

RESEARCH ARTICLE

Nabag (*Zizyphus spina-christi*) Extract Prevents Aberrant Crypt Foci Development in Colons of Azoxymethane-Treated Rats by Abrogating Oxidative Stress and inducing Apoptosis

Nejib Guizani, Mostafa Ibrahim Waly, Vandita Singh, Mohammad Shafiur Rahman*

Abstract

Zizyphus spina-christi (ZSC) fruit is a rich source of bioactive compounds but any medicinal properties in chemoprevention of colon cancer have hitherto not been studied. The aim of the present study was to examine *in vivo* protective effects of ZSC water extract on colon carcinogenesis in azoxymethane (AOM)-treated rats. Our results showed that ZSC significantly reduced AOM-induced colonic aberrant crypt foci development and AOM-induced oxidative stress as indicated by restoration of endogenous glutathione depletion and abrogating the impairment of total antioxidant capacity. Caspase-3 cleavage, which has been considered as an apoptotic index, was almost undetectable in AOM-treated rats and ZSC exhibited pro-apoptotic effects evidenced by increased levels of cleaved caspase-3. In the studied model, our findings provide the first *in vivo* evidence that ZSC extract could inhibit the early stage of colon carcinogenesis by preventing oxidative stress and inducing apoptosis.

Keywords: Azoxymethane - colon cancer - zizyphus spina-christi - glutathione - colonic cells apoptosis

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Introduction

Azoxymethane (AOM) is a specific colon carcinogenic agent that induces the development of aberrant crypt foci in animal models (Hangen and Bennink, 2002; Leonardi et al., 2010). A recent study has shown that AOM induces oxidative stress in colonic homogenates as it was evident by glutathione (GSH) depletion and impairment of total antioxidant capacity (TAC) (Al-Numair et al., 2011).

Colon carcinogenesis is a multistep process, and several lines of evidence proposed that AOM-induced colon cancer development partly by inhibiting the tumor suppressor gene p53-mediated apoptosis and by causing aberrant cell survival, thus contributed to oncogenesis (Steller, 1995; Arai et al., 1996; Wu et al., 2004). Wild type p53 has multiple functions as a tumor suppressor, including cell cycle arrest in response to DNA damage and induction of apoptosis (Giaccia and Kastan, 1998; Hirao et al., 2000). Cysteine proteases and caspases are essential for the execution step in apoptosis (Cohen, 1997; Nicholson and Thornberry, 1997). Among the caspases, caspase-3 that is activated during apoptosis by being cleaved into p20 fragments and subsequently cleaves several intracellular substrates in a cascade-signaling pathway (Fernandes-Alnemri et al., 1994; Nicholson et

al., 1995; Tewari et al., 1995).

The impact of diet in the prevention of colon cancer pathogenesis has been receiving an escalating attention in the past two decades. Epidemiological studies have indicated that high consumption of dietary antioxidant nutrients is associated with low risk of many oxidative stress-mediated diseases. There is increasing use of plant derived natural compounds as alternative sources of drugs or herbal extracts for therapeutic uses (Olatunde, 2003). *Zizyphus spina-christi* (ZSC) fruit is commonly used for the treatment of many human diseases (Kirtikar and Basu, 1985; Han and Park, 1986). Previous studies revealed that ZSC fruits were found to have medicinal properties such as hypoglycemic, hypotensive, anti-inflammatory, antimicrobial, antioxidant, anti-tumour, hepatoprotective and as an immune system stimulant (Said et al., 2006; Yossef et al., 2011). Aberrant crypt foci (ACF) are considered to be precancerous lesions in the colorectum of humans and rodents (Bird, 1987; Al-Numair et al., 2011). The number of crypts/foci has been shown to increase with time following carcinogenic AOM-treatment, and ACF demonstrate increased colonic cells proliferation (Bird, 1987). The aim of the present study was to investigate the *in vivo* antiproliferative effect of ZSC extract on colon carcinogenesis and the mechanisms involved.

Materials and Methods

Preparation of ZSC extract

ZSC freeze dried powder (50 g) was extracted with 750 ml of water at room temperature (25°C for 24 hrs). The extracts were then filtered and centrifuged (Sanyo Harrier 18/80) at 6000 RCF for 30 mins at 3°C. The supernatant was decanted and crude extract was stored at -40° C until used for the subsequent experiments.

Total phenolic content of ZSC extract

Total phenolic of ZSC extract were measured by the Folin-Ciocalteu assay (Singleton and Rossi, 1965), with slight modifications. Ten μ l of ZSCF extract was taken in a test tube and 3 ml of distilled water followed by 250 ml Folin-Ciocalteu reagent were added to it and vortex mixed. After a short incubation of 5 min, 750 ml of sodium carbonate (1.9 M) was added, then 990 ml of water was added to make up the total volume up to 5 ml. The mixture was then incubated for 2 h and absorbance at 765 nm was measured. The phenolic content was determined considering gallic acid standard curve. The concentration of phenolic in nabag extracts was expressed as mg gallic acid equivalents mg GAE/100g dry-nabag solids. All the measurements were taken in triplicate and the mean values were calculated.

Animals

Male Sprague-Dawley rats were used in this experiment. The rats were housed in individual polypropylene cages and were provided with basal diet (standard laboratory chow diet from Oman Mills, Muscat, Oman) and normal tap water ad-libitum. Rats were housed under standard conditions of temperature (22±2°C), humidity (60%) and a 12 hr light:dark cycle. This protocol in this study was approved by the Sultan Qaboos University Animal Ethical Committee, and was conducted in accordance to international laws and policies (EEC Council directives 86/609, OJL 358, 1 December, 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

Experimental design and treatment protocol

Forty weanling male Sprague-Dawley rats, 4 weeks of age, with an average body weight of 50±5 g were randomly assigned to 4 groups (10 rats/group); the control group and treatment groups (ZSC, AOM and ZSC plus AOM). After acclimatization for 1 week (at 5 weeks of age), the rats were fed ad-libitum on a basal diet and divided into four groups: *i*) control rats; *ii*) AOM-treated rats were given an intra-peritoneal injections of AOM once a week for 2 weeks (week 8 and 9) at a dose of 15 mg AOM/kg body mass; *iii*) and *iv*) ZSC and ZSC plus AOM-treated groups, each rat was daily administered the ZSC extract by oral gavages, at a dose of 1.5 ml/day (from week 6 to week 16).

The rats in the control group were given 1 ml intra peritoneal injection of 0.9% physiological saline once a week for 2 weeks and the rats in the AOM-injected group were given 2 intra peritoneal injections of AOM (Sigma

Chemical Co., St. Louis, MI) dissolved in physiological saline once a week (15 mg/kg body weight) for 2 weeks. At the end of the experiment (16 weeks of age), all rats were euthanized and the colon tissues were examined microscopically for lesions of aberrant crypt foci (ACF) and colonic measurements of glutathione (GSH), total antioxidant capacity (TAC) and the apoptotic index, cleaved caspase-3.

Colon preparation

The colons were carefully removed from rats and were kept on a glass plate in ice jackets. The colons were then opened longitudinally, rinsed with ice-cold physiological saline, and sectioned longitudinally into two halves of equal width and were spread out with flat mucosal side up. The mucosal layer from one half was removed by scraping and immediately homogenized. The other half was fixed flat in 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ) between two filter papers for one week before aberrant crypt foci (ACF) enumeration.

Aberrant crypt foci (ACF) enumeration

ACF are commonly accepted precursor lesions for colonic tumors and the method used for ACF enumeration was followed as described in previous studies (Bird, 1987; Al-Numair et al., 2011). Fixed colons were stained with 0.2% methylene blue in Krebs's ringer bicarbonate buffer for 20 minutes in a petri dish and rinsed with physiological saline. After staining, the colons were placed with the mucosal surfaces up on a slide, examined with a light microscope under 40X magnification and then scored for ACF. In brief, the ACF were distinguished from normal crypts by their darker stain, enlarged and slightly elongated size, thick epithelial lining, slightly elongated cryptal opening and often slit shaped. The total number of ACF was recorded for all examined colons.

Scraped colonic mucosa homogenization

The colon mucosal layer scrapings of each rat (~50 mg) were immediately homogenized in 1 mL of 100 mM potassium phosphate buffer (pH 7.2) by a glass-teflon homogenizer with an ice-cold jacket and centrifuged at 10,000 g at 4°C for 60 minutes. The resulting supernatant was used for determination of protein content, the apoptotic index (cleaved caspas-3), and oxidative stress indices (glutathione and total antioxidant capacity).

Western blot analysis for caspase-3 and its cleaved fragment

Sequential 1 cm sections of rat colon (proximal, middle or distal) were prepared by lysis in a polytron homogenizer (Brinkman Instruments, Westbury, NY) for 30s in cold lysis buffer (Cell Signaling Technologies, U.S.A) in the presence of protease inhibitor cocktail (Sigma, Germany). After centrifugation, 10 micrograms of total protein of each sample were loaded on a 15% SDS-PAGE gel. After electrophoresis, protein was transferred to nitrocellulose membrane. Blots were blocked with 5% nonfat milk in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) and probed with

1:1500 dilutions of either caspase-3 or caspase-3p20 antiserum in 5% nonfat milk/TBST. Immunoblots were then processed with horseradish-peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) using the enhanced chemiluminescence (ECL) Western blotting detection system kit (Amersham, Arlington Heights, IL). The blots were exposed to Hyperfilm (ECL, Amersham) at room temperature. Quantification of the total protein from all colonic lysates were done by using BCA protein assay system (Pierce, USA). Densitometry analyses of bands were adjusted with β -actin as loading control.

GSH measurements

Aliquots of supernatant (100 μ L) were transferred to fresh eppendorf tubes and 2 μ L of monochlorobimane (25 mmol/L) and 2 μ L of glutathione-S-transferase reagent were added, as provided by a commercial kit (Biovision, Mountain View, CA, USA, Catalog # K251). After 30 mins of incubation at 37°C, the samples and standards were read in a fluorescence plate reader at 380/460 nm. GSH content was determined by comparison with values from a standard curve using freshly prepared GSH and normalized to the protein content of the assayed colon mucosal tissue homogenates.

Total antioxidant capacity (TAC) measurements

A colorimetric method using Randox Assay Kit (Randox Laboratories Ltd, UK) was used to measure the TAC. The assay was based on the incubation of samples with 2, 2'-azino-di-[3-ethylbenzthiazoline sulphonate (6)] diaammonium salt (ABTS) with a peroxidase (methmyoglobin) and hydrogen peroxide to produce the radical cation ABTS⁺ which has a relatively stable blue-green color. The absorbance of the mixture was measured at 600 nm. Antioxidants present in the assayed colonic mucosal tissue homogenate samples inhibited the oxidation of ABTS to ABTS⁺ (cause suppression of the color production), which was proportional to their concentration. The capacity of the assayed samples antioxidants was compared with that of standard Trolox, a water soluble tocopherol analogue, which is widely used as a traditional standard for TAC measurement assays. The assay results are normalized to the protein content of the assayed colonic tissue homogenates.

Analysis of protein content

Protein content of colonic mucosa homogenates was assayed by the method of Lowry et al. (1951), using bovine serum albumin as standard and protein content was expressed as mg/ml of sample.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.03; GraphPad Software Inc. San Diego, CA). The results are expressed as means \pm standard deviation (SD). The statistical analysis was performed using one way analysis of variance (ANOVA) followed by Tukey's test, Student's unpaired t-test for means comparisons, and a P-value of less than 0.05 was considered significant.

Results

Phenolic compounds in extract

The fresh nabag contained phenolic as 1644 mg GAE/100 g dry-nabag solids and the extract prepared for the animal feed trial contained 0.843 mg GAE/ml extract. This produced a daily dose of phenolic content of 25 mg GAE/kg BW (i.e. 175mg GAE/kg BW for a week).

Food consumption of animals

The average daily food intake for the rats of the four groups was similar with no statistical significant differences and the mean value was 12.7 \pm 3.5g/day, (p>0.05), indicating that AOM-injection and or ZSC-administration have no effect on the food intake.

Body weight gain of animals

Body weight was recorded weekly for the whole duration of the experiment. All rats from all groups grew at a similar rate throughout the study, and no mortality occurred in any group. At the beginning (week 1), the average body weight of all rats was 50 \pm 5g. At the end of the experiment (week 16), the final body weight for all experimental groups were not significantly different, 310.40 \pm 11.9, 312.3 \pm 10.7, 311.7 \pm 9.9 and 310.9 \pm 7.7g for control, AOM, ZSC and ZSC+AOM injected groups respectively (p>0.05).

Aberrant crypt foci (ACF) enumeration and distribution

In the present study we investigated the effect of ZSC extract administration on the AOM-induced colon tumors by counting ACF. As presented in Table 1, the rats from both the control and ZSC groups did not show any development of ACF. Meanwhile all the examined colon

Table 1. Aberrant crypt foci (ACF) Enumeration and Distribution

Groups	ACF Count in		
	Proximal Colon	Middle Colon	Distal Colon
Control	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
AOM	6.89 \pm 2.61**	17.25 \pm 4.78**	39.64 \pm 11.23**
ZSC	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
ZSC+AOM	0.48 \pm 0.23*	4.43 \pm 1.78*	13.74 \pm 5.98*

*Significantly lower than corresponding (AOM) group, Based on one way ANOVA test, p<0.05; Values were expressed in means \pm standard deviation; **significantly higher than control group

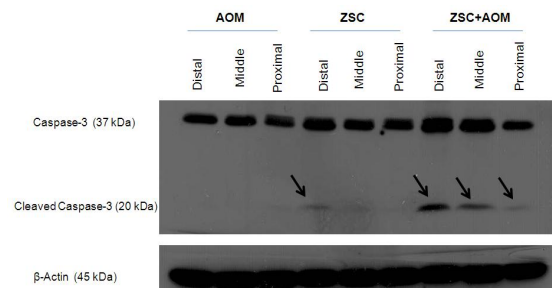


Figure 1. Colonic Cells Apoptosis and Caspase-3 Cleavage. Lysates were prepared from distal, middle and proximal colons from AOM, ZSC, ZSC+AOM -injected rats. Top panel. Western blotting for caspase-3 and cleaved caspase-3. Bottom panel, β -Actin loading protein

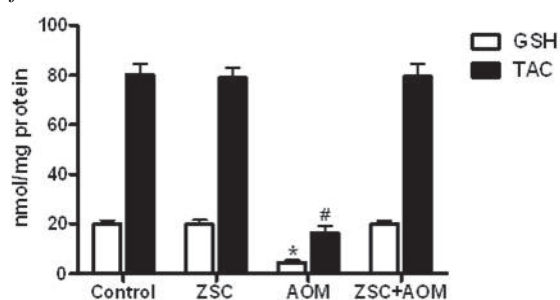


Figure 2. AOM-Treatment Induced Colonic Oxidative Stress as Evident by GSH Depletion and Impairment of TAC. ZSC extract abrogated the observed oxidative stress. *, #significantly lower than corresponding control group. Based Student's unpaired t-test, $p < 0.05$

of rats with AOM injection developed ACF, the highest density and count were observed in the distal part of colon rather than middle and proximal colon ($p < 0.05$). The ACF enumeration of AOM-injected rats were significantly decreased with concomitant administration of ZSC extract ($p < 0.05$). The size of the examined ACF was medium, 4-6 aberrant crypt/foci.

Cleavage of caspase-3 as an apoptotic index

Western blotting was conducted to detect caspase-3 (37 kDa) and its cleaved fragment (20 kDa) produced by caspase cleavage. As anticipated in Figure 1, Caspase-3 cleavage was almost undetectable in AOM-treated rats. Meanwhile, ZSC exhibited pro-apoptotic effects evidenced by increased levels of cleaved caspase-3. Caspase-3 cleavage was detected in a gradient with highest levels in colonic lysates in distal colon of rats administered with ZSC plus AOM injection.

Intracellular GSH-depletion and TAC impairment by AOM-injection

As presented in Figure 2, the intracellular GSH measurements in colonic mucosa tissue homogenates for both the control and ZSC groups were comparable with no statistical significant difference; meanwhile the AOM-injected group showed significant reduction in the endogenous GSH levels. It was observed that, ZSC administration and injected restored the AOM-induced GSH depletion to a level that is comparable to control group. The mean TAC values among the control and ZSC groups were observed insignificant ($p > 0.05$). ZSC-administration abrogated the AOM-induced TAC impairment.

Discussion

Epidemiological studies have indicated that high consumption of dietary antioxidant nutrients is associated with low risk of many oxidative stress-induced diseases (Esfahani et al., 2011; Gülçin, 2012). These dietary antioxidant nutrients synergize with cellular reductants in scavenging of free-radicals and chelating transition metals that are catalysts in lipids peroxidation. Cellular reductants are thiol groups (cysteine and glutathione) that are predominant at the active sites of various enzymes and proteins. GSH is the major intracellular reductant that

spare cellular proteins from oxidative damage by oxidizing their sulfhydryl groups (cysteine can be oxidized to cystine and two molecules of glutathione can be oxidized to a disulfide) (Shafer and Buettner, 2001).

The AOM dose (30 mg/kg rat body weight) was deliberately chosen as such was used in previous studies as a potent carcinogenic dose that induced ACF and colon tumors in rats (Bird, 1987; Al-Numair et al., 2011). Our data suggests that the AOM treatment created oxidative milieu and induced the carcinogenic in colon cells by depleting the GSH. This depletion was restored by ZSC administration which also ameliorated the AOM-induced TAC impairment. Treatment of rats with 175mg GAE/kg body wt (per week) ZSC extract for 16 weeks produced no functional disturbances in liver and kidney and no haematological changes were detected as well (data not shown). ZSC significantly ameliorated the AOM-induced ACF development and suppress the AOM-induced inactivation of caspase-3 cleavage.

The observed AOM-induced GSH depletion and TAC impairment represents an evidence of colonic oxidative stress and a low colonic redox capacity that is characterized by inability to counterbalance the reactive oxygen species (ROS) and other prooxidants compounds that are generated from either exposure to various environmental insults or generated as by-products of normal cellular oxidative metabolism. ROS is involved in the pathogenesis of many diseases in a mechanism that includes cellular damage and apoptosis in rodents (Tsunada et al., 2003; Shiraishi et al., 2009). ROS oxidize (remove electrons) from nucleic acids, resulting in mutations, and polyunsaturated fatty acids of the phospholipids that comprise cell membranes and other cellular lipid structures, this would be followed by a loss of intracellular membrane integrity of cell organelles, a change in extracellular membrane permeability, and eventually cell damage and apoptosis.

Biochemical indicators of cells death include caspase-3 that is crucial mediator of programmed cell death (apoptosis). Caspase-3 is also required for some typical hallmarks of apoptosis, and is indispensable for apoptotic chromatin condensation and DNA fragmentation in all cell types examined (Arai et al., 1996; Nicholson and Thornberry, 1997). Caspase-3 activation, by cleavage, has been considered as an apoptotic index, and this is essential for the formation of apoptotic bodies and commitment to loss of cell viability.

AOM-treated rats showed almost undetectable caspase-3 cleavage, which has been considered as an inhibition of colonic cell apoptosis and this promoted cells proliferation leading to malignant cell growth in both colonic mucosa and tumors, and this effect was significantly suppressed by ZSC extract administration. Similarly Hibasami et al. (2003) also demonstrated that phenolic compounds in tea extracts antitumor activity by triggering apoptosis and suggested the proliferation of colon cancer cells was suppressed by the presence of phenolic compounds. However, the specific effect of ZSC in apoptosis is remained unclear.

In conclusion, ZSC extract prevents ACF development in colonic cells of AOM-treated rats by abrogating oxidative stress and inducing apoptosis. These findings

suggest a chemopreventive effect of ZSC in a mechanism that involves combating oxidative damage induced by AOM. Further in vivo studies are required to shed light on the synergistic effect of ZSC on AOM-mediated apoptosis.

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