaf that exerts cancer preventive properties.

Oxidative stress is associated with the carcinogenic process and induces gene mutations and cell injury (Cerutti et al., 1991). Nitric oxide (NO) induces superoxide anion of leukocytes (Nathan et al., 1994; Tsujimoto et al., 1993), and the overexpression of inducible NO synthase (iNOS) is found in colon cancer of human and rat (Moochhala et al., 1996; Takahashi et al., 1997). In addition, we found that tropical plants *Terminalia catappa* and P*eucedanum japonicum* both inhibit the occurrence of carcinogen-induced colonic preneoplastic lesions and that these plats also have antioxidative activities (Morioka et al., 2004; Morioka et al., 2005). In the present study, we found that the Neem extract also has antioxidative activity, suggesting that this activity of Neem may contribute to the inhibition of the occurrence or induction of preneoplastic lesions in the rat colon.

It was demonstrated that the aqueous extract of Neem leaf was not toxic to mice up to the dose level of 1000 mg/kg (Subapriya et al., 2005). In addition, the acute toxicity of the Neem extract occurred in the range of 13 g/kg in mice (Okpanyi et al., 1981). In the present study, the dose level (250 mg/kg body weight) of the Neem extract did not cause any acute toxicity. After treatment of rats with the extract, the liver and kidney revealed no morphological changes. It is of interest whether Neem is useful in the prevention of human colon cancer. Thus, important consideration is whether or not, following oral administration, sufficient blood and tissue levels can be achieved and the extract exerts significant adverse side effects.

In conclusion, aqueous extracts of Neem leaves can inhibit the occurrence of colonic preneoplastic lesions and cell proliferation and also have antioxidative activity. Therefore, Neem extract may contain a compound(s) that has cancer preventive activity and this plant may therefore be useful for the prevention and therapy of human colon cancer.

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