The effects of Gingko biloba extract on acetic acid-induced colitis in rats

Ratlarda asetik asit ile oluşturulmuş kolitte Gingko biloba ekstraktının etkileri

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Background/aims: *Gingko biloba is an antioxidant substance which has antagonistic activity on platelet-activating factor. We aimed to investigate the antioxidant effect and the histopathologic changes caused by Gingko biloba on acetic acid-induced colitis.* **Methods:** *Totally 22 rats were divided into three groups. Group 1 (n=7) served as the control group. Group 2 (n=7) and Group 3 (n=8) were given 2 ml/day of 4% acetic acid by intracolonic instillation for three days. Gingko biloba (100 mg/kg) was then given only to Group 3 intraperitoneally for three days. Oxidative stress was assessed by determinate tissue and serum malondialdehyde (MDA) levels, and colonic damage was assessed by histologic examination.* **Results:** *Depth of necrosis, extent of necrosis, degree of inflammation, extent of inflammation, fibrosis and total histologic scores in Group 2 were significantly higher than in the control group (p<0.05). The same parameters were lower in Group 3 versus Group 2, but the difference was not significant. Tissue and serum MDA levels in Group 2 were significantly higher than Group 1 (p<0.01 and 0.05, respectively). Again, the same parameters in Group 3 were lower than in Group 2, but the difference was not significant statistically.* **Conclusions:** *Gingko biloba did not significantly affect histopathological and oxidative stress parameters in experimental colitis.*

Key words: Gingko biloba, colitis, oxidative stress, malondialdehyde, acetic acid

Amaç: *Gingko biloba platelet aktivatör faktör antagonistik etkiye sahip antioksidan bir maddedir. Biz ratlarda asetik asit ile oluşturulmuş kolitte Gingko biloba'nın antioksidan ve histopatolojik etkilerini araştırmayı amaçladık.* **Yöntem:** *Toplam 22 rat üç gruba ayrıldı. Grup 1 (n=7) kontrol grubuydu. Grup 2 (n=7) ve grup 3 (n=8)'e 3 gün kolon içerisine rektal yoldan 2 ml/gün %4 asetik asit verildi. Grup 3'e eş zamanlı 3 gün 100 mg/kg intraperitoneal şekilde Gingko biloba verildi. Oksidatif stres doku ve serumda malondialdehit düzeylerinin ölçülmesiyle, kolon hasarı ise histopatolojik incelemeyle değerlendirildi.* **Bulgular:** *Grup 2'de nekroz derinliği, nekroz yaygınlığı, inflamasyon derecesi, infalamsyon yaygınlığı, fibrozis ve total histolojik skorlar kontrol grubundan anlamlı yüksekti (p<0.05). Aynı parametreler grup 3'de grup 2'den daha düşüktü ancak fark anlamlı değildi. Grup 2'de doku ve serum malondialdehit düzeyleri grup 1'den anlamlı yüksekti (p<0.05). Aynı parametreler grup 3 de grup 2'den daha düşüktü ancak fark istatistiksel olarak anlamsızdı.* **Sonuç:** *Gingko biloba deneysel kolitte histopatolojik ve oksidatif stres parametrelerini anlamlı etkilemedi.*

Anahtar kelimeler: Gingko biloba, kolit, oksidatif stres, malondialdehit, asetik asit

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are collectively known as inflammatory bowel disease (IBD). Although the pathophysiology of IBD is not known with certainty, immunological processes and reactive oxygen species (ROS) have been proposed to contribute considerably in development of tissue injury (1-4). A growing body of experimental and clinical data suggests that chronic gut inflammation may result from a dysregulated immune response to normal bacterial antigens.

This uncontrolled immune system activation results in the sustained overproduction of reactive metabolites of oxygen and nitrogen. It is thought that some of the intestinal and/or colonic injury and dysfunction observed in IBD is due to elaboration of these reactive species (5). Attenuating oxidative stress in IBD patients has already been a therapeutic strategy for 50 years. Commonly used drugs, in particular sulfasalazine and its active moiety 5-aminosalicylic acid, are potent ROS

scavengers (6). In many studies, it has been reported that antioxidants show beneficial effects on experimental colitis (7-9).

Reactive oxygen species cause impairment of cellular membrane stability and cell death by lipid peroxidation (10). Malondialdehyde (MDA) is an end product of the lipid peroxidation process (11). An increase in free radicals causes overproduction of MDA. MDA level is commonly known as a marker of oxidative stress (12).

Platelet-activating factor (PAF) is a mediator that plays a role in the pathogenesis of IBD. It has been demonstrated that PAF receptor antagonists had beneficial effects on experimental colitis (13-16). Mucosal production of PAF is elevated in UC (17). Therapeutic efficacy of PAF receptor antagonists were reported in animal models of IBD (3).

Gingko biloba extract (EGb 761) is a dioecious tree with a history of use in traditional Chinese medicine. Gingko biloba contains many different flavone glycosides and terpenoides. It has an antioxidant action as a free radical scavenger (18, 19). It had been reported in many studies that Gingko biloba improved tissue damage in various organs by its antioxidant effect (18-20). Gingko biloba has an antagonistic activity on PAF. The drug is well tolerated (21).

Acetic acid has been used extensively in many experimental studies, since it causes colitis in rats (22-24). Oxidative stress and PAF are important factors that play a role in the pathogenesis of IBD. As Gingko biloba is known to have antioxidant and antagonist activity on PAF receptors, we aimed to investigate the effects of Gingko biloba on tissue injury and oxidative stress in an experimental model of acetic acid-induced colitis.

MATERIALS AND METHODS

Animals

Healthy male Wistar rats weighing 200-250 g were used in the study. Animals were harbored on a 12-h light/dark cycle (lights on from 08:00 am) at a constant ambient temperature ($24\pm1°C$) with normal rat chow and water available ad libitum. The study protocol was in accordance with the guidelines for animal research and it was approved by the Ethical Committee of our hospital.

Induction of Colitis

After rats were anesthetized with a dose of 75 mg/kg ketamine (Ketalar, Parke-Davis and Eczacibasi, Istanbul) injected intraperitoneally, a flexible plastic catheter with an outer diameter of 2 mm (Bıçakçılar, Istanbul, Turkey) was inserted rectally into the colon with the aim to place the catheter tip 8 cm proximal to the anus. Colitis was induced by intracolonic instillation of 2 ml/day 4% acetic acid with 24-hour intervals for three days, as previously described (24). The rats were inspected for the presence of diarrhea.

Experimental Protocol

Twenty-two rats were randomly divided into three groups. Group 1 (n=7) served as the control group and 1 ml 0.9% saline was given by intracolonic instillation. Groups 2 (n=7) and 3 (n=8) were given 2 ml/day of 4% acetic acid by intracolonic instillation for three days. Following induction of colitis, Group 3 alone was then administered intraperitoneally a dose of 100 mg/kg Gingko biloba (supplied as a dry powder, obtained from Abdi Ibrahim Drug Company, Turkey) starting 24 hours after the last dose of acetic acid and maintained for three consecutive days. Gingko biloba treatment regimen was chosen in accordance with the treatment procedures used by other studies done with Gingko biloba (18,25). Gingko biloba solution was prepared daily by dissolving 1 g of the extract in 100 ml of 0.9% saline.

On the 7th day of the study, all animals were sacrificed by ketamine administration in overdose. A piece of distal colon 10 cm in length was removed via laparotomy for histological and biochemical examinations. Concomitantly, blood samples were taken from the right ventricle for MDA analysis. All colon tissues were washed two times with cold saline solution, placed into glass bottles, labeled, and stored at -20°C until processing.

Assessment of Colonic Damage

A piece of distal colon 10 cm in length was fixed in phosphate-buffered formaldehyde and embedded in paraffin, and 5μm sections were prepared. Tissues were routinely stained with hematoxylin and eosin and were evaluated by light microscopy by a pathologist unaware of the experiments being performed.

The histologic scoring of induced colitis was determined by examining each specimen for the following features and allocating increasing points according to the severity of the findings:

A: Depth of necrosis: none = 0; mucosal = 1; mucosal and submucosal = 2; mucosal, submucosal, and muscularis propria = 3; full thickness = 4.

B: Extent of necrosis: none = 0; small area = 1; moderate area = 2; large area = 3; extensive = 4.

C: Degree of inflammation: none = 0; minimal = 1; mild = 2; moderate = 3; severe = 4.

D: Extent of inflammation: none = 0; mucosal = 1; mucosal and submucosal = 2; mucosal, submucosal, and muscularis propria = 3; full thickness = 4.

E: Fibrosis: none = 0; mucosal = 1; mucosal and submucosal = 2; mucosal, submucosal and muscularis propria = 3; full thickness = 4.

The scores for each category examined were calculated for each specimen in the different groups. These were then added to obtain the total score, which was then divided by the number of rat colons examined in each group to obtain the average histologic score of induced colitis for the group, as previously described (26).

MDA Analysis

On the processing day, tissues were first thawed and washed with 0.9% saline again and cut into small pieces with scissors. Approximately 200-250 mg colonic tissues were then homogenized in 10 volumes (w/v) of ice-cold 150 mM KCl using a glass-Teflon homogenizer for 2 min at 5000 rpm (Tempest Virtishear, Model 278069; The Virtis, Gardiner, NY). The MDA level was measured in the homogenate. The protein concentration of the homogenates was determined according to Bradford at this stage (27).

Tissue and serum MDA levels were determined by the method of Buege and Aust (28). Briefly, 250 μL serum or tissue homogenate; 500 μL of TBA reagent (3.7 g/L in 0.25 mol/L HCl); and 1.5 ml of 15% trichloroacetic acid (in 0.25 mol/L HCl) were combined in a 10 ml screw-cap Pyrex centrifuge tube, mixed, and heated for 30 min in boiling water. After cooling in an ice bath, 3 ml of n-butanol was added, mixed and centrifuged, and the chromogen extracted. The absorbance of the organic phase was determined spectro-photometrically at 535 nm against a blank. As a calibrator, tetramethoxypropane (TMOP) was used and absorbance of standards was converted to mmol MDA from a calibration curve generated with 1,1,3,3-TMOP. Lipid peroxidation levels were expressed as nanomoles per gram wet colonic tissue protein (nmol/g protein) for tissue homogenates or nanomol/L for serum samples.

Statistical Analysis

Data are expressed as mean ± SD. Statistical calculations were done with SPSS 11.0 statistical software package. Results were analyzed by Kruskal-Wallis and Mann-Whitney U tests. P values <0.05 were considered as significant.

RESULTS

In our study, colitis was successfully produced by intracolonic instillation of 2 ml/day 4% acetic acid with 24-hour intervals for three days. Because severe diarrhea in all rats except the control group was observed on the third day, administration of acetic acid was ceased.

Histologic Results

Table 1 shows mean ± SD values of depth of necrosis, extent of necrosis, degree of inflammation, extent of inflammation, fibrosis and total histologic scores in colon tissue for each group. These parametres were first compared with Kruskal-Wallis test and a statistically significant difference between groups was detected excluding necrosis (p=0.32, 0.28, 0.013, 0.006, 0.012 and 0.001, respectively).

Mann-Whitney U test was used to compare the groups regarding the parameters above. In Group 2, depth of necrosis, extent of necrosis, degree of inflammation, extent of inflammation, fibrosis and total histologic scores were higher than in Group 1 (p<0.01, 0.01, 0.05, 0.01, 0.01 and 0.01, respectively). Acetic acid increased tissue damage, as seen in Figure 1.

Table 1. Mean ± SD values of depth of necrosis, extent of necrosis, degree of inflammation, extent of inflammation, fibrosis and total histologic scores of groups

Groups	DN	EN	DI	EI	FIB	THS
Group 1 (n=7)	0.00±0.00	0.00±0.00	1.57±0.53	1.29±0.48	0.00±0.00	3.14±0.69
Group 2 (n=7)	1.43±1.27[a]	1.43±1.27[a]	2.57±0.53[b]	2.71±0.75[a]	1.14±0.90[a]	9.29±3.54[a]
Group 3 (n=8)	1.00±1.19	0.88±0.99	2.25±0.46	2.25±0.70	1.00±0.75	7.38±2.44

Group 1: Control, Group 2: Acetic acid-induced colitis, Group 3: Acetic acid-induced colitis + Gingko biloba
DN: Depth of necrosis, EN: Extent of necrosis, DI: Degree of inflammation, EI: Extent of inflammation, FIB: Fibrosis, THS: Total histologic score
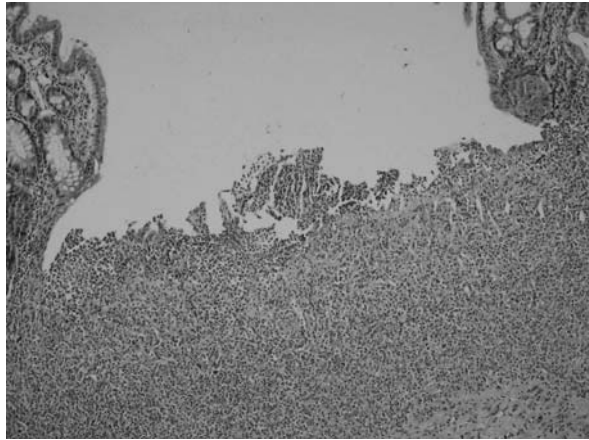[a]compared with control p<0.01, [b]compared with control p<0.05

Figure 1. This image demonstrates the damage caused by acetic acid on colonic tissue

In Group 3, depth of necrosis, extent of necrosis, degree of inflammation, extent of inflammation, fibrosis and total histologic scores were lower than in Group 2, but the differences were not statistically significant (p=0.46, 0.36, 0.22, 0.28, 0.71 and 0.26, respectively). Gingko biloba did not alter tissue damage significantly (Figure 2).

Figure 2. This images shows that Gingko biloba does not alter tissue damage

Biochemical Assessment

Table 2 presents mean ± SD values of tissue and serum MDA levels (nmol/g protein and nmol/L) of groups. Tissue and serum MDA levels were first compared with Kruskal-Wallis test and statistically significant difference between groups was detected (p=0.001 and 0.014, respectively). Mann-Whitney U test was used to compare the groups regarding these parameters. Tissue and serum MDA levels in Group 2 were found significantly higher than in Group 1 (p<0.01 and 0.05, respectively). The same parameters in Group 3 were found lower than in Group 2, but the differences were not statistically significant (p=0.90 and 0.95, respectively).

Table 2. Mean ±SD of tissue and serum malondialdehyde (MDA) levels of groups

Groups	Tissue MDA (nmol/g protein)	Serum MDA (nmol/L)
Group 1 (n=7)	2.02±0.84	1.41±0.27
Group 2 (n=7)	37.14±12.10[a]	2.32±0.95[b]
Group 3 (n=8)	33.75±13.98	2.23±0.63

Group 1: Control, Group 2: Acetic acid-induced colitis, Group 3: Acetic acid-induced colitis + Gingko biloba, [a]compared with control p<0.01, [b]compared with control p<0.05

DISCUSSION

Inflammatory bowel disease is characterized by the involvement of ROS in tissue damage (29-31). Increased granulocyte accumulation in inflammatory lesions of gut mucosa in patients with IBD has been shown (32, 33). Those activated cells release a number of inflammatory mediators such as toxic oxygen metabolites, lysosomal enzymes, and derivatives of arachidonic acid metabolism. It has been proposed that inflammation of mucosa causes impairment of the antioxidant defense mechanism, and makes tissue more susceptible to oxidative damage (34, 35). In turn, superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals, secreted by neutrophils and phagocytes accumulating in the inflammatory lesion, cause impairment of cellular membrane stability and cell death by leading lipid peroxidation (10). Colonic biopsy from specimens of patients with active IBD had enhanced levels of lipid peroxidation products. These findings suggest that chronic gut inflammation promotes an imbalance between pro-oxidant and antioxidant mechanisms, leading to the net accumulation of oxidatively modified proteins and lipids (36). In many studies, it has been

reported that antioxidants show beneficial effects on experimental colitis (7-9).

Since oxygen radicals have high reactivity and short lifetime, it is difficult to assess the involvement and extent of tissue damage induced by oxygen radicals. Therefore, this problem has been bypassed by measuring the effects of radical reactions with biological substances (i.e., lipid peroxides and/or their products as MDA) (18).

Platelet-activating factor is a mediator that plays a role in the pathogenesis of IBD. It had been demonstrated that PAF receptor antagonists had beneficial effects on experimental colitis (13-16). Gingko biloba is an antioxidant substance and also has antagonistic activity on PAF. By such mechanisms, Gingko biloba may be useful in lowering the inflammatory process in experimental colitis.

In our study, we successfully produced distal colitis with acetic acid. Acetic acid increased tissue damage significantly. In Group 2, depth of necrosis, extent of necrosis, degree of inflammation, extent of inflammation, fibrosis and total histologic scores were higher than in Group 1 ($p<0.01$, 0.01, 0.05, 0.01, 0.01 and 0.01, respectively). In Group 3, depth of necrosis, extent of necrosis, degree of inflammation, extent of inflammation, fibrosis and total histologic scores were lower than in Group 2, but differences were not significant statistically ($p=0.46$, 0.36, 0.22, 0.28, 0.71 and 0.26, respectively). These results show that Gingko biloba does not attenuate intestinal damage.

Tissue and serum MDA levels in Group 2 were found significantly higher than in Group 1 ($p< 0.01$ and 0.05, respectively). Tissue and serum MDA levels in Group 3 were found lower than in Group 2, but differences were not statistically significant ($p=0.90$ and 0.95, respectively). In our study, we found that Gingko biloba did not alter tissue damage and MDA levels significantly. But it has been reported in many studies that Gingko biloba improved tissue damage in various organs by its antioxidant effect (18-20). Zeybek et al. reported that Gingko biloba significantly decreased MDA levels and histopathologic scores of the pancreatitis in rats (18). Bridi et al. reported that Gingko biloba had antioxidant activity in the hippocampus, striatum and substantia nigra of rats (19). Gingko biloba has an antioxidant activity as a free radical scavenger, a relaxing effect on vascular walls, an ameliorating effect on blood flow and microcirculation, and a stimulating effect on neurotransmitters. Besides a direct scavenging effect on ROS, Gingko biloba exerts an anti-inflammatory effect on inflammatory cells by suppressing the production of reactive oxygen and nitrogen species (19).

Our results may depend on administration route and dosage of Gingko biloba. In our study, we produced distal colitis with acetic acid and administered Gingko biloba intraperitoneally. At the present time, topical agents have been widely recommended in treatment of distal colitis. Tissue damage may be better improved with the installation of Gingko biloba extract into the colon and/or with different dosage and timing applications.

In conclusion, it is difficult to judge the effects of Gingko biloba with our results. There is no report in the literature concerning the effects of Gingko biloba on experimental colitis. Gingko biloba extract is a well-tolerated antioxidant substance that has antagonistic activity on PAF receptors. We believe that further studies are needed to investigate the effects of Gingko biloba in experimental colitis.

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