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The Effects of Low-dose Methotrexate On Endothelial Dysfunction and Programmed Cell Death (Apoptosis) in 2,4,6 Trinitrobenzensulphonic Acid (TNBSA) - Induced Model of Experimental Colitis

2,4,6 Trinitrobenzensulfonik Asid (TNBSA) ile İndüklenmiş Deneysel Kolit Modelinde Düşük Doz Metotreksatın Endotelial Disfonksiyon ve Programlanmış Hücre Ölümü (Apoptosis) Üzerine Etkileri

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ÖZET

Amaç: Günümüzde iltihabi barsak hastalıkları (İBH) için yürütülen farklı ilaç tedavileri üzerine yapılan çalışmalar giderek artmaktadır.Metotreksat (Mtx) antienflamatuar etkileri nedeniyle İBH'nın tedavisinde kullanılmakta olan bir ilaçtır. Bu çalışmada MTX'in deneysel kolit modelinde endotelial disfonksiyon ve kolon mukozası üzerine etkilerinin araştırılması amaçlanmıştır.

ABSTRACT

Purpose: Currently, the medical investigations on alternative drug therapies in inflammatory bowel disease (IBD) have been increased. Methotrexate (Mtx) has been used in the management of IBD due to its anti-inflammatory effects. It's aimed to investigate the effects of Mtx on endothelial dysfunction and colonic mucosa in experimental acute colitis.

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Materyal ve Metod: Bu çalışma 2,4,6 trinitrobenzensulfonik asit (TNBS) ile indüklenmiş kolitli 36 sıçan üzerinde gerçekleştirildi. Denekler 3 gruba ayrıldı: Grup I: TNBS+Mtx, Grup II: TNBS ve Grup III: Serum fizyolojik lavman. Grup I'e tek doz Mtx uygulanırken, Grup II'de sadece kolit oluşturuldu. III.gruba ise 1 ml/kg/gün serum fizyolojik lavman uygulandı.Deneyin yedinci günü denekler feda edilerek distal kolon kısımları immunohistokimyasal ve biyokimyasal analizler için çıkartıldı.
Bulgular: Bu deneysel akut kolit modelinde Mtx, yangılı kolon mukozasındaki MPO aktivitesi, TNF-α, NO, iNOS ve eNOS salınımını azaltmış ve mukozal hasarı iyileştirmiştir. Bunun yanında Mtx uygulanımı ile apoptotik ve proliferatif süreç baskılanmıştır.
Sonuç: Mtx özellikle iltihabi barsak hastalığının akut alevlenme dönemlerinde alternatif bir ilaç olarak düşünülebilir.

Anahtar Kelimeler: Metotreksat, İltihabi barsak hastalığı, Apoptosis

Material and Methods: This study was carried out on 36 rats induced for colitis by 2,4,6 trinitrobenzensulfonic (TNBS) acid, and they were divided into 3 groups: Group I: TNBS+Mtx, Group II: TNBS, Group III: saline enema. While single dose of Mtx administered as an intramuscular injection in the drug group (Group I), only colitis was induced in the second group (Group II). 1 ml/kg/daily of saline enema was administered intrarectally to the third group. On the seventh day of the experiment, the animals were sacrificed, and the distal colon was removed surgically for immunohistochemical and biochemical analyses. Colonic mucosal NO, iNOS, eNOS, MPO activity, macroscopic changes and histomorphology were evaluated.
Results: Mtx reduced MPO activity, TNF-α, NO, iNOS and eNOS release from inflamed colonic tissue, and it healed mucosal damage in experimental model of acute colitis. Additionally, Mtx suppressed apoptotic and proliferative processes in the inflamed colonic tissue.
Conclusion: Mtx may be regarded as a valid agent especially in acute attack episodes of inflammatory bowel disease.

Key words: Methotrexate, Inflammatory bowel disease, Apoptosis

Introduction

Despite the fact that the exact etiology of inflammatory bowel disease (IBD) remains unknown, advances in the understanding of the immune system and the control of intestinal inflammation have led to the promise of more precise therapies.[1] Gut tissue injury is the result of an abnormal immune response and involves multiple non-immune cellular systems, including intestinal microvascular endothelial cells.[2-5] Endothelial dysfunction has shown to play an important role in the pathogenesis of vascular diseases.[5] An imbalance characterized by reduced NO production or increased reactive oxygen species production may promote endothelial dysfunction.[6,7] In IBD, NO plays a role in vasodilatation.[6-8] The deficiency in anti-inflammatory and antioxidant effects of NO and excessive production of superoxide compromises vascular endothelial function. Thus, endothelial dysfunction becomes an expected finding in the patients with IBD. Kocaman *et al* have also showed that significant endothelial dysfunction was found in patients with severe and moderate ulcerative colitis (UC) as compared with patients with mild UC and normal control subjects. There is a large body of evidence that the inducible form of iNOS that is responsible for high-output production of NO from L-arginine is up-regulated in various forms of mucosal inflammation. Multiple detection strategies have demonstrated that iNOS expression, enzymatic activity and NO production are increased in human IBD tissues.[7,8,10] There is also evidence that the level of iNOS-derived NO correlates well with disease activity in ulcerative colitis.

The homeostatic balance between pro and anti-inflammatory factors has been shown to be markedly disturbed in the intestinal mucosa of patients with IBD.[11,12] Increasing evidence suggests that cytokines, released locally from infiltrating immune cells recruited

to the sites of active inflammation, mediate the pathologically altered intestinal epithelial cell turnover and the high apoptosis rate observed under these inflammatory conditions.[12] As the epithelium is a site of iNOS expression and marked cell death in gut inflammation, we hypothesized that NO may induce apoptosis in colonic epithelial cells. Therefore, the effects of Mtx with anti-proliferative, cytotoxic and anti-inflammatory properties on the histomorphology of colonic mucosa, apoptosis and mucosal NO production in experimentally induced colitis were investigated.

Material and Methods

Thirty-six of Sprague-Dawley rats weighing 150-190 g were used. All animals were kept in a temperature-controlled room with a 12-h light-dark cycle and given a standard laboratory cow and tap water ad libitum. This study was approved by the local animal ethics committee of Ege University.

Induction of colitis

Experimental colitis was induced using 2,4,6 trinitrobenzenesulfonic (TNBS) acid (P2297, SIGMA Chemical, USA) as described in the literature.[13] All animals fasted for 12 h were anesthetized with Ketamine (i.p.,90 mg/kg). The 10F gauge pediatric feeding tube was inserted into the rectum nearly 10 cm proximal to the anus. Then, 0.60 ml of TNBS 5% w/v in 0.25 ml of 50% ethanol was instilled into the lumen of the colon in trandelenburg position and tube was kept inside for 5 minutes in order to get a prompt inflammation with uniform spread along the colon of the rat.All animals were weighted prior to induction of colitis. TNBS colitis was induced as previously described. All animals were randomized into three groups: Group I= TNBS colitis+ Single dose of Methotrexate (0.3 mg/kg, i.m., n=12), Group II= Only TNBS colitis and saline enema had been used in Group III as control (n=12). On the seventh consecutive day, rats were reweighted and then sacrificed by cervical dislocation method. All animals underwent inferior-median laparotomy and distal colons of the rats were removed. Extraluminal changes of colon were examined macroscopically and noted as intrabdominal adhesions, edema, stricture, ulcers, dilatation or petechial focuses. Three representative specimens were taken from the colon from a region 2 to 4 cm proximal to the anus. First segment was taken for immunohistochemical analysis for tissue content of iNOS and eNOS. The second was frozen in 0.05M phosphate buffer, pH6, containing 0.5% hexadecyl-trimethylammonium bromide for myeloperoxidase (MPO), and nitric oxide analysis. And the last specimen was fixed in 10% formalin solution for routine histological examination.

Macroscopic and histomorphologic evaluations of mucosal damage were made as previously described in the literature.[14] For histomorphological examination; mucosal damage was scored as follows: 0=no lesion, 1=edema, hyperemia and minimal inflammatory cell infiltration (ICI), 2=1+crypt abscess or irregularity, 3= 2+multifocal ICI and necrosis. And the macroscopic scoring was made as: 0=no lesion, 1=edema+hyperemia, 2=petechia, small ulcer, 3=large ulcer, necrosis, 4= megacolon, perforation, stenosis. The presence or absence of intraabdominal adhesions, edema and etc. were noted.

Histochemical analysis

All specimens were fixed in 10% formalin during 24 h. Specimens were washed and soaked in a graded series of ethanol. Then they were embedded in paraffin. Sections (5 mm thick) were cut and prepared for both histochemical and immunohistochemical staining. Hematoxylin-eosin staining was used for histological diagnosis.

NO, eNOS and iNOS analyses

Since nitrite (NO2-) and nitrate (NO3-) levels can be used to estimate NO production, we measured the concentration of these stable NO oxidative metabolites in homogenized tissue. Determination of NO2- and NO3- was based on the Griess reaction, in which a chromophore with a strong absorbance at 545 nm is formed by reaction of NO2- with a mixture of naphthlethylenediamine and sulfanilamide.[14] A standard curve was established with a set of serial dilutions (100 µmol/L to µmol/L) of sodium nitrite. The resulting equation was then used to calculate the unknown sample concentrations.

The sections were incubated at 60 °C overnight and then dewaxed in xylene for 30 min. After soaking in a decreasing series of ethanol, sections were washed distilled water. They were then treated with 2% trypsin in 50 mM Tris buffer (pH 7.5) at 37 °C for 15 min, and washed with PBS. Sections were delineated with a Elite

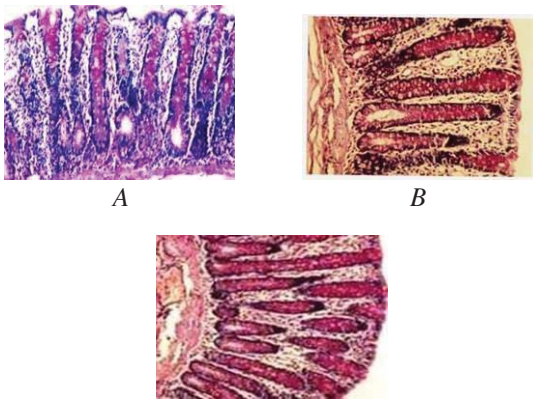

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Figure 1.
A. Local PMNL infiltrations and normal mucosal cript formation (PASX20, Control Group)
B. Minimal edema with local PMNL infiltrations (PASX20,TNBS+Mtx Group)
C. Extensive polymorphonuclear leukocyte (PMNL) infiltration, irregularity of the crypts (PASX20,Only TNBS Group)

Pap pen (DBS, Pleasanton, CA, USA) and incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity to inhibit endogen peroxidase. They were washed 3 times for 5 min with PBS and incubated with primary antibodies, to endothelial NOS (anti-eNOS RB-1711, Neomarkers, Fremont, CA) diluted 1:200 and inducible nitric oxide synthase (anti-iNOS RB-1605, Neomarkers, Fremont,CA) diluted 1:100 for 18 h. Next, the sections were incubated with biotinylated IgG (supplied ready to use by Zymed) for 30 min, followed by three washes in PBS and then with streptavidin-peroxidase conjugate (supplied ready to use by Zymed) for 30 min (Histostain-Plus Bulk Kits; Zymed, San Francisco, CA) and washed with PBS three times. They were then incubated with a solution containing diaminobenzidine (DAB, supplied ready to use by Zymed) 50 µl for each sections (Histostain-Plus Bulk Kits; Zymed,San Francisco, CA) for 5 min to visualize immunolabeling, and finally counterstained with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany). After rinsing with distilled water, counter staining was performed in Mayers Hematoxylin. The sections were dehydrated with 80% and 95% alcohol and immersed in xylene and covered with entellan (01730 Surgipath, Bretton, Peter Borough, Cambridgeshire). Color photographs were taken with 100 ASA color film (Fujifilm, Tokyo, Japan). Control samples were processed in an identical manner, but in order to antibodies IgG which same type with primary antibodies were used. Serial sections were examined and immunolabelling patterns were compared.

Detection of the apoptotic cell death in situ using the TUNEL method

Fragmentation of the DNA in the nucleus is one of the first morphological changes of the apoptotic process and can be detected in histological sections using a terminal deoxynucleotidyltransferase-biotin nick end-labelling method (TUNEL) performed with a commercial kit (DeadEnd Colorimetric TUNEL system, Promega G7130) according to the manufacturer's instructions. Briefly, after proteinase K treatment for 10 min, the sections were incubated at 37 °C with TdT for 60 min.

Apoptotic and Proliferative Index

The quantitative of apoptosis and proliferation were carried out in a blind fashion by two independent observers. Each observer counted at least 100 cells in more than 15 randomly chosen fields per case, and the number of positive cells per 100 cells was designated as the apoptotic index on TUNEL-stained sections and was expressed in percentage. The Ki-67 labelling index was determined by counting at least 100 cells in more than 15 different areas of each section and was expressed in percentage.

MPO activity

The method described by Wei was used in tissue MPO assay15. In this method, 1.3 ml of 25 mM 4-aminoantipyrine 2% phenol solution and 1.5 ml of 1.7 mM H_2O_2 were added and equilibrated for 3-4 min. After establishing the basal rate, a 0.2 ml sample of filtered supernatant was added to the cuvettes and quickly mixed. Increases in absorbance at 510 nm per min were recorded. One unit of MPO activity is defined as that which degrades 1 µmol of H_2O_2 per min at 25 °C. Data are expressed as U/g protein.

Statistical analysis

All values were expressed as the mean ± SEM. The Mann-Whitney U test for non-parametric data evaluation, student T test and one way ANOVA variance analysis

Table 1. Summary of indicators of colitis

Groups	Microscopic score	Macroscopic score	Pretreatment body weight(g)	Posttreatment body weight(g)
Colitis+Mtx (Group I)				
N=	12	12	12	12
SEM±	0.45	0.62	3.34	2.92
Mean	1.25	0.25	165.42	162.92
Median	1.00	0.00	162.50	160.00
Only colitis (Group II)				
N=	12	12	12	12
SEM±	0.51	1.13	3.45	3.65
Mean	2.58	1.00	184.58	168.75
Median	3.00	1.00	185.00	167.50
Saline (Group III)				
N=	12	12	12	12
SEM±	0.51	0.45	2.54	4.94
Mean	0.42	0.25	190.00	182.5
Median	1.00	0.00	190.00	187.5
P value	0.0001^{a},0.003^{b}, 0.0001^{c}	0.045^{a},0.799^{b},0.060^{c}	0.001^{a},0.0001^{b},0.482^{c}	0.581^{a},0.005^{b},0.05^{c}

P < 0.05 values were statistically significant. [a]:Mtx/TNBS, [b]:Mtx/Control, [c]:TNBS/Control

with Tukey's multiple correlations were used for parametric data. A value of p<0.05 was regarded as significant.

Results

When the groups were examined macroscopically, there were two animals (2/12) one with edema and hyperemia and the other with a few petechias on their colonic walls in Mtx group. In contrast, only colitis group showed higher macroscopic injury scores of colon. In fifty percent of this group, hyperemia and edema of colon were the most commonly observed macroscopic finding. Colonic perforation in 1 mm was observed in one of the animals in only colitis group. When Mtx group was compared to groups II and III (control) in terms of macroscopic injury scores, there was statistically difference (p=0.045, p=0.799). Mtx treatment reduced colonic damage macroscopically (Table1 and Fig.3).

Histological assessment demonstrated extensive polymorphonuclear leukocyte (PMNL) infiltration, irregularity of the crypts and ulcerations especially in only colitis group. In contrast, minimal edema with local PMNL infiltrations was seen in Colitis+Mtx group. This group of animals showed better results when compared to only colitis group as for histomorphologic mucosal damage. In Colitis+Mtx group; 75% (n=9) of the animals had grade-1 and 25 %(n=3) had grade-2 mucosal damage. In saline group; 58.3% (n=7) had grade-2 and

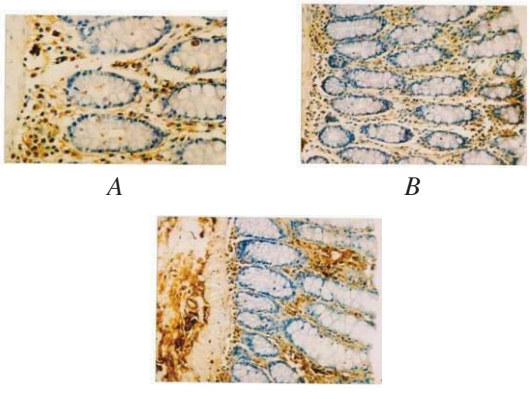

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Figure 2.
A. Mild staining of eNOS in Control Group
B. Moderate staining of eNOS in TNBS+Mtx Group
C. Severe staining of eNOS, TNBS Group

42.7% (n=3) had grade-3 mucosal damage. In the present study, Mtx increased the healing of colonic mucosal injury due to 2,4,6 TNBSA when compared to other two groups with statistically significance (p=0.0001, p=0.003) (Fig 1).

In the present study, administration of Mtx reduced MPO activity in colon of rats with colitis (Table 2). While, in untreated group, mean MPO activity was 0.55 ± 4.953E-02 U/g, it was 0.60±6.348E-02 U/g in Mtx group with statistically significance (p=0.018). The beneficial effects of Mtx were also evident as mean post-treatment body weights of the animals which were reduced almost 45% in group II when compared to group I (Mtx group) but this was not statistically significant (p=0.581) (Table 2).

Table 2. Data for cytokins and NO status

Groups	MPOa	TNF-α	NO	iNOS	eNOS
Colitis+Mtx (Group I)					
N=	12	12	12	12	12
SEM±	0.91	0.85	0.20	0.90	0.16
Median	0.51	6.97	0.79	1.00	2.00
Mean	0.84	7.33	0.66	2.15	2.72
P value	SS	SS	SS	NS	SS
Only colitis (Group II)					
N=	12	12	12	12	12
SEM±	1.10	0.89	0.26	0.67	0.13
Median	0.87	13.35	0.57	2.00	3.00
Mean	1.25	12.75	0.83	2.25	2.87
P value	SS	SS	NS	NS	SS
Control (Group III)					
N=	12	12	12	12	12
SEM±	0.63	0.66	0.19	0.10	0.11
Median	0.52	4.16	0.70	1.00	1.00
Mean	0.60	4.62	0.60	1.78	2.85
P value	0.018^{a},0.918^{b},0.011^{c}	0.0001^{a},0.05^{b},0.0001^{c}	0.05^{a},0.180^{b},0.806^{c}	0.014^{a}, 0.178^{b}, 0.0001^{c}	0.0001^{a},0.0001^{b},0.0001^{c}

P < 0.05 values were statistically significant. [a]:Mtx/TNBS, [b]:Mtx/Control, [c]: TNBS/Control, MPOa:Myeloperoxidase activity (U/mg protein), IL: Interleukin-6, NO:Nitric oxide, iNOS: inducable NO, eNOS: endothelial NO

Inflamed colonic tissue contained increased amounts of NO compared with control group (p=0.014). Tissue treated with Mtx contained 0.66±0.2 nmol/g of NO and it was statistically significant when compared with only colitis-Group II (p=0.05). In the immunohistochemical evaluation of the specimens; Mtx group showed less iNOS and eNOS staining in colon mucosa when compared to group II with statistically significance. While iNOS and eNOS staining of colonic tissues in group II were moderate to severe, Mtx given animals showed better results as they were mildly stained. Table 2 In the present study, experimental colitis induced apoptosis in colon mucosa. Mtx inhibited apoptosis with statistically significant against Group II in which only colitis was induced with any drug administration (p=0.0001). All data were given in details in Table 3.

Discussion

Several clinical trials and analyses have examined the role of Mtx in patients with IBD.[16] Kozarek *et al.*,[17] had initially reported the use of Mtx in IBD patients. Nearly two thirds of patients had improvement in symptoms and this had been demonstrated by colonoscopy in one third of the patients with Crohn's disease, whereas no

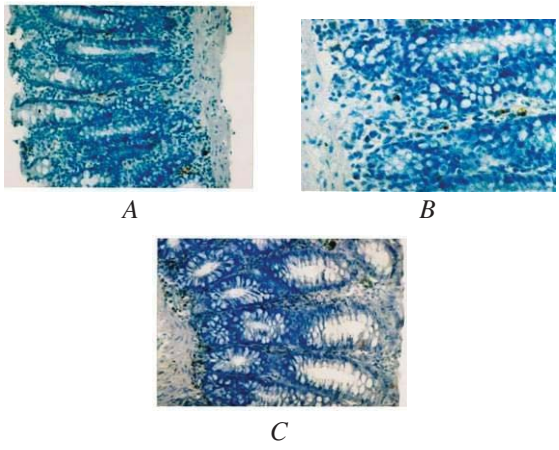

Figure 3.
A. A few apoptotic cells in colonic mucosa (Control Group-Tunel method).
B. Moderate apoptosis in colonic mucosa (TNBS+Mtx Group-Tunel method).
C. Severe apoptosis in colonic mucosa (Only TNBS Group-Tunel method).

such beneficial effect was seen in the patients with ulcerative colitis (UC).[17] Although Kozarek *et al*.[17] suggested a 40% response rate in UC, no reliable data from controlled trials currently exist to support the use of Mtx as therapy for UC. On the other hand, Mtx has also been shown to be effective in the treatment of steroid-dependent IBD. Early studies showed a good clinical response in 39-76% of patients with disease remission usually achieved in the first 3-6 months of therapy.[17-19] The most common side effects of Mtx are gastrointestinal in nature, such as anorexia, nausea, stomatitis, and diarrhea.[20,21] More serious adverse effects include hepatotoxicity, bone marrow suppression, pulmonary hypersensitivity, opportunistic infections, anaphylactoid reactions, and possibly, neoplasms.[21-23] Te *et al*.[24] concluded that cumulative Mtx doses up to 5410 mg given up to 281 wks in patients with IBD are associated with little hepatotoxicity. However, the side effects of Mtx such as hepatotoxicity, bone marrow suppression etc. were not examined in the present study. Our data showed that Mtx administration reduced the mucosal damage in the colon due to experimental colitis. Proliferation, differentiation, and apoptosis of intestinal epithelial cells are tightly regulated by a variety of growth factors, cytokines, hormones, luminal nutrients, and mesenchymal structures. The homeostatic, physiologic intestinal epithelial cell turnover is markedly disturbed in the acutely inflamed intestinal mucosa of patients with immune-mediated bowel disorders.[25] Macroscopically, the intestinal mucosa is characterized by an inflammatory reaction along with erosions or ulcerations.[26] In UC and celiac disease, histological analyses revealed a dramatically increased number of dying intestinal epithelial cells at the site of acute inflammation, characterized by an inflammatory infiltrate in the form of activated lymphocytes, neutrophils and monocytes.[27-29] While apoptosis is historically not linked to states of inflammation, it has become increasingly apparent that inflammatory mediators, e.g., oxidants and cytokines, can induce apoptosis.[30] Inflammation in intestines has been shown to be associated with enhanced production of NO while inhibition of NOS ameliorates gut inflammation especially in IBD.[30-34] NO has been demonstrated to induce apoptosis in macrophages.[35-38] In gut inflammation NO synthesis is augmented via iNOS expression in both experimental animals and humans.[33] Exaggerated NO synthesis in enterocytes of an inflamed mucosa may be regarded as a mechanism to establish a chemical barrier which is not conducive for translocation by intestinal flora.[35] In so doing, the enterocyte is at risk of DNA damage. Apoptosis may be a defense against the survival of transformed cells.[35] Our data led us to suggest that during gut inflammation, the overproduction of NO by iNOS in epithelia and infiltrating leukocytes may result in epithelial cell death via apoptosis. Nevertheless, IBD is a predisposing risk factor for colon cancer and it would be no more illogical to speculate that this may result from a failure to initiate programmed cell death in cells transformed by oxidant stress, excess nitric oxide or related species. Briefly, Mtx administration suppressed apoptosis in colon mucosa in our study and one can speculate that, with specific stimulation, intestinal epithelial cells activate their intracellular apoptosis machinery to commit programmed cell death. The normal, physiologic pattern of apoptosis in intestinal epithelial cells and in lamina propria T cells seems to be markedly disturbed under immune-mediated conditions.[36]

Overproduction of NO by iNOS has been implicated in colitis and induction of iNOS seemed to act as a critical toxic effecter molecule in the pathogenesis of colonic

Table 3. Apoptotic indexes for groups

Groups	Apoptotic index(AI)	Ki-67 labelling index(PI) AI/PI	
Colitis+Mtx (Group I)			
N=	12	12	12
SEM±	8.71E-03	2.70E-03	0.29
Mean	6.75E-02	3.16E-02	2.22
Median	7.00E-02	3.00E-02	2.25
Only colitis (Group II)			
N=	12	12	12
SEM±	2.50E-02	4.32E-03	0.63
Mean	0.53	8.66E-02	5.81
Median	0.53	8.50E-02	6.31
Saline (Group III)			
N=	12	12	12
SEM±	1.16E-02	2.87E-03	1.46
Mean	7.75E-02	1.91E-02	5.89
Median	7.50E-02	2.00E-02	3.72
P value	0.0001[a], 0.0001[b], 0.915[c]	0.0001[a], 0.045[b], 0.0001[c]	0.037[a], 0.032[b], 0.998[c]

P < 0.05 values were statistically significant. [a]:Mtx/TNBS, [b]:Mtx/Control, [c]:TNBS/Control, AI:Apoptotic index, PI:Proliferative index

inflammation.[15] In IBD, inflammatory damage is associated with increased production of pro-inflammatory cytokines and NO through the iNOS pathway. In the model of acute experimental colitis, amelioration of inflammation with highly selective iNOS inhibitor has been reported.[37-49] Kocaman *et al.* suggested that the endothelial dysfunction may underlie a part of the pathophysiology related to the hypothesis that the severity of UC may parallel the severity of some extraintestinal complications of UC.[9] In their study, they concluded that endothelial dysfunction is associated with moderate and severe UC; moreover, endothelial dysfunction was significantly worse in patients with severe UC. Peroxynitrite, derived from the reaction of NO with superoxide O_2, is a potent nitrating and oxidizing agent that can induce apoptosis in a variety of different cell types.[38,46] Yue *et al.*[50] has reported that the local elevated level of peroxynitrite produced from increased iNOS activity is a major contributor to colon epithelial cell apoptosis during colon inflammation. In our study, Mtx markedly reduced eNOS and iNOS as well with NO production in colon mucosa. We did not measure peroxynitrite levels but also suggest that iNOS activity may also be affected by this interaction. In conclusion, we suggest that single low dose of Mtx has shown to ameliorate histopathologic damage and endothelial dysfunction via the regulation effect on NO pathway and anti-inflammatory effects in the colon mucosa of rats with colitis. Surely, further experimental and clinical studies will not only help us to understand the physio-pathologic mechanisms but will also assist us in designing novel strategies to treat IBD. Therefore in the need of further randomized clinical trials, Mtx may be useful and be the choice of treatment modalities especially in acute form of in IBD.

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