The Protective Effect of Vitamin C on Azoxymethane-induced Oxidative Stress in Colon of Mice

Vitamin C'nin Farelerin Kalın Barsağında Azoxymethane ile Oluşturulan Oksidatif Stres Üzerindeki Koruyucu Etkisi

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Abstract
Purpose: The Azoxymethane model for colon carcinogenesis is often used to study on the initiation and promotion stages of colon carcinogenesis in mice. Our aim in this study was to evaluate the effects of vitamin C on levels of oxidative stress parameters in azoxymethane induced colonic oxidative stress in mice.
Material and Methods: In this purpose thirty Swiss albino mice (weighing 31.49±3.1 g), aging 12 weeks were randomly divided into three groups of ten each. Animals of group I and II were treated with weekly doses of subcutaneous 5 mg/kg azoxymethane for 7 weeks, whereas, the mice in group II were further treated with 500 mg/kg vitamin C administered intraperitoneally during the same period. Group III served as the control group.
Results: In the vitamin C treated mice, levels of MDA and NO were significantly lower than those of azoxymethane only group (p=0.031, p<0.001). The levels of GSH in the vitamin C treated mice were significantly higher than that of azoxymethane only group (p=0.004).
Conclusion: The present study demonstrates that intraperitoneal administration of vitamin C reduces azoxymethane induced oxidative stress in colon of mice.

Key words: **Ascorbic acid; Azoxymethane; Colon; Mice; Oxidative stress.**

Özet
Amaç: Farelerde kolon karsinogenezisinin oluşumu ve gelişiminin izlenmesinde azoksimetanla oluşturulan kolon karsinogenezisi modeli kullanılmıştır. Bu çalışmadaki amacımız vitamin C'nin fare kalın barsağında azoxymethane ile oluşturulan oksidatif strese karşı antioksidan aktivitesini araştırmaktır.
Gereç ve Yöntemler: Bu sebeple 12 haftalık 31.49±3.1 g ağırlığında 30 adet Swiss Albino fare onarlı üç gruba ayrıldı. Birinci ve ikinci gruptaki farelere 7 hafta boyunca haftada bir defa subkütanöz 5 mg/kg azoxymethane verildi. Aynı zaman peryodunda ikinci gruptaki farelere 500 mg/kg vitamin-C intraperitoneal uygulandı. Üçüncü gruptaki farelere kontrol grubu olarak herhangi birşey verilmedi.
Bulgular: Vitamin C verilen grupta MDA ve NO seviyesi sadece azoxymethane verilen gruba göre anlamlı derecede daha düşük idi (p=0.031, p<0.001). Vitamin C verilen grupta GSH seviyesi sadece azoksimetan verilen gruba göre anlamlı derecede daha yüksek idi (p=0.004).
Sonuç: Bu çalışma; farelerde intraperitonel verilen vitamin C'nin azoxymethane ile oluşturulan oksidatif stresi azaltığını göstermektedir.

Anahtar kelimeler:**Askorbik Asit; Azoksimetan; Kolon; Fare; Oksidatif stress.**

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Introduction

Colon cancer is one of the most common neoplasm, especially in the Western world (1). Epidemiologic and experimental studies suggest that colon cancer has a close relation with dietary factors (2). Diets with high content of animal fats are associated with increased fecal bile acids and have been implicated in the increased occurrence of this malignancy (2, 3). In order to reduce the influence of risk factors, change of diet among the risk groups are considered.

Increased amount of reactive oxygen species (ROS) can be produced in intracolonic cavity due to the effect of bacteria and dietary metabolites. Numerous studies showed that ROS such as oxygen radicals, hydroxyl radicals and hydrogen peroxide played a role in the pathogenesis of colon's tumor genesis (5). Aberrant crypt foci (ACF) are the earliest identifiable putative precursors of both human and experimental colon cancers and have been used to evaluate the chemo preventive efficacy of many agents (3).

Vitamin C is a water soluble vitamin that is richly found in fruits and vegetables. It is thought that the antioxidant activity of Vitamin C might contribute to its possible cancer preventive potential (6, 7). As a free radical scavenger, vitamin C is thought to have a protective effect on the cellular biopolymers and might be beneficial as a preventive measure in the cancer development. Another beneficial effect of vitamin C might be its ability to impede nitrosamine formation by reacting with nitrite to convert it to nitrous oxide, thus preventing the genetic material from the damaging effect of mutagenic nitrosamines (8, 9). The aim of this study is to determine the effects of vitamin C on antioxidant activity in azoxymethane (AOM) induced oxidative stress development in mice.

Materials and Methods

Thirty inbred BALB/c Swiss Albino mice (weighing 31.49±3.1 g), aging 12 weeks were randomly divided into three groups (five male and five female mice for each group kept on separate cages) -of ten each. Animals were kept in individual wire cages in a well ventilated room with controlled humidity (50±10 %), temperature (+24±2 ^{0}C), and a 12 hr light/dark cycle. Mice were obtained from Kafkas University, Animal Laboratory (Kars, Turkey). Experiments were performed at the Inonu University Experimental Research Center. All experiments in this study were performed in accordance with the guidelines for Animal Research from the National Institute of Health and were approved by the Committee on Animal Research at Kafkas University. Drinking water and diet were supplied ad libitum. Animals of group I and II were treated with bi-weekly doses of subcutaneous 5 mg/kg azoxymethane for 7 weeks, whereas, the mice in group II were further treated with 500 mg/kg vitamin C administered intraperitoneally during the same period. Group III served as the control group.

By the end of the 7th week animals were sacrificed and tissue specimens from the large intestine were obtained. NO is rapidly oxigenated to NO_2 and further to NO_3, therefore direct assessment of NO is almost impossible (in vivo). So the combined production of NO_2 and NO_3 can be used to evaluate NO synthesis in vitro and in vivo. Nitrate was assayed by a modification of the cadmium-reduction method (10). The produced nitrite was determined by diazotization of sulfanilamide and coupling to naphthylethylene diamine (NNDA). After samples were deproteinized with somogy reagent, the nitrate was reduced by Cu-coated Cd in glycine buffer at pH 9.7. The reduction followed pseudo-first order reaction kinetics, a convenient time intervalfor assay being 90 minute. Mixed and then read absorbances against the blank at 545 nm after 20 to 60 minute. Results are expressed as µmol/mg tissue.

MDA in tissues were determined by the method of Uchiyama and Mihara (11). A 3 ml aliquot of 1 % phosphoric acid and 1 ml of 0.6 % thiobarbituric acid solution were added to 0.5 ml of 10 % tissue homogenate pipetted into a tube. The mixture was heated in boiling water for 45 min. After cooling, the color was extracted into 4 ml of n-butanol. The absorbance was measured in spectrophotometer (Ultraspec Plus, Pharmacia LKB Biochrom, UK) with 532nm. The amounts of lipid peroxides were calculated as thiobarbituric acid reactive substances of lipid peroxidation and are given as nmol/mg tissue.

Glutathione (GSH) was determined by the spectrophometric method which was based on the use of Ellman's reagent (12). Briefly, after centrifugation at 3000 rev./min for 10 min, 0.5 ml of supernatant was added to 2 ml of 0.3 mol/l $Na_2HPO_4.2H_2O$ solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. Glutathione levels

were calculated using an extinction coefficient of 13600 M-1 cm-1. Results are expressed in nmol GSH/g tissue.

Statistical analysis: The statistical package for social sciences (SPSS) version 10.0 was used for statistical analysis. Individual group parameters were assessed using the Mann–Whitney t test. The results are given in the text as means ± STD for all comparisons; statistical significance was defined as $P < 0.05$.

Results

The results of MDA, GSH and NO of the three groups are shown in Table I. In the vitamin C treated mice, levels of MDA (66.76 ± 31.77) and NO (9.3±2.9) were significantly lower than those of azoxymethane only group (108.89 ± 28.6 and 18.1 ± 4.5; p=0.031 and p<0.001) respectively. The levels of GSH in the vitamin C treated mice (13.08 ± 7.7) were significantly higher than that of azoxymethane only group (6.3 ± 1.6, p=0.004).

Table I. Tissue levels of MDA, GSH, and NO activities in groups

Groups	MDA (nmol/g tissue)	GSH (nmol/g tissue)	NO (μmol/g tissue)
I. Sham (n=10)	56.3 ±23.6	6.4±1.2	9.8±2.0
II. AOM (n=10)	108.89±28.6	6.3±1.6	18.1±4.5
III. AOM+Vit C (n=10)	66.76±31.77	13.08±7.7	9.3±2.9
P values			
I versus II	0.003	>0.05	<0.001
II versus III	0.031	0.004	<0.001

MDA, Malaondialdehyde; GSH, Reduced glutathione; NO, Nitric Oxide; AOM, Azoxymethane. *p<0.05 was considered to be statically significant.

Discussion

The AOM model for colon carcinogenesis is often used to study on the initiation and promotion stages of colon carcinogenesis in mice. AOM is an intermediate of a colonic carcinogen DMH 1, 2-dimethylhydrazine and metabolized by cytochrome P-450. It has been shown that administration of the colon specific carcinogen AOM increases both iNOS and COX-2 activities in colonic mucosa (13). The present study indicates that intraperitoneal administration of vitamin C at a dose of 500 mg/kg reduced MDA and NO and increased GSH levels in colon tissues of AOM given mice. It has been suggested that higher NO or higher activity of İNOS increases the colon carcinogenesis (14).

The role of NO in the initiation of carcinogenesis is complex and involves the formation of reactive nitrogen oxide species (RNOS). Oxidation of NO with superoxide forms peroxynitrite and nitrosating species such as NO_3, NO_2 and N_2O_3. N_2O_3 leads to the formation of carcinogenic *N*-nitroso compounds, DNA strand breaks and cross-linking of DNA. Reactive nitrogen species such as NO and peroxynitrite are also cytotoxic and can cause DNA damage, and these are generated by the macrophages during inflammatory bowel disease, which is a predisposant factor for colon cancer (15). Several studies showed that higher NO or higher activity of iNOS increases colon carcinogenesis (2, 10, 16, 17). In the present study, levels of NO were significantly lower in vitamin C treated mice, than those of azoxymethane only group (p<0.05). Several studies carried on various laboratory animals have proven that vitamin C has the potential of preventing colon cancer development (3). In our study, protective effect of vitamin C against AOM induced oxidative stress in mice was not revealed because of there was not any AFC which is accepted primary of colon carcinoma in histopathologic examination of AOM induced mice colon.

Several mechanisms may contribute a reduced level of NO in Vitamin C injected mice. For instance, vitamin C stimulates the immune system and it is suggested that it might prevent cancer development by enhancing the immune surveillance (3). Furthermore, by inhibiting the production of nitrosamines and nitrosamides, vitamin C can reduce nitrites, which induce tumor development in experimental animals and possibly in humans (3). Vitamin C might be beneficial with its ability to impede nitrosamine formation by reacting with nitrite to convert it to nitrous oxide, thus preventing the genetic material from the damaging effect of mutagenic nitrosamines (7).

MDA a major product of lipid peroxidation, is known to have mutagenic and carcinogenic effects. It is a known fact that lipid peroxidation has an important role at the initiation and promotion phases of cancer, and lipid peroxidation increases in the malignant tissues and during the advanced stages of cancer (14, 18, and 19). In our study, MDA concentration was significantly increased in AOM administered group in comparison to vitamin C supplemented group. These results are in accordance with previous studies (18-20). Although tissue MDA levels were clearly decreased by vitamin C, its exact mechanism is not clear. Reports that vitamin C directly scavenges hydroxyl radicals and thereby inhibits lipid per oxidation are well documented (20). Reduction in MDA levels in the vitamin C treated mice is probably due to vitamin C's antioxidant and free radical scavenging effects. Vitamin C is readily absorbed and can be administered via any route. With ease, it seems to enter peritoneal tissues where it prevents oxidative damage, preserves mitochondrial function, and it has low toxicity (21).

Glutathione is the most abundant intracellular non-protein thiol. This tripeptide is an important antioxidant against free radicals and can be significantly lowered during oxidative stress. Depletion of intracellular GSH is one of the initial steps leading to apoptosis. As most cells do not have GSH uptake mechanism, they depend on re-synthesis to prevent cell death (22). In the present study, significantly higher GSH levels were detected in vitamin C treated mice. This increased GSH is consistent with the protective effects of vitamin C against oxidative damage induced by AOM, which is expected to reflect the fact that colon tissue is better protected by vitamin C against oxidative damage induced AOM in mice.

In conclusion, the present study demonstrates that intraperitoneal administration of vitamin C reduces azoxymethane-induced oxidative stress in mice colon. However, more investigations are required to evaluate vitamin C's antioxidant protective effect in clinical and experimental models.

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