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*Original Article*

# Protective effect of 2-aminoethyl diphenylborinate in rat colitis model induced by acetic acid

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**Abstract:** Some of the diseases like ulcerative colitis, Crohn disease and certain types of intestinal cancers are not treatable effectively. Our aim was to investigate the protective effects of 2-aminoethyl diphenylborinate (2-APB) on the rats with acetic acid induced colitis. Twenty four Sprague-Dawley rats were randomly divided into the following four groups: (1) control group, (2) colitis group, (3) 2-APB group, (4) colitis+2-APB group. Twenty four hour after the acetic acid administration blood samples were collected under the ether anesthesia. After the collection of all blood samples rats were euthanized by cervical dislocation under the anesthesia and abdomen was opened and the colon was taken. Superoxide dismutase activities, total antioxidant capacity, malondialdehyde, ceruloplasmin, total cialic acid and iron ($Fe^{2+}$) levels were measured using blood samples. Superoxide dismutase activities, total antioxidant status, malondialdehyde and DNA fragmentation levels were evaluated at colon tissues. Paraffin sections of colon tissue were subjected to: a) immunohistochemistry (Bcl-2), b) TUNEL-staining (apoptotic cells), c) histopathological (masson's trichrome staining) examinations. Sections were evaluated semiquantitatively. Induction of colitis caused pathological and biochemical alterations in rat colon. Rates of apoptosis increased concomitantly with the levels of oxidants in colitis group, while activities of antioxidant enzymes decreased remarkably. Administration of 2-APB however, ameliorated the biochemical and pathological alterations in rats caused by colitis. In the light of the data obtained from the present study it could be recommended that using of 2-APB in colitis could be considered as a remedy at least as a complementary drug.

**Keywords:** Apoptosis, 2-aminoethyl diphenylborinate, colitis

## Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disorder of unknown origin. Several studies showed that the pathogenesis and progression of the disease is depend on the multiple genetic, environmental and immune factors [1-4]. Usually, the mucosal inflammation involves the rectum, but it may extend proximally, resulting in procto-sigmoiditis, left-sided colitis, or pancolitis. In addition, affected people may suffer from extraintestinal manifestations of colitis, including affections of the skin, eyes, joints, or the liver in the form of primary sclerosing cholangitis. Furthermore, longstanding inflammation may increase the risk for colorectal cancer [5]. Bloody diarrhea, rectal urgency, tenesmus, and abdominal cramping are some clinical manifestations of UC. Ulcerative colitis follows a relapsing and remitting course necessitating therapy for induction of remission as well as maintenance of remission [6]. Several factors such as ischemia, infection, and inflammation can cause the loss of mucosal integrity and circulatory, immunologic, and inflammatory response to injury are the leading causes of necrosis of the affected area [7]. It has been a well known fact that inflammatory mediators like cytokines, eicosanoids and reactive oxygen species (ROS) are important components of those inflammatory pathways. These molecules play an important role in both the initiation and

the evolvement of UC [8, 9]. The production of these metabolites which are shown to be the final common mediators of inflammation has been found to occur in the gastrointestinal tract [7]. In this respect, taking antioxidants and dietary supplements supporting body's defence system against oxidants like hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^{\cdot}$) and others may be beneficial.

Accumulation of cytosolic $Ca^{2+}$ is important in the progression of cell death. Oxygen deficiency in ischemia results in failure of the respiratory chain, lack of ATP and disfunction of ion pumps. Cellular homeostasis can't be maintained anymore and $Ca^{2+}$ begins to leak from intracellular stores, causing activation of store-operated $Ca^{2+}$ (SOC) channels in the cell membrane, both of which result in large increases in cytosolic $Ca^{2+}$ concentration [10]. The excess cytosolic $Ca^{2+}$ is resulted the release of cytochrome c, apoptosis-inducing factor, and other molecules that lead to mitochondria-associated caspa se-dependent and caspase-independent cell death [11].

2-Aminoethoxydiphenyl borate (2-APB) was originally characterized as a cell-permeable inhibitor of inositol 1,4,5-trisphosphate (IP3)-induced $Ca^{2+}$ release. In several studies 2-APB has been used to inhibit the release of intracellular $Ca^{2+}$. Specificity of 2-APB as a blocker of $Ca^{2+}$ release via the IP3 receptor in the endoplasmic reticulum of several different cell lines [12]. In another study, it was confirmed that 2-APB has a direct inhibitory effect on store operated calcium channels (SOCCs) [13, 14]. Some studies indicate that 2-APB also plays an important role in antiapoptotic and antioxidant status in many disease [15, 16].

Reactive oxygen species (ROS) induce lipid peroxydation. Malondialdedyde (MDA) also known as a thiobarbituric acid-reactive substance is end product of lipid peroxidation. Malondialdedyde is investigated as an indirect marker of lipid peroxidation [17, 18]. In recent studies serum MDA concentration has been shown to be significantly higher in colitis models [19, 20]. Total antioxidant status (TAS) assay indicates the total antioxidant capacity of samples used to analyse. In the presence of antioxidants divalent copper (II) is reduced to monovalent copper (I) and reduced copper ions react with chromogen to produce a colorful product with maximum absorbance at 490 nm. Reactive oxygen molecules (ROMs) are potent inflammatory mediators likely to be involved in tissue injury and acute attacks seen in UC [21]. It is known that antioxidants decrease the harmful effects of oxidative damage caused by ROMs, and increase TAS in colitis models [22, 23]. Furthermore, sialic acid plays an important role in inflammation. Biochemical parameters are benefical indicators for diagnosis and evaluation of periparturient disease. There have been e few studies to assess the lipid peroxidation, acute phase response, antioxidant substances and apoptosis in the UC.

The development of modern treatment of UC started with the introduction of sulfasalazine (SASP) by the Swedish physician Nanna Svartz in 1942 [24]. After realization that conventional drugs used in the treatment of UC are not always effective and may cause some serious side effects [25, 26]. Overall, it is evident that there is a need to try a new, more effective and less toxic agents in the treatment of UC. Using antioxidant agent scould be considered one of the therapeutic strategies in the treatment of UC. Hence, the present study was undertaken to evaluate the putative protective effect of 2-APB on the experimental model of UC induced by acetic acid in Sprague-Dawley rats.

## Materials and methods

### *Animals*

Tweny four male Sprague-Dawley rats (8 weeks old, weighing 200-260 g each) were obtained from Firat University, Experimental Research Centre (Elazig, Turkey), and housed under standard laboratory conditions (24±3°C, 40-60% humidity, a 12-h light and dark cycle). A commercial pellet diet (Elazig Food Co., Elazig, Turkey) and fresh tap water were given *ad libitum*.

### *Chemicals*

2-APB was obtained from Sigma-Aldrich (Sigma-Aldrich, Inc, Louis, MO).

### *Induction of ulcerative colitis and treatment protocols*

The animals were randomly divided into the following four groups and each group has six rats: (1) control group, (2) colitis group, (3) 2-APB

group, (4) colit+2-APB group. The rats in group 1 was not given anything but commercial pellet diet and fresh water. The animals in group 2 were administered 0.9% NaCl orally for 3 days by using pediatric catheter and at the end of third day to induce an acute colitis 2 mL of a 3% solution of acetic acid is infused intrarectaly. Briefly, following ketamin anesthesia, a soft polyethylene catheter with an outer diameter of 2 mm was inserted rectally into the colon with the aim of placing the catheter tip at 8 cm proximal to the anus and 2 mL acetic acid was carefully infused. To spread acetic acid completely into the colon 2 ml of air was applied and then catheter was taken out. To prevent leakage rats were kept in head-down position for 25 seconds. Animals in group 3 were given daily 2-APB (2 mg/kg) only by intraperitonal injection for 3 days. In group 4 following 3 days administration of 2-APB colitis is induced in rats by infusion of 2 ml acetic acid (3%) solution intrarectaly. The effective dose for 2-APB was 2 mg/kg which was reported [27].

*Sample collection*

Twenty four hour after the acetic acid administration blood samples were collected under ether anesthesia and the samples were kept for 2 h at room temperature to proper clotting. The blood samples were then centrifuged at 2500 g at 4°C for 15 min and stored at -20°C until analyses. After the collection of all blood samples rats were euthanized by cervical dislocation under the anesthesia and abdomen was opened and the colon was exposed. The distal 8 cm of the colon was excised and opened by longitudinal incision. Half sections of colon tissues was preserved with 10% formalin for histopathologic evaluation and the other sections were used for biochemical analysis (MDA, TAS, SOD and apoptosis rate).

*Biochemical analysis*

In serum specimens MDA, total sialic acid, TAS and $Fe^{2+}$ levels as well as SOD and ceruloplasmin activities were measured. In colon tissues, however in addition to SOD activity MDA and TAS concentrations and the amount of DNA fragmantation were detected.

$Fe^{2+}$ (Archem, A2241, Istanbul, Turkey) levels were measured by commercial available kits using a Biochemistry Auto Analyzer (Sinnowa D280, China). Lipid peroxidation was determined using the procedure described [28] in which MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid substance to form a colored complex with a maximum absorbance at 532 nm.

The samples were incubated with xanthine oxidase solution for 1 h at 37°C to measure SOD activity in serum. Absorbance was read at 490 nm to generate superoxide anions. Superoxide dismutase activity was determined as the inhibition of chromagen reduction. In the presence of SOD, superoxide anion concentration is reduced, yielding less colorimetric signal. Superoxide dismutase activity was expressed in percent.

Total antioxidant capacity was determined using an automated measurement method with a commercially available kit developed by Rel (Total Antioxidant Status Assay kit, Rel Assay Diagnostics, RL0017, TURKEY). The antioxidative effect of the sample against the potent-free radical reactions initiated by the reduced hydroxyl radical is measured using this method. The results were expressed as millimoles of Trolox equivalent per liter.

Ceruloplasmin (CPN) concentrations were determined by the method of Sunderman and Nomoto [29]. In this method; CPN and p-phenylendiamine forms a colored oxidation product and this product is proportional to the concentration of serum CPN.

Total sialic acid values of the rats were measured at 549 nm by the method of Warren in which Sialic acid is oxidized to formylpyruvic acid which reacts with thiobarbituric acid to form a pink colored product [30].

The extent of apoptosis was evaluated by the measurement of DNA fragmentation. This was assessed by quantification of cytosolic oligonucleosome-bound DNA by using the Cell Death Detection ELISA plus kit (Roche, Mannheim, Germany). Colon of the rats were treated with a homogenizer (Stuart SHM1, UK). The 0.2 g homogenate was made with the lysis buffer and then centrifuged at 20000 g for 10 min at 4°C. The supernatant fraction was used as the antigen source for the immunoassay. This assay is based on the quantitative sandwich ELISA principle using mouse monoclonal anti-

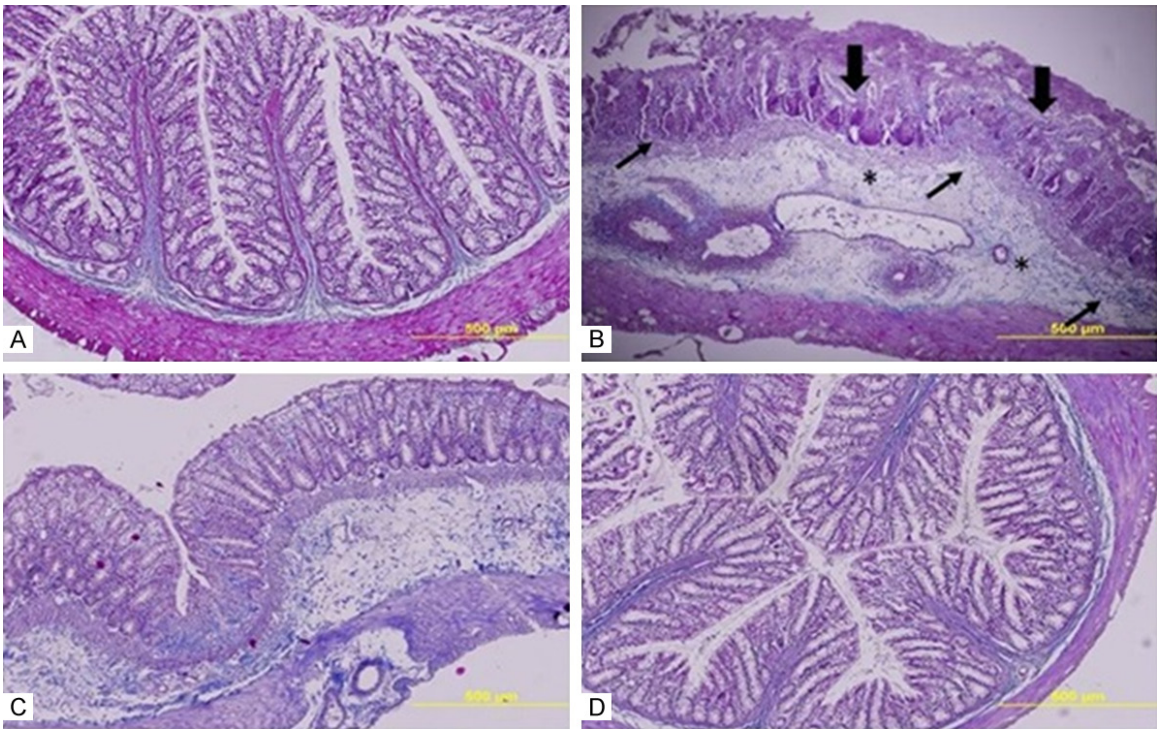


**Figure 1.** Representative photomicrographs of masson's trichome-stained colon tissue sections from experimental groups. Colon microscopic image of normal rat with intact epithelial and mucosal layer (A). The colitis rat with extensive damage including cellular infilteation (arrow), edema in submucosa (*) and ulceration (thick arrow), dilated vessels (arrow head) (B). Smaller ulcers covered with regenerated epithelium in the 2-APB treated group (C). APB treated rat has normal colon archtitecture (D) (masson's trichrome; original magnification ×20).

bodies directed against histones (coating antibody) and DNA (peroxidase-labelled antibody) respectively. The amount of peroxidase retained in the immunocomplex is determined photometrically with ABTS (2,29-azino-di-(3-thylbenzthiazoline sulfonate) as a substrate (Thermo Multiskan FC Microplate Photometer, USA). This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates.

*Histopathologic evaluation*

For histopathological examination colonic specimens were fixed in 10% formalin and embedded in paraffin for serial sections through routine tissue examination stages and six colon rings were obtained from each colon. Then, tissues were processed routinely, embedded in paraffin and cut into 5 μm sections. Paraffin sections were deparaffinized with xylene, hydrated and all sections stained with masson's trichrome. Sections were evaluated for histopahological alterations using a light microscope and selected areas were photographed (Olympus BX-51, Tokyo, Japan).

*Quantitative immunohistochemistry*

Bcl-2 and TUNEL staining were performed at the paraffin sections. TUNEL staining was performed using a assay kit according to the manufacturer's instructions (apopTaq Peroxidase In Situ Apoptosis Detection Kit, S7101, Millipore, USA).

After 2 hours incubation at 40°C, sections were deparafinnized in xylene, hydrated through graded alcohol and endogenous peroxidase blocked with 0.3% methanol-$H_2O_2$ solution for 30 min. After washing 3 times with PBS, the tissues were treated with 2% bovine albumin serum (BSA, Sigma) for 30 min to prevent nonspecific binding and then were incubated with antibodies to Bcl-2 (Santa Cruz Biotechnology), each diluted to 1:100, for 30 min at room temperature. After incubation for 10 min with biotinylated secondary antibodies, they were incubated with an avidin biotin complex enzyme solution for 45 min, and 3,3'-diaminobenzidine (DAB) was applied as the chromogen for 5 min and hematoxylin was used for counter staining.

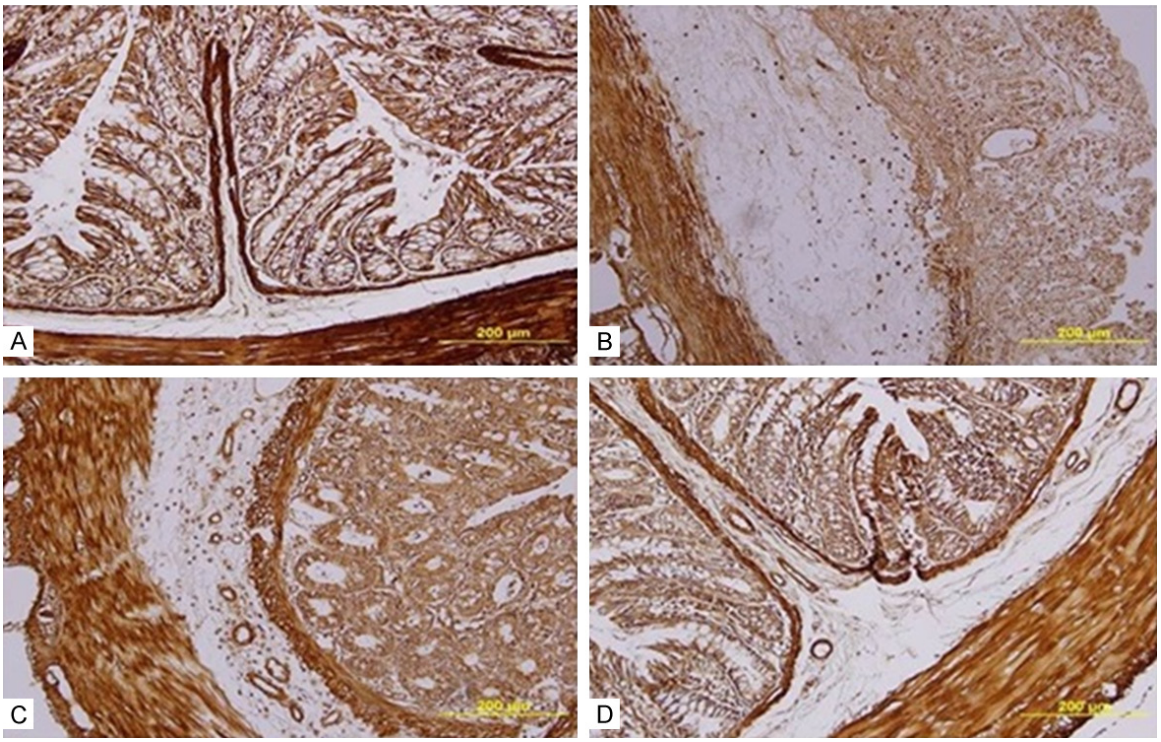


**Figure 2.** Immunhistochemical detection of antiapoptotic protein Bcl-2 in rat colon. A strong staining was observed in control animals (A). The staining intensity for Bcl-2 decreased remerkably in colon of colitis group (B). Administration of 2-APB to colitis induced rats increased the staining intensity of Bcl-2 (C). No significant alteration were detected in the staining for Bcl-2 in 2-APB group only (D).

Sections were semiquantitatively evaluated for Bcl-2 and TUNEL immunostaining and localization using a light microscope and selected areas were photographed.

*Statistical analyses*

All statistical analyses were performed on SPSS 19.0 (IBM SPSS, Inc, Chicago, IL). All the assays were performed at least three times. All data were expressed as the mean ± standard error of the mean (S.E.M.) from 6 rats per group, The one-way analysis of variance was used to test the differences between the groups. The tukey's honestly significant difference (HSD) test was used for multiple comparisons. In all analyses, $P$ values <0.05 were considered statistically significant.

## Results

*Histologic findings*

Colon of the control (**Figure 1A**) and 2-APB-treated rats (**Figure 1D**) showed healthy colon morphology with mucosa and submucosa. In colon of colitis group (**Figure 1B**) however, several alterations such as loss of mucosal architecture with glandular destructions and complete loss of goblet cells, massive mucosal and submucosal inflammatory infiltration, edema in submucosa, vascular dilatation, thickening of the colon wall and ulcerous areas were observed in all sections. Inflammatory cell infiltration was observed predominantly in the mucosa and submucosa. Microscopic examinations in 2-APB+colitis group (**Figure 1C**) revealed that ulcerous parts of colon were covered with regenerated epithelium. These observations suggest that 2-APB alleviated histopathologic damage in colon.

*Immunohistochemistry and TUNEL*

Effects of 2-APB on the apoptosis rate in colitis were evaluated by detection of Bcl-2 protein and using the terminal deoxynucleotidyl transferase dUTP nick end labeling staining (TUNEL) method.

As shown in **Figure 2** a diffuse staining was observed for Bcl-2 throughout the colon at

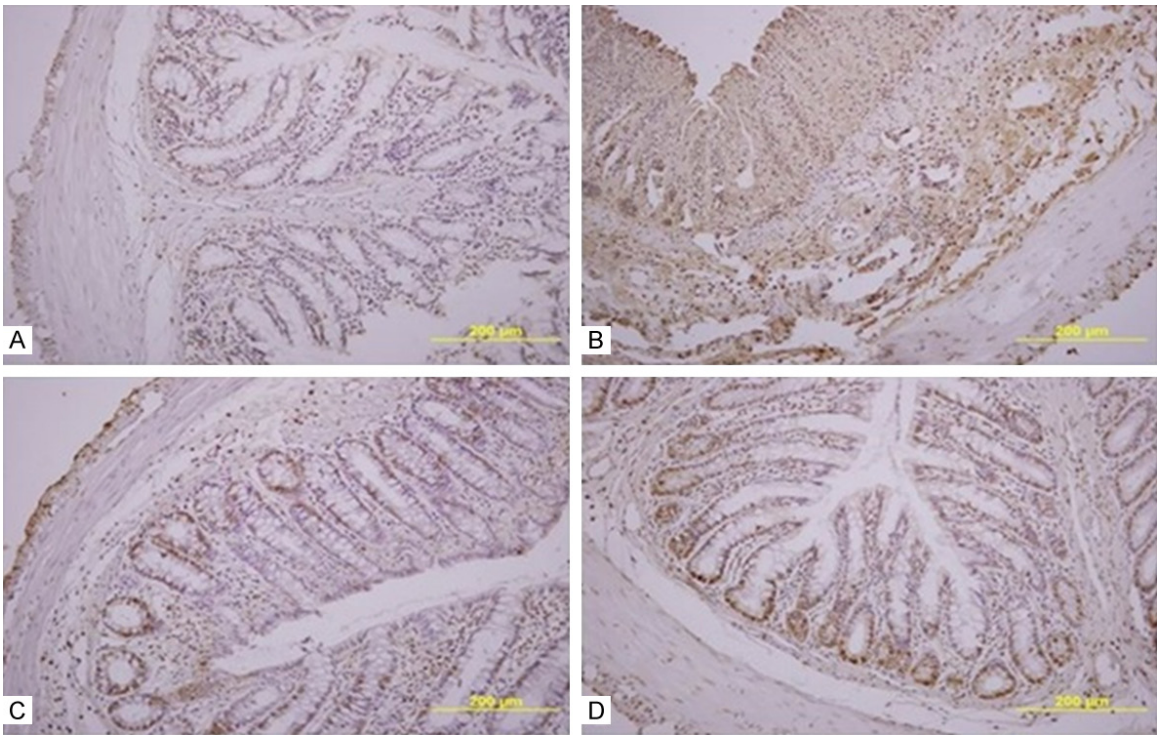


**Figure 3**. Apoptosis rates detected by using the terminal deoxynucletidyl transferase dUTP nick end labeling staining (TUNEL) method. Number of apoptotic cells in control animals (A) was moderate, while inducing of colitis by acetic acid incerased the number of apoptotic cells (B). Adminstration of 2-APB to colitis induced rats increased the staining intensity of Bcl-2 (C). No significant alteration were detected in the staining for Bcl-2 in 2-APB group only (D).

each experimental groups. However, the staining intensity in control (**Figure 2A**) and 2-APB groups (**Figure 2D**) were stronger than in colitis (**Figure 2B**) and 2-APB+colitis (**Figure 2C**) groups. Apoptotic cells also visualized using TUNEL technique. In control and 2-APB given animals number of apoptotic cells in colon was modarate (**Figure 3A** and **3D**). Inducing colitis by acetic acid however, increased the number of apoptotic cells (**Figure 3B**). On the other hand using 2-APB in rats with colitis reduced the number of apoptotic cells (**Figure 3C**).

*Biochemical findings*

As seen in **Table 1** serum MDA levels in colitis group were significantly ($P<0.05$) higher than that of controls. Administration of 2-APB in group 4 decreased serum MDA concentrations significantly ($P<0.05$). Furthermore, serum SOD activity in colitis group was significantly ($P<0.05$) lower than that of controls. However, as shown in **Table 1** administration of 2-APB in group 4 increased serum SOD activity in a significant manner ($P<0.05$). In comparison to control animals serum TAS in colitis group was significantly ($P<0.001$) lower, while administration of 2-APB in group 4 ameliorated decreased TAS in group 2. Serum ceruloplasmin concentrations in colitis group were significantly ($P<0.05$) lower than that of controls. Likewise, administration of 2-APB in group 4 increased serum ceruloplasmin levels remerkably. Serum $Fe^{2+}$ concentrations in colitis group was significantly ($P<0.001$) lower than that of control animals. Administration of 2-APB group 4, however didn't have any significant effect on serum $Fe^{2+}$ concentrations. Concerning the serum total sialic acid concentrations, induction of colitis increased serum sialic acid levels significantly ($P<0.05$) and i.p. injection of 2-APB reduced the serum total sialic acid levels significantly ($P<0.05$).

Similar to the serum samples tissue MDA levels in colitis group were significantly ($P<0.05$) higher than that of controls. Administration of 2-APB in group 4 decreased MDA levels significantly ($P<0.05$). As shown in **Table 2** tissue SOD activity in colitis group were significantly ($P<0.05$) lower than that of controls. As seen in serum samples administration of 2-APB in group 4

**Table 1.** Biochemical parameters of serum samples and statistical results in each groups

| Parameters | Control $\overline{X} \pm S_{\overline{x}}$ (n=6) | Colit $\overline{X} \pm S_{\overline{x}}$ (n=6) | Colit+2APB $\overline{X} \pm S_{\overline{x}}$ (n=6) | 2APB $\overline{X} \pm S_{\overline{x}}$ (n=6) | P |
|---|---|---|---|---|---|
| MDA (μmol/L) | $16.91\pm0.47^{b,c}$ | $25.73\pm1.98^{a}$ | $17.56\pm0.39^{b,c}$ | $14.96\pm0.43^{d}$ | * |
| SOD (% inhibition) | $60.37\pm1.09^{a}$ | $50.38\pm1.39^{c}$ | $57.26\pm1.34^{b}$ | $61.65\pm0.97^{a}$ | * |
| TAS (mmol trolox Equiv./L) | $1.29\pm0.04^{a,b}$ | $0.98\pm0.04^{c}$ | $1.22\pm0.02^{b}$ | $1.37\pm0.02^{a}$ | ** |
| Ceruloplasmin (g/dl) | $41.27\pm0.63^{a}$ | $23.27\pm1.76^{c}$ | $32.58\pm1.44^{b}$ | $40.40\pm0.87^{a}$ | * |
| Total cialic acid (μg/ml) | $491.29\pm13.58^{c}$ | $697.23\pm18.13^{a}$ | $602.18\pm13.43^{b}$ | $505.81\pm13.30^{c}$ | * |
| ALP (U/L) | $73.25\pm4.96^{a}$ | $41.53\pm11.27^{b}$ | $74.66\pm2.56^{a}$ | $82.00\pm6.38^{a}$ | * |
| Fe (μg/dl) | $139.51\pm\pm9.53^{a}$ | $31.27\pm3.23^{b}$ | $49.56\pm4.04^{b}$ | $146.18\pm9.55^{a}$ | *** |

*P<0.05: statistically significant, **P<0.01, ***P<0.001: statistically significant, a, b, c, d: The difference between groups in the same row including different letters are important.

**Table 2.** Biochemical parameters of tissue samples and statistical results in each groups

| Parameters | Control $\overline{X} \pm S_{\overline{x}}$ (n=6) | Colit $\overline{X} \pm S_{\overline{x}}$ (n=6) | Colit+2APB $\overline{X} \pm S_{\overline{x}}$ (n=6) | 2APB $\overline{X} \pm S_{\overline{x}}$ (n=6) | P |
|---|---|---|---|---|---|
| MDA (μmol/mg protein) | $6.91\pm0.29^{b}$ | $11.13\pm0.32^{a}$ | $8.01\pm0.14^{b}$ | $7.53\pm0.06^{b}$ | * |
| SOD (% inhibition/mg protein) | $64.28\pm0.87^{a}$ | $50.48\pm1.57^{c}$ | $57.25\pm0.52^{b}$ | $61.54\pm0.70^{a}$ | * |
| TAS (mmol trolox Equiv./mg protein) | $0.64\pm0.014^{a}$ | $0.42\pm0.016^{c}$ | $0.55\pm0.009^{b}$ | $0.63\pm0.014^{a}$ | * |
| DNA Fragmentation (U/mg protein) | $0.78\pm0.06^{c}$ | $1.98\pm0.07^{a}$ | $1.57\pm0.10^{b}$ | $0.83\pm0.07^{c}$ | ** |

*P<0.05: statistically significant, **P<0.01, ***P<0.001: statistically significant, a, b, c: The difference between groups in the same row including different letters are important.

increased tissue SOD activity significantly (P<0.05). Tissue TAS in colitis group was significantly (P<0.05) lower than that of controls and administration of 2-APB in group 4 amelioreted decreased tissue TAS. Induction of colitis in rats increased the DNA fragmentation significantly (P<0.001) and administration of 2-APB in group 4 decreased the elevated levels of apoptosis in colitis group (**Table 2**).

## Discussion

Several studies showed that the pathogenesis and progression of the colitis is depend on the multiple genetic, environmental and immune factors [1-4]. Over the past decate, there has been substantial interest in the oxidative stress and its role in the development of numerous diseases. Over production of ROS and their roles in the developmnet of colitis has also been detected [7]. Using of conventional drugs in the treatment of colitis is not efficient and may cause some serious side effects [25, 26]. Therefore, there is a need to develop a new, more effective and less toxic agents in the treatment of colitis. Using antioxidants could be taken into concideration one of the therapeutic strategies in the treatment of colitis. In this respect, we used a rat model to examine the probable antioxidant, protective and acute phase response effects of 2-APB on acetic acid induced colitis.

Histological examinations of the present study revealed that alterations such as loss of mucosal architecture, massive mucosal and submucosal inflammatory infiltrations, edema in submucosa, vascular dilatation, thickening of the colon wall and ulcerous areas in colitis group partly regenerated by administration of 2-APB. These findings showed that 2-APB alleviated histopathologic damage caused by acetic acid in colon.

Effects of 2-APB on the apoptosis rate in colitis were evaluated by detection of antiapoptotic Bcl-2 protein as well as using the terminal deoxynucleotidyl transferase dUTP nick end labeling staining (TUNEL) method and DNA-fragmentations technique. All these three methods informing about the apoptosis rate confirmed that colitis induces cells to apoptotic death and using 2-APB in colitis protects cells to a certain extend from programmed cell

death. 2-Aminoethoxydiphenyl borate probably exhibits this protective effect through preventing intracellular calcium accumulation during colitis by blocking store-operated $Ca^{2+}$ channels.

Malondialdehyde is an indicator of lipid peroxidation associated with oxidative stress [31]. Endogenous antioxidats such as SOD protect the cells against ROS damage [32]. In the present study, intraperitonal administration of 2-APB to rats with colitis decreased both serum and tissue MDA levels and as might be expected SOD activities and TAS levels increased by the administration of 2-APB. Antioxidant properties of 2-APB shown in the present study are in line with the findings of previous studies [15, 16, 33].

Ferroxidase activity of ceruloplasmin inhibits-$HO^{-}$ generation from $H_2O_2$. Several studies have reported that ceruloplasmins antioxidant activity may play an important role in severe disorders [34, 35]. Low levels of ceruloplasmin in colitis group and concominat increase in ceruloplasmin with SOD activities and TAS concentrations indicates that ceruloplasmin has an antioxidant activity in colitis and administration of 2-APB supports its role as an antioxidant.

Serum sialic acid has been reported as a marker of the acute phase response [36]. In the present research sialic acid values in colitis group was found to be higher than in control group and administration of 2-APB smoothen acute phase response of sialic acid in colitis. Furthermore, serum $Fe^{2+}$ levels in colitis group decreased significantly and administration of 2-APB didn't show any significant effect on the serum $Fe^{2+}$ levels. Infiltrating cells seen in colitis may enter into the blood and use serum iron for their proliferations. Low levels of serum iron may be due to the withdrawal of serum iron by iron storage proteins.

The results of this study demonstrate that 2-APB has a protective effect against acetic acid induced colitis and it could be recommended that using of 2-APB in colitis could be considered as a remedy at least as a complementary drug.

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## Disclosure of conflict of interest

None.

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