Nicotine alleviates colitis-induced damage in rats via its anti-oxidative activity

Suçlanlarda kolite bağlı hasarı nikotin antioksidatif aktivitesi ile hafifletir

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ABSTRACT

Objective: Previous studies have demonstrated a higher incidence of ulcerative colitis in non-smokers. We investigated the beneficial effects of nicotine treatment on colitis-induced anxiety and oxidative colonic damage on rats.

Materials and Methods: Wistar Albino (250-300 g) rats (n=40) were randomly divided into 5 groups as saline-treated colitis group, nicotine pre-treated colitis group, nicotine post-treated colitis group, continuously nicotine-treated colitis group and control group. Groups received intraperitoneal injections of saline or nicotine (0.1 mg/kg/day) for 15 days prior to and for 3 days following the colitis induction. Malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO), superoxide dismutase (SOD) and catalase (CAT) activities, histological evaluation and DNA fragmentation were studied in colonic samples. Anxiety levels were evaluated with the hole-board test. Results were evaluated using ANOVA and Mann-Whitney-U tests.

Results: The saline-treated colitis group had increased MPO and MDA levels, DNA fragmentation and histological damage scores when compared with the control group. In the nicotine-treated groups MPO and MDA levels and DNA fragmentation were reduced, with lower histologic damage scores. Reduced SOD, CAT and GSH levels were also increased in nicotine-treated groups.

Conclusion: This study demonstrates antioxidant effects of nicotine treatment in the acetic acid-induced colitis model including an increased antioxidant capacity, reduced migration of neutrophils to the inflamed colon and a reduction of membrane damage.

Key words: Ulcerative colitis, Anxiety, Myeloperoxidase, Superoxide dismutase, Glutathione, Catalase

ÖZET

Amaç: Epidemiyolojik çalışmalar ülseratif kolitin sigara kullanmanınla daha sık görüldüğünü bildirmektedir. Bu çalışmada kolite oluşan oksidan hasar ve anksiyete üzerine nikotin ön tedavisinin ve/veya kolit sonrası tedavinin olması yararlı etkilerinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Wistar Albino (250-300 g) sıçanlar (n=40), serum fizyolojik (SF) verilen, ön-tedavili, tedavili ve ön-tedavili kolit grubu olmak üzere beş gruba ayrıldı. Kolit öncesinde 15 gün ve kolit sonrasında 3 gün sıçanlara intraperitoneal SF ya da nikotin (0,1 mg/kg/gün) uygulandı. Kolon örneklerinde oksidan hasar belirleme için malondialdehid (MDA), miyeloperoksidad (MPO) aktivitesi, glutatyon (GSH), süperoksit dismutaz (SOD), katalaz ve DNA fragmentasyonu ölçümleri ile histolojik skorlama yapıldı. Delikli kutu testi ile anksiyete seviyeleri belirlendi. Sonuçlar ANOVA ve Mann Whitney U testi ile analiz edildi.

 Bulgular: Kolitte artan anksiyetenin nikotin ile değişmediği görüldü. SF verilen kolit grubunda kolon MPO ve MDA düzeyleri ile DNA fragmantasyonunun ve hasar skorunun kontrol grubuna kıyasla artış, nikotin verilen tüm kolit gruplarında ise bu hasar göstergelerinin anlamlı şekilde düştüğü görüldü. Histolojik doku hasarı skorunun nikotin uygulanan kolit grubunda daha az olduğu görüldü. SOD, katalaz ve GSH düzeylerindeki azalmının nikotin tedavisile anlamlı şekilde arttığı görülüldü.

Sonuç: Çalışmada acısdan asitli oluşturulan kolit modelinde, nikotin tedavisinin doku antioksidan kapasitesini artırmak, inflamasyon sonucu nötrofil göcünü ve membran hasarını azaltarak antioksidan etki oluşturduğu gösterildi.

Anahtar kelimeler: Ulseratif kolit, Anksiyete, Miyeloperoksidadz, Süperoksit dismutat, Glutatyon, Katalaz

Introduction

Inflammatory bowel disease (IBD) is an idiopathic chronic disease with probable genetic heterogeneity affecting nearly 30 million people worldwide [1]. Although it is well-documented that smoking is associated with several diseases and with an increased morbidity and mortality of smokers [2], several epidemiological observations have demonstrated that the risk of developing ulcerative colitis (UC) is greater in both ex-smokers and non-smokers [3,4] than in smokers. Published evidence also suggests that as compared to a healthy population, a higher proportion of...
IBD patients suffer from anxiety and depression [5], and studies of smokers have shown that nicotine can relieve stress and reduce anxiety [6]. Based on these observations, nicotine, as the primary causative and addictive substance in tobacco, has been used as an alternative therapeutic agent for treating UC in some clinical trials [7-9].

Major pathogenic characteristics of UC include the infiltration of neutrophils into the inflamed colon. When the recruitment of polymorphonuclear cells (PMN) and macrophages into the colon is increased, a marked increase in the production of reactive oxygen metabolites (ROM) and pro-inflammatory cytokines will result as a secondary amplification of the inflammatory responses [10]. It has been shown that in the mucosa of IBD patients chronic and relapsing inflammation is characterized by an increased expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α in immune cells [11, 12]. In contrast to this, nicotine was shown to suppress the synthesis of eicosanoids [13-15] and pro-inflammatory cytokines, interleukin (IL)-1β and TNF-α, and this may explain the beneficial effects of smoking and nicotine in ulcerative colitis [16]. Moreover, it has also been proved that chronic nicotine administration to experimental animals results in a significant decrease in the antibody response [17] and that chronic in vivo nicotine exposure leads to T-cell anergy and may contribute to nicotine/cigarette smoke-induced immunosuppression [18].

Based on these findings, the present study investigated the beneficial effects of nicotine treatment on colitis-induced anxiety and oxidative colonic damage in rats.

Materials and Methods

Animals
Male Wistar albino rats (250-300 g) supplied by the Marmara University (MU) Animal Center (DEHAMER) were housed in an air-conditioned room with 12-h light and dark cycles, where the temperature (22±2oC) and relative humidity (65-70%) were kept constant. All experimental protocols were approved by the MU Animal Care and Use Committee.

Induction of colitis and experimental protocol
Before the induction of colitis, animals were fasted for 18 h. Colitis (n=32) was induced by a modification of the method of MacPherson and Pfeiffer [19]. Under light ether anesthesia, a polyethylene catheter (PE-60) was inserted into the colon with its tip positioned 8 cm from the anus. To induce colitis, a solution of 1 ml of 5 % (v/v) acetic acid diluted in saline (pH 2.3) was instilled. After a 30 sec period of exposure, excess fluid was withdrawn, and the colon was then flushed with 1.5 ml of phosphate-buffered saline (pH 7.4). The rats in the control group (n=8) were subjected to the same procedure with the exception that isotonic saline was substituted for acetic acid. The time of acetic acid or saline instillation and decapitation was identical in all experimental groups.

The rats were randomly divided into 5 groups. Animals in all groups received intraperitoneal injections of either saline or nicotine (nicotine bitartrate, 0.1 mg/kg/day; Sigma, St. Louis, MO) for 15 days prior to the colitis induction, and injections were continued with saline or nicotine for the 3 consecutive days following the colitis induction. The saline-treated colitis group (n=8) received saline injection before and after the colitis induction. In the nicotine pre-treated colitis group (n=8), nicotine injections were given for 15 days before the colitis induction and were replaced with saline for 3 days following the colitis induction. The nicotine post-treated colitis group (n=8) had saline injections prior to the colitis induction, but received nicotine during the following 3 post-colitis days. Nicotine was continued for 18 days in the continuously nicotine-treated colitis group (n=8). The intracolonic saline instilled control group received saline injections for 18 days (n=8).

On the third day of colonic instillation, all rats were decapitated and the last 8 cm of the colon were removed. Samples taken from this distal colon region were stored at -80°C for subsequent measurement of glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) levels and myeloperoxidase (MPO) activity. Additional tissue samples were obtained for histological evaluation and for the determination of DNA fragmentation in the colonic mucosa.

Evaluation of anxiety
Hole-board tests were performed to evaluate the level of anxiety on the first day of the experiment before any treatments were started. They were repeated on the 15th day before colitis induction and on the 3rd day of colitis induction. It is well known that an increase in anxiety reduces exploratory behavior in rats, which can be tested by using the hole-board test [20]. The hole-board apparatus, providing a measure of directed exploration in rats [21], consisted of a wooden board (40×40 cm) with 16 equally spaced holes (each 3 cm in diameter). The hole-board test was performed by placing the rat in the center of the wooden board, and the test was recorded by a video camera for 5 min. Then the number of head-dipping into the holes was counted from the videotape [22]. A number of head-dippings below normal indicated a reduction in the exploratory behavior and increased anxiety.

Assessment of severity of colitis
The rats were decapitated 72 h after the colonic instillation of saline or acetic acid and the distal 8 cm of the colons were opened down their mesenteric borders and cleansed of...
luminal contents. For light microscopic investigations, the tissues were fixed in 10% formaldehyde and then underwent routine histologic preparation before embedding in paraffin. For each animal, four randomly taken tissue sections (5µm) were cut on a rotary microtome, mounted on slides and stained with hematoxylin and eosin. Sections were examined under an Olympus BX51 photomicroscope for observation of morphological changes. All tissue sections were examined by an experienced histologist, who was unaware of the treatments. At least five microscopic areas were examined to score each specimen. Histological scoring was made as 0, none; 1, mild; 2, moderate; 3, severe for each of the following criteria: epithelial damage, submucosal edema, inflammation and damage in muscularis externa. The maximum score calculated was 12.

**GSH and MDA assays**

Colonic samples were homogenized in ice-cold 150 mM KCl for determination of MDA and GSH levels. The MDA levels were assayed for products of lipid peroxidation [23]. Results were expressed as nmol/g MDA tissue. GSH was determined by the spectrophotometric method using Ellman's reagent [24]. Results are expressed as µmol/g GSH tissue.

**MPO activity**

Tissue-associated MPO activity was determined in the colonic samples as an indication of accumulation of neutrophils. MPO is a natural constituent of primary granules of neutrophils, and a direct relationship between the MPO activity measured on tissue samples and the number of neutrophils has previously been shown [25]. All reagents for MPO assay were obtained from Sigma. The tissue samples (0.2–0.3 g) were homogenized in 10 volumes of ice-cold potassium phosphate buffer (50mM K2HPO4, pH 6.0) containing hexadecyltrimethylammonium bromide (HETAB; 0.5%, w/v). The homogenate was centrifuged at 30,000 g for 10 min at 4°C, and the supernatant was discarded. The pellet was then resuspended with an equivalent volume of 50mM K2HPO4 containing 0.5% (w/v) hexadecyltrimethylammonium bromide and 10mM ethylenediaminetetraacetic acid (EDTA, Sigma). MPO activity was assessed by measuring the H2O2-dependent oxidation of o-dianisidine 2HCL. One unit (U) of enzyme activity was defined as the amount of MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

**SOD and CAT activities**

SOD activity in the colonic samples was measured according to a previously described method [26]. Briefly, measurements were performed in cuvettes containing 2.8ml of 50mM potassium phosphate (pH 7.8) with 0.1mM EDTA, 0.1mM of 0.39 mM riboflavin in 10 mM potassium phosphate (pH 7.5), 0.1ml of 6 mM of o-dianisidine 2HCl in deionized water, and tissue extract (50, 100 ml). Cuvettes with all their components were illuminated with 20-W Sylvania Grow Lux fluorescent tubes that were placed 5cm above and to one side of cuvettes maintaining a temperature of 37°C. Absorbances were measured at 460 nm with a Shimadzu UV-02 model spectrophotometer. A standard curve was prepared routinely with bovine SOD (Sigma–Aldrich; S-2515-3000 U) as reference. Absorbance readings were taken at 0 and 8 min of illumination and the net absorbances were calculated.

The method for the measurement of CAT activity was based on the catalytic activity of the enzyme that catalyzes the decomposition reaction of H2O2 to give H2O and O2 [27]. Briefly, the absorbances of the tissue samples containing 0.4 mL homogenate and 0.2 mL H2O2 were read at 240 nm and 20°C against a blank containing 0.2 mL phosphate buffer and 0.4 mL homogenate for about 1 min.

**Colonic DNA fragmentation assay**

Mucosal samples (0.10-0.15 mg) from colon were homogenized in 10 volumes of a lysis buffer (5 mM Tris HCl, 20 mM ethylene diamine tetraacetic acid [EDTA], 0.5% (v/v) t-octylphenoxypolyethoxyethanol [Triton-X 100]; pH=8.0). Two separate samples of 1 mL were taken from the mucosal samples and centrifuged at 25,000 g for 30 min to separate the intact chromatin in the pellet from the fragmented DNA in the supernatant. The pellet was resuspended in 1 mL of Tri-EDTA buffer (pH=8.0), 10 mM:1 mL. Both the supernatant and the resuspended pellet were assayed for the DNA content by the diphenylamine reaction described by Burton [28]. Tricarboxylic acid (TCA, 25%) was added to both tubes containing the resuspended pellet and supernatant and the tubes were kept at 4°C overnight. The samples were then centrifuged at 4°C and 25,000 g for 30 min. The supernatants were discarded and 5% of freshly prepared TCA was added to the pellets in both tubes. The tubes were shaken for 20 min at 95°C and a freshly prepared diphenylamine (DPA) solution (15 mg DPA dissolved in 1 mL glacial acetic acid with the addition of 1 mL concentrated sulphuric acid) was added in a volume twice that of the samples. After the samples were left at 37°C for 4 h, optical densities were read at 595 nm.

**Statistics**

Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). All data were expressed as means ± SEM. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey’s
multiple comparison tests, and the histological data were compared with the non-parametric Mann Whitney U test. Values of p<0.05 were regarded as significant.

Results

Effect of nicotine treatments on the anxiety levels of rats

Head-dipping numbers that were recorded using the hole-board test at the beginning of the experiment were not different among the experimental groups. Numbers of head-dippings were significantly reduced in all rats with colitis (p<0.05), indicating reduced exploratory behavior and increased anxiety, but no differences were evident among the treatment regimens (data not shown).

Effect of nicotine treatments on colonic MDA levels, MPO activities and DNA fragmentation ratios

In the saline-treated colitis group, colonic MDA level, an index of lipid peroxidation, was found to be significantly elevated as compared to the control group (p<0.001; Fig. 1A). Nicotine, given either as a pre- or post-treatment or as a continuous treatment, significantly abolished the elevation in MDA levels (p<0.001). Induction of colitis in saline-treated rats caused a significant increase in colonic levels of MPO activity, a marker of neutrophil infiltration into the tissue, compared with that of the control group (p<0.05), while only continuous nicotine treatment given to the rats with colitis decreased the enzyme activity (p<0.05; Fig. 1B). In accordance with increased MDA levels and MPO activities, DNA fragmentation indicating apoptosis, was significantly increased in the saline-treated colitis group as compared to the control group (p<0.01; Fig. 1C). However, this colitis-induced increase in apoptosis was suppressed by nicotine treatments (p<0.05-0.01), while the reduction by nicotine pre-treatment did not reach statistical significance.

Effect of nicotine treatments on the extent of colonic damage

Histological analysis by light microscopy revealed that the saline-treated colitis group showed moderate damage in the epithelium and muscularis externa layer with mild submucosal edema and inflammation, as compared to the control group, which demonstrated a regular colonic mucosa and submucosa (Fig. 2). Similarly, in the nicotine pre-treated colitis group, moderate epithelial damage and submucosal edema along with mild damage in the muscularis externa and mild inflammation were observed. In the nicotine post-treated and the continuously-treated colitis groups, mild epithelial and damage of the muscularis externa with submucosal edema and moderate inflammation were evident in the post-treatment group. Histological damage scores in the saline-treated and nicotine-treated colitis groups were not statistically different (Fig. 1D).

Figure 1. (A) The malondialdehyde (MDA) levels, (B) myeloperoxidase (MPO) activities, (C) percentage of DNA fragmentation and (D) histological damage scores of the experimental groups. *p < 0.05, **p<0.01, compared to vehicle-treated control group; +p < 0.05, ++p<0.01, +++p=0.001, compared to vehicle-treated colitis group.
Effect of nicotine treatments on colonic GSH contents

Induction of colitis in the saline-treated rats caused a significant reduction in colonic GSH level when compared with that of the control rats (p<0.01). In contrast to this, nicotine treatment in either regimen abolished this reduction in GSH content (p<0.01; Fig. 3A). Similarly, SOD activity in the saline-treated colitis group demonstrated a significant decrease (p<0.05), but the enzyme activity was replenished only in the nicotine post-treated colitis group (p<0.05; Fig. 3B). When compared with the control group, CAT activity...
in the saline-treated colitis group showed a tendency to decrease, but the reduction did not reach statistical significance (Fig. 3C). However, treatment with nicotine with different regimens elevated the CAT activity significantly (p<0.05-0.001).

Discussion

The results of the present study reveal that nicotine treatment ameliorates colitis-induced oxidative damage as assessed by depressed colonic MDA level, MPO activity and mucosal apoptosis concomitant with increased tissue GSH content, SOD and CAT activities. Application of a 15-day pretreatment was equally effective as the 3-day post-treatment or the continuous nicotine treatment on most of the parameters. However, the effect of nicotine on neutrophil infiltration was only evident when nicotine was given both before and after the colitis induction. However, colitis-induced anxiety demonstrated by reduced head-dipping at hole-board test was not altered by nicotine treatment. The amount of nicotine injected into the rats in the current study (0.1 mg/kg/day) was chosen to correlate with the daily intake of nicotine during smoking in humans, which was shown to vary widely among subjects (10.5 to 78.6 mg, i.e. 0.15 to 1.1 mg/kg/day for a 70-kg man) [29].

Ulcerative colitis is a chronic idiopathic IBD with diffuse, recurrent inflammation of the colon and the rectum, which is characterized by cycles of acute inflammation, ulceration, and bleeding of the colonic mucosa [30]. Epidemiological studies have shown that there was a distinct lack of current smokers in a cohort of UC patients when matched with control subjects [3, 31], while UC patients with a smoking history usually acquire their disease within 1 or 2 years after they have quit smoking [32-34]. Based on this observation, numerous studies have been conducted on the relationship between smoking and UC, and these have further demonstrated that smoking not only protects against the development of UC, but also ameliorates the clinical course of the disease [35-41]. Although the specific mechanisms underlying the therapeutic effect of nicotine are not clear yet, several mechanisms were suggested to mediate the beneficial actions of nicotine. In support of the anti-inflammatory potential of nicotine, it was observed that nicotine inhibited the release of the pro-inflammatory cytokine TNF-α from immune cells and altered the capacity of the cells to respond to TNF-α [42, 43]. Nicotine was shown to have potentiating effects on gut barrier permeability through an increased expression of tight junction proteins [44]. An immunomodulatory effect of nicotine is mediated via the α7 subunit of the nicotinic ACh receptor (α7 nAChR) on various cells of the immune system including T-cells and B-cells, dendritic cells, and macrophages [45]. Accordingly, Kolgazi et al [46] have recently shown that modulation of the cholinergic system by nicotine or ACh esterase inhibition acetic acid-induced colonic inflammation and reversed the pro-inflammatory cytokine response. The current study supports the view that nicotine, by modulating the oxidant and antioxidant status of the colonic tissue, ameliorates colonic damage induced by acetic acid.

The etiology of UC is not clearly understood, but inflammatory mediators, such as excessive production of ROM and an increased expression of pro-inflammatory cytokines by the inflamed mucosa have been proposed to contribute significantly to the development of tissue injury [47]. In patients with active episodes of ulcerative colitis, it is shown that superoxide and hydrogen peroxide, generated by activated leukocytes, are involved in the amplification of the inflammatory response and subsequent mucosal damage. If the production rate of toxic oxidants exceeds the capacity of the endogenous antioxidant enzymes (e.g. superoxide dismutase, catalase and glutathione peroxidase) exacerbation of the inflammation is inevitable. Thus, enhanced oxidative stress and impaired antioxidant defense systems due to the deleterious effect of ROM contribute to the pathogenesis of colitis. Despite the detrimental effects of nicotine as a result of cigarette consumption, nicotine was shown to exert protective action via its direct antioxidant effect or by stimulating the antioxidant systems. Accordingly, in the current study colitis induction resulted in reduced levels of SOD and GSH, while nicotine treatment not only replenished these antioxidant stores but also increased catalase levels, verifying the supportive role of nicotine on the cellular antioxidant capacity.

There are several possible sources of ROM production by the inflamed colon, including the colon epithelium, the microvascular endothelium and/or the inflammatory cells. During acute attacks of UC, massive infiltration of neutrophils and mononuclear cells into the lamina propria and submucosa occurs [48]. Nicotine was discovered to enhance neutrophil sequestration, possibly by scavenging other oxidants in tobacco smoke [49]. It was previously shown that nicotine treatment depresses colonic neutrophil recruitment in acetic acid-induced colitis [47]. Exposure to tobacco smoke as well as different regimens of nicotine administration have attenuated activated colonic MPO activity in different models of colitis [50,51]. In accordance with these, the present findings also demonstrate that nicotine treatment suppresses colonic neutrophil recruitment, suggesting that the actions of nicotine involve the inhibition of the major source of oxidants, neutrophils.

There is a long history of observations suggesting that psychological stress and anxiety contribute to the course of IBD [52, 53], whereas chronic stress increases the severity of intestinal inflammation [54]. In parallel with previous studies demonstrating that psychological stress might amplify intestinal inflammation [55], the present data...
demonstrate that colitis increased the anxiety of the animals when placed on the hole-board. However, the degree of anxiety was not changed in nicotine-treated rats despite the significant attenuation of colonic inflammation. Studies on smokers have shown that nicotine can reduce anxiety and relieve stress [56], which may be responsible for smoking more cigarettes when under stress. Based on anxiety experiments in rodents, it was reported that nicotine can act as an anxiolytic and an antidepressant, while adaptation that occurs by its chronic use may result in increased anxiety and depression following withdrawal [56]. Thus, in the current study, the beneficial effect of nicotine given in the indicated protocol lacked an anxiolytic action.

In conclusion, the results demonstrate that the beneficial effects of nicotine in rat acetate acid-induced colitis include the inhibition of neutrophils infiltrating the injured colon and the maintenance of antioxidant enzymes via the direct antioxidant actions of nicotine.

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