

Resveratrol Pretreatment Ameliorates TNBS Colitis in Rats

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Abstract: Inflammatory bowel disease (IBD) is a chronic intestinal inflammatory disease in humans constituting a major health concern today whose prevalence has been increasing over the world. Production of reactive oxygen species (ROS) and disturbed capacity of antioxidant defense in IBD subjects have been reported. Antioxidants may play a significant role in IBD treatment. This study aimed at evaluating ameliorative effects of intraperitoneal resveratrol pretreatment on trinitrobenzene sulphonic acid (TNBS)-induced colitis in rats. Thirty five Wistar-Albino female rats were divided equally into five groups. Inflammation was induced by the intrarectal administration of TNBS under anesthesia. Intraperitoneal administration of resveratrol (RSV) at a concentration of 10mg/kg/day for 5 days before the induction of colitis significantly reduced microscopy score and malondialdehyde (MDA) levels and increased glutathione peroxidase (GSH Px) activity compared to TNBS and vehicle groups. Also an insignificant increase in catalase (CAT) activity was observed in the RSV treated group compared to TNBS and vehicle groups. In this paper, the most recent patent on the identification and treatment of IBD was indicated. In conclusion, antioxidant RSV proved to have a beneficial effect on TNBS colitis in rats. In light of these advantageous results, the RSV can be considered as adjuvant agent in IBD treatments.

Keywords: Antioxidant system, inflammatory bowel disease, neutrophil infiltration, proinflammatory cytokines, resveratrol, TNBS.

1. INTRODUCTION

Inflammatory bowel disease (IBD), which the main forms are Ulcerative colitis (UC) and Crohn's disease (CD), is a chronic intestinal inflammatory disease caused by multifactorial conditions such as environmental, genetic and immunoregulatory factors. The incidence of IBD has been increasing over the world and higher prevalence rates of IBD are seen in the developed countries [1].

Neutrophil infiltration, proinflammatory cytokines, adhesion molecules, eicosanoids and the ROS are clearly involved in the pathogenesis of IBD [2-5]. Neutrophils, macrophages, and cytotoxic T cells destroy the intestinal mucosa either directly through physical contact or indirectly through the release of many active molecules such as the ROS, cytotoxic proteins, lytic enzymes, or cytokines such as tumour necrosis factor (TNF- α), interleukin 1 (IL-1) and 6 (IL-6) [6].

Under normal physiologic conditions, there is a balance between oxidative stress factors and antioxidant system entities. The toxic oxidants may destroy cells if their rate of

production exceeds the capacity of endogenous antioxidant defense systems [7, 8]. Antioxidant enzymes such as superoxide dismutase (SOD), the CAT and the GSH-Px constitute antioxidant defense systems.

Resveratrol (RSV) is a natural polyphenol found in various foods, including mulberries, peanuts, and grapes. Previous reports have showed that the RSV exhibits many health beneficial effects, including anti-inflammatory, cardioprotective, neuroprotective and cancer-protective activities [9-11]. Resveratrol has been also demonstrated to modulate lipid oxidation and lipoprotein metabolism [12]. Resveratrol and its derivatives are also potent inhibitors of myeloperoxidases, which causes tissue damage in chronic inflammatory diseases such as inflammatory bowel disease [13]. The anti-inflammatory mechanism of the RSV is not understood completely, but it could be through many various ways such as inhibition of release of IL-1, IL-6 and TNF- α from macrophages, inhibition of prostaglandine production and inhibition of COX enzyme, iNOS expression and subsequent NO production as well as inhibition of apoptosis and MPO activity [14-16].

The NF- κ B pathway has been shown to contribute to colitis and colon cancer associated with colitis [17]. Also, inflammation in the colon down-regulates silent information regulator 1 (SIRT-1) and enhances nuclear transcription fac-

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tor-kappa B (NF- κ B) activity. Taken together, Hofseth *et al.* reported that the RSV plays a critical role in the regulation of inflammation that controls colitis and colon cancer by inducing SIRT-1 and down-regulating NF- κ B activation [18, 19].

Recently, Li P *et al.* reported that resveratrol effectively decreased collagen I expression in IGF-1-stimulated fibroblasts by inhibiting IGF-IR/ERK1/2 signaling in a SIRT1 independent manner. However, resveratrol alone inhibited collagen I synthesis by activating SIRT1. These findings provide novel insight of resveratrol as a therapeutic agent for intestinal fibrosis, which is an incurable complication of Crohn's disease [20]. In another study, it has been reported that the anti-inflammatory and apoptotic effects of resveratrol could be attributed to its inhibitory effect on sphingosine kinase 1 (SphK1) providing a useful therapeutic tool to break the link between inflammation and carcinogenesis risk in ulcerative colitis [21].

In the present study, we aimed to investigate ameliorative effects of intraperitoneal the RSV pretreatment on TNBS-induced colitis in rats.

2. MATERIALS AND METHODS

2.1. Chemicals

Resveratrol (RSV) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were purchased from Santa Cruz Biotechnology (sc-200808; Santa Cruz, CA, USA) and Sigma Chemical Company (P2297; St. Louis, MO, USA) respectively. The RSV were dissolved in dimethyl sulfoxide (DMSO) and given intraperitoneally in final volume of 0.2ml.

2.2. Animals

Wistar Albino female rats (250-300g) were kept in a light-and temperature-controlled room on a 12:12-h a light-dark cycle, where the temperature (22 ± 0.5 C) and relative humidity (65-70%) were kept constant and fed a standard diet and water ad libitum.

2.3. Study Groups

Thirty-five Wistar-Albino female rats were divided equally into five groups: The RSV, Sham, TNBS, TNBS+DMSO (vehicle control) and TNBS+RSV (n=7). Rats in the Sham group received an enema of physiological saline instead of the TNBS solution, and TNBS+DMSO group received 0.2ml of the DMSO. The RSV was suspended in the DMSO solution and administered intraperitoneally at a dose of 10mg/kg/day for 5 days (96, 72, 48, 24 and 1hr) before the induction of colitis. Rats in the RSV group were treated by the RSV without induction of colitis. While rats in the RSV and the RSV treatment groups received the RSV, rats in vehicle group received the DMSO at the same time and volume.

2.4. Induction of Colitis

By a slightly modifying, colitis was induced according to the method described by Morris *et al.* [22]. After fasting, the animals overnight and emptying the colons on the morning of experiment, inflammation was induced by the intrarectal administration of 0.8ml of a 25-mg TNBS solution dissolved in 37% ethanol using an 8cm long cannula under ether anesthesia. Animals were sacrificed by toxic dose ether 24 hours

after induction of colitis. After decapitation, the last 10cm of the colon was excised, opened longitudinally. Longitudinal colon segments were subjected to biochemical and histopathological examinations.

2.5. Histological Analysis

The intestinal samples were fixed in 10% neutral formaldehyde solution at 4°C for 24 hours. The tissue samples then underwent routine histological procedure (dehydration in ethanol and clearing in xylene) and were embedded in paraffin blocks. The samples in paraffin blocks were randomly cut into 5 μ m sections by a microtome (Leica RM 2135). These sections were stained with haematoxylin-eosin and mounted with entellan. The pictures were taken with Olympus DP20 Digital camera attached onto an Olympus BX51 microscope. The extent of TNBS induced colitis were graded by using a histological grading scale, which each parameter was graded in a range from 0 to 3, which depends on the degree of changes (no change 0; mild 1; moderate 2; severe 3). Damage/necrosis, inflammatory cell infiltration, submucosal edema, and hemorrhage of mucosa were used in the assessment of histological score [23, 24]. An observer unaware of the groups performed the microscopic scoring of tissue samples.

2.6. Biochemical study

Tissues were homogenized in 0.1M phosphate buffer, pH 7.4 at 0-4°C. In order to determine Cu, Zn-SOD activity, the supernatant was used after a portion of the homogenate was centrifuged at 10000g for 15min at 0-4°C, whereas in order to measure the MDA, the GSH-Px and the CAT activities, the supernatant was used after the remaining homogenate was centrifuged at 700g for 10min. Determination of protein level was carried out by using Lowry method [25].

2.7. Determination of Myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) activity in the colonic tissues, an index of leukocyte recruitment, was measured with a rat MPO assay kit according to the manufacturer's instructions (E0601r, EIAab, Wuhan, China). Using a spectrophotometer at 450nm, the concentration of the MPO in the specimens was determined by comparing the OD of the specimens to the standard curve. Data are expressed as ng/mg.

2.8. Determination of Malondialdehyde (MDA) level

The MDA production and lipid peroxidation were determined in the tissues by the method of Ohkawa [26]. The MDA forms a colored complex in the existence of thiobarbituric acid (TBA), which is detectable by measurement of absorbance at 535nm. The results were expressed as nmol/mg wet tissue.

2.9. Determination of Catalase (CAT) activity

The method described by Goth [27] was used for the assessment of the CAT activity in colonic tissue samples. 0.2ml supernatant obtained from the tissue homogenization were incubated in 1ml substrate (hydrogen peroxide) in sodium-potassium phosphate buffer (pH: 7.4) at 37°C for 1 minute and 1ml ammonium molybdate was then added to terminate the reaction. The concentration of hydrogen perox-

ide was measured at 405nm using a spectrophotometer. One unit of the CAT decomposes 1 μ mol of hydrogen peroxide per min. Values of CAT activity were expressed as kU/g.

2.10. Determination of Superoxide Dismutase (SOD) activity

The method of Sun *et al* was used to measure Cu, Zn-SOD activity [28]. A total 3.0ml reaction mixture included 0.1mmol of xanthine, 0.1mmol of EDTA, 50mg of bovine serum albumin, 25 μ mol of NBT, 9.9nmol of xanthine oxidase, and 40mmol of Na₂CO₃ (pH 10.2) from each per liter. The formation of formazan was detected at 560nm and 25°C. One unit of the SOD refers to the amount of protein that inhibits the rate of NBT reduction by 50%. Data are expressed as U/g protein.

2.11. Determination of Glutathione Peroxidase(GSH-Px) Activity

The GSH-Px activity was carried out using Glutathione Peroxidase Cellular Activity Assay Kit (Sigma CGP1, USA). 940 μ l and 890 μ l Glutathione Peroxidase assay buffer were pipetted into blank and sample cuvettes respectively. Then 50 μ l of NADPH assay reagent were added into each cuvette. After 60 μ l tissue homogenate were added into sample cuvette and all cuvettes were mixed by inversion, the reaction were started by addition of 10 μ l of 30mM tert-Butyl Hydroperoxide (t-Bu-OOH) solution and mixed by inversion. Then the decrease in absorbance at 340nm using a kinetic program recommended by kit was followed. The activity of Glutathione Peroxidase in the sample was calculated using the formula given by Kit. The activity per extract was expressed as mmol/min/g.

2.12. Statistical Analysis

All results were expressed as means \pm SE of the mean. The data were analyzed using SPSS 17.0 program. After ensuring of normal distribution of data, the analytic assessment of comparisons between twain groups was carried out by Mann Whitney U test. Differences of $p < 0.05$ were regarded as significant.

PATIENT CONSENT & ANIMAL PROTECTION

All experiments on animals were performed in accordance with the ethical guidelines for the Care and Use of Laboratory Animals of the United States National Institutes of Health. The study protocol, including procedures for animal handling and husbandry, was reviewed and approved by the Animal Care and Use Committee of Adnan Menderes University.

3. RESULTS

In biochemical analyses, the MDA levels of TNBS (25.30 \pm 3.62) and TNBS+DMSO groups (26.61 \pm 3.97) were higher than the RSV (10.97 \pm 1.06) and Sham control groups (11.45 \pm 1.80, $p < 0.01$). The RSV treatment (19.40 \pm 4.54) decreased the MDA levels compared to TNBS and TNBS+DMSO groups ($p < 0.05$).

The GSH-Px activity in treatment group (57.35 \pm 2.24) was higher than all other groups ($p < 0.05$). There was no difference for the GSH-Px activity ($p > 0.05$) among the RSV

control (46.8 \pm 1.66), Sham control (42.70 \pm 2.35), TNBS (47.54 \pm 3.68) and TNBS+DMSO groups (47.89 \pm 2.34).

The RSV and Sham Control groups (13.38 \pm 2.69 and 19.24 \pm 4.61) had the highest the CAT activity level, whereas TNBS group (10.36 \pm 2.78) had the lowest the CAT activity level. The CAT activity in TNBS+RSV group (12.02 \pm 4.04) was higher compared to TNBS and TNBS+DMSO (11.49 \pm 4.76) groups. However, this increase in the CAT activity of treatment group was not significant ($p > 0.05$).

While the MPO activities of TNBS and TNBS+DMSO groups (1.29 \pm 0.20 and 2.13 \pm 0.36) were higher than the RSV and Sham Controls (0.81 \pm 0.11 and 0.60 \pm 0.14), there was no decrease in the MPO activity in TNBS+RSV group by the RSV treatment (2.34 \pm 0.26) compared to TNBS group.

The highest the SOD activity level was observed in TNBS+DMSO group (0.78 \pm 0.13). TNBS group (0.66 \pm 0.07) had higher the SOD level compared to the RSV and Sham control groups (0.26 \pm 0.02 and 0.30 \pm 0.06), whereas it was not significantly different from TNBS +DMSO group (0.78 \pm 0.13). Although the SOD activity of TNBS+RSV group (0.55 \pm 0.05) was higher than the RSV and Sham Controls [$p < 0.01$, Fig. (1)], but it was lower than TNBS and TNBS+DMSO groups.

The results of the histological examination showed that while there was no difference between the microscopic injury scores of the TNBS colitis (9.43 \pm 1.51) and the vehicle (TNBS + DMSO) groups (9.29 \pm 0.76, $P > 0.05$), the microscopic injury scores of the RSV (0.83 \pm 0.75) and Sham (0.83 \pm 0.41) groups were significantly different from the microscopic injury scores of TNBS colitis and the vehicle groups ($P < 0.01$) Fig. (1 & 2). Treatment with the RSV (6.29 \pm 1.50) resulted in a substantially lower microscopic injury score compared to that in the TNBS-induced colitis and vehicle-treated groups ($P < 0.01$) Fig. (1 & 2). There was no marked difference for the microscopic injury scores between the RSV and Sham groups ($P > 0.05$), Fig. (1).

4. DISCUSSION

Because of its resemblance to human IBD, the TNBS-induced colitis model has been widely used as an experimental model and has led to its widespread use in the investigation of IBD pathogenesis. TNBS colitis is characterized by oxidative damage and mucosal infiltration of the inflammatory cells [22]. Various antioxidant agents such as vitamin E, selenium and melatonin have been used to prevent colitis [29, 30]. In the present study, we used resveratrol (RSV) to investigate the effects on TNBS-induced colitis. Previous studies have shown the antioxidant effects and anti-inflammatory action of resveratrol [14-16].

Oxydative stress, lipid peroxidation and free radical chain reactions break down integrity of intestinal mucosa barrier, and activate inflammatory mediators, resulting in increased colonic MDA contents. A number of experiments have demonstrated that the MDA level in rats with TNBS colitis decreased after treatments using antioxidant and anti-inflammatory agents [31, 32]. In our study, the levels of the MDA in the colitis and vehicle control groups significantly increased compared to level of the RSV and Sham control groups ($p < 0.01$). Treatment with the RSV resulted in a marked decrease in the MDA levels of treatment group, sug-

gesting that the RSV successfully inhibited lipid peroxidation induced by TNBS ($p < 0.05$) Fig. (1).

The balance between oxidative stress factors and antioxidant system entities is important for IBD pathogenesis, especially for propagation of the tissue damage. Enzymatic antioxidants such as the GSH-Px, the SOD and the CAT constitute the antioxidant system and these defense systems protect the cells against oxidative stress. Using an antioxidant agent, Xing *et al.* and Wang *et al.* observed a higher the GSH-Px levels in the treatment groups compared to TNBS groups in their TNBS colitis studies [32, 33]. In our study, the GSH-Px level was also higher in the RSV treated group compared to TNBS and TNBS+DMSO groups ($p < 0.05$). Among our biochemical results, the enzyme showing the best antioxidant activity for prevention of tissue injury was the GSH-Px, Fig. (1).

Superoxide dismutase (SOD) is one of the significant antioxidant enzymes in the protection of the tissue against oxida-

tive damage. The SOD is a first enzyme to detoxify superoxide anion to hydrogen peroxide. Then, the CAT and the GSH-Px enzymes try to convert hydrogen peroxide to water to prevent the ROS injury in the cell. In the literature, a number of studies have shown a higher activity for the SOD and the CAT enzymes in TNBS colitis in rats [32-35]. On the contrary, Beno *et al.* found the lower activities for the SOD and the CAT enzymes in colonic mucosa of 17 patients with idiopathic proctocolitis after 5-aminosalicylic acid treatment compared to activities of the same enzymes before the treatment [36]. In the present study, we observed a reduction in the SOD activity and an increase in the CAT activity in the RSV treated animals compared to TNBS group. However, these changes in the activity of both enzymes were not significant ($p > 0.05$). The reason for the insignificant change in the CAT activity could be removal of H_2O_2 by other cellular antioxidant molecules such as the GSH-Px, Fig. (1).

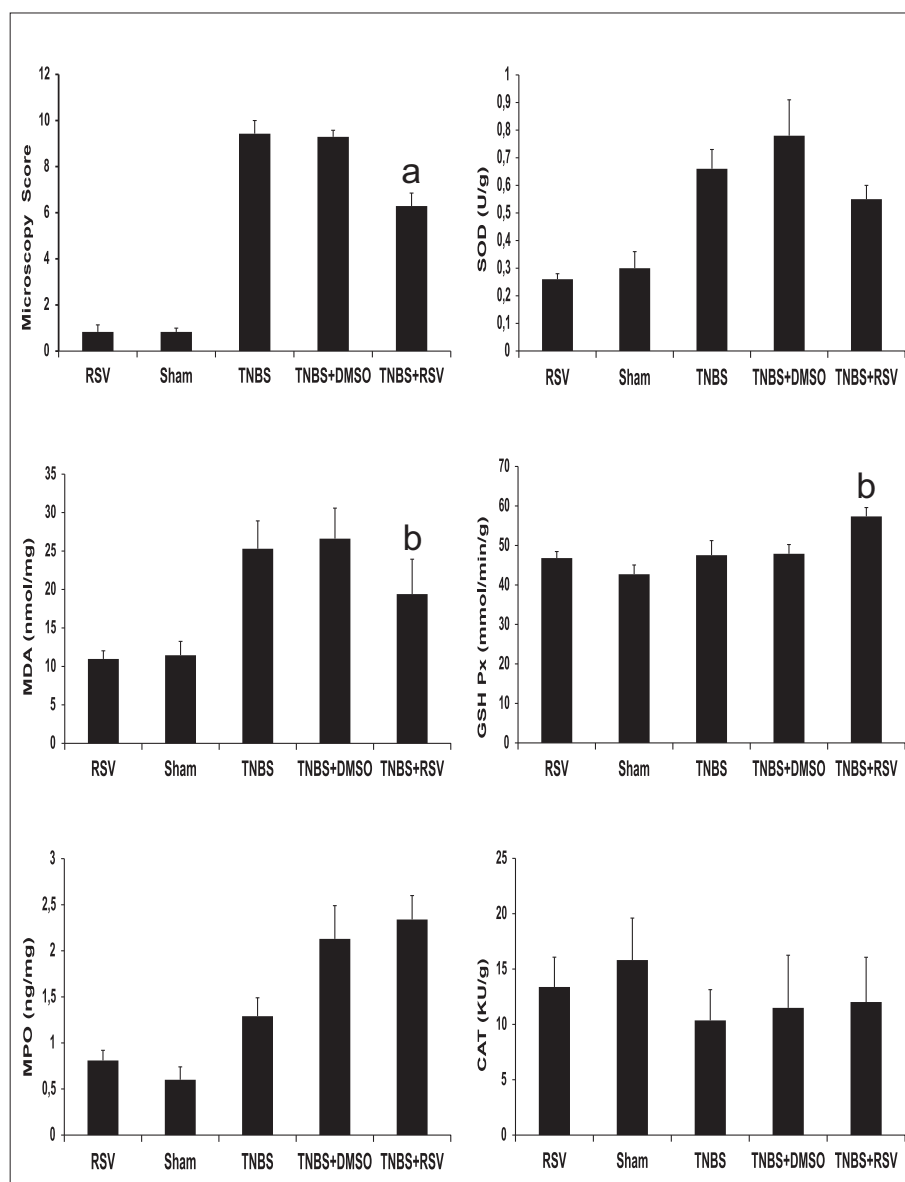


Fig. (1). Effects of RSV (10mg/kg) administered intraperitoneally for 5 sequential days before the induction of colitis on microscopy scores, the MDA, the MPO, the SOD, the GSH-Px and the CAT activities in rat colonic mucosa. Data are presented as means \pm SE of the mean. Letters of a and b above columns refer the significance for $P < 0.01$ and $P < 0.05$ respectively. All P values versus TNBS and vehicle control.

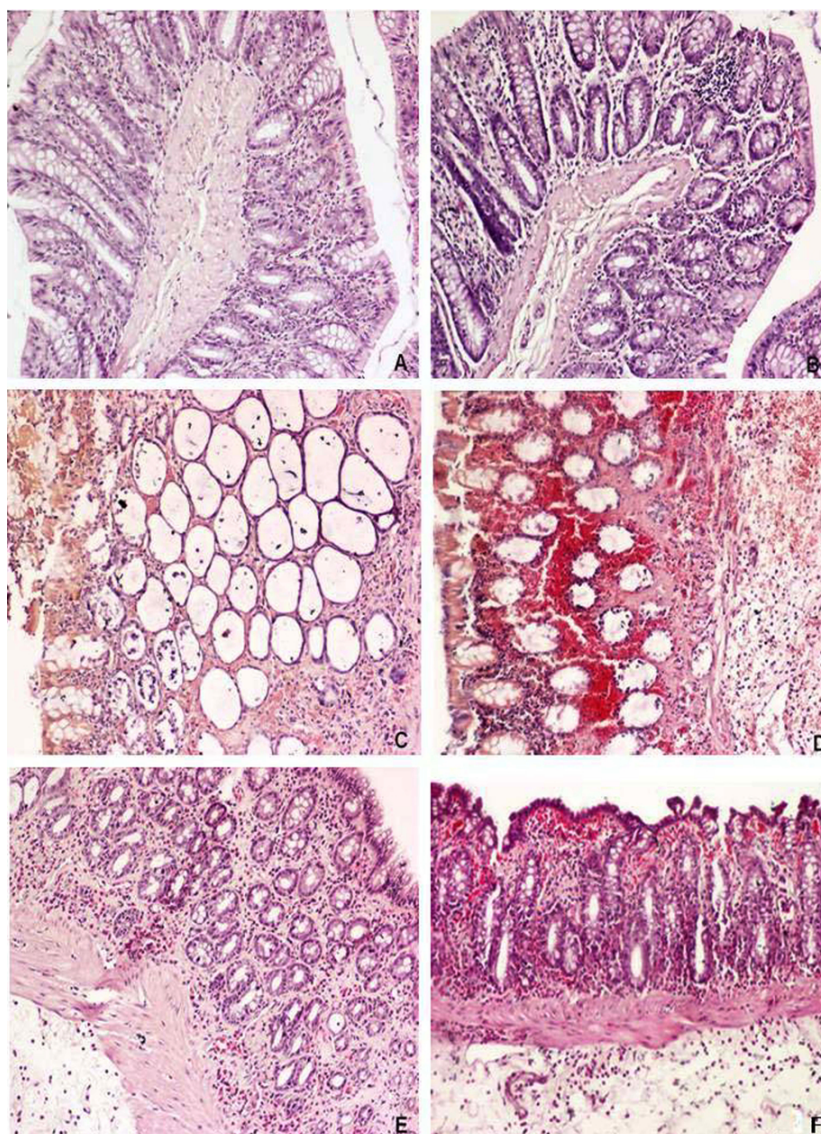


Fig. (2). Histopathological features of the colon associated with colitis and the effects of Resveratrol (RSV) on colon injury. (A) Resveratrol (noncolitis) group (normal histological appearance of rat colonic mucosa), (B) Sham group (no histological modification), (C) TNBS-induced colitis group (widespread necrosis and diffuse infiltration of inflammatory cells in the mucosa- submucosa and submucosal edema), (D) TNBS colitis + DMSO group (similar pattern to TNBS colitis with evident diffuse hemorrhage), (E) TNBS colitis + Resveratrol (10mg/kg) group (diminished findings of the tissue injury, inflammation and edema in the colonic mucosa). Mucosal injury was produced by TNBS administration (25mg/animal), characterized by necrosis of epithelium, focal ulceration of the mucosa and diffuse infiltration of inflammatory cells in mucosa and submucosa as well as submucosal edema. Treatment with Resveratrol (10mg/kg) reduced the the morphological changes associated with TNBS administration protecting the mucosal architecture (Fig. 2E & 2F). Original magnifications was 100 x for all groups (A-E) on Hematoxylin and eosin stained slides.

Colonic the MPO activity indicates neutrophil activation and inflammation. During acute inflammation, activated neutrophils pass out of the blood stream and enter the inflamed mucosa and submucosa of the large intestine, leading to the overproduction of the ROS, proteases, lactoferrin, and lipid mediators, all of which can contribute to intestinal injury [6]. In the biochemical results of our study, MPO activity was increased in all TNBS treated rats. However, this increase in the MPO activity was not reduced in rats treated with the RSV, Fig. (1).

In the present study, microscopic injury score were higher in all colitis (TNBS, vehicle and treatment) groups

compared to the RSV and Sham control groups ($p < 0,05$). Using the RSV, we observed a marked reduction in the microscopic injury score in animals treated with 10mg/kg of the RSV ($p < 0,05$), Fig. (1). Because the RSV treatment substantially decreased the inflammatory cell infiltration in TNBS-induced colitis, inhibitory effects of the RSV observed in histology results likely depend on anti-inflammatory effect as well as antioxidant effects even though the failure of reduction in the MPO activity by the RSV in treatment group, Fig. (1 & 2).

In addition, because we used the RSV totally before induction of the colitis, the beneficial effects of the RSV may

result from a combination of balancing the microflora, immunomodulation on intestinal macrophages and enhancement of antioxidant activity of the colonic tissues, which was previously suggested by Fukuda *et al.* [37]. But, it remains to be demonstrated.

The effects of the RSV on TNBS-induced colitis were studied by Martin *et al.* in detail in two studies mimicking acute and chronic inflammatory conditions [15, 16]. They demonstrated that the RSV had substantial anti-inflammatory and antioxidant effects on TNBS-induced colitis as demonstrated in the inflammations of other different tissues [9-11]. They administered the RSV before and after the induction of colitis in these studies. In their pretreatment study, Martin *et al.* administered the RSV by gavage 48, 24 and 1hr before the induction of colitis and 24hr later, whereas in their post-treatment study they administered the RSV by gavage daily at a dose of 10mg/kg during 2weeks after the induction of colitis [15, 16]. In the present study, we administered the RSV at the same dose (10mg/kg) for 5 days (96, 72, 48, 24 and 1hr) intraperitoneally and as a just pretreatment without any post-treatment part. Similar to Martin *et al.* pretreatment study, our results have shown that the RSV were effective on TNBS colitis, Fig. (1 & 2).

Our study is different from Martin *et al.* pretreatment study by six aspects: i) treatment period (totally pretreatment, no injection after TNBS administration versus 1 injection after TNBS administration), ii) pretreatment time (daily total 5 dose versus daily total 3 dose), iii) delivery way of the RSV (intraperitoneal versus intragastric), iv) the dose of the TNBS per animal (25mg TNBS in 37% ethanol versus 10mg TNBS in 50% ethanol), v) efficiency term (24hours versus 48hours) and vi) gender of animal (female versus male). As known, it may not be always possible to take the medicines orally during therapy of diseases and parenteral ways sometimes are necessary. Our study has shown that it was possible. In addition, while the TNBS solution was including 25mg TNBS dissolved in 37% ethanol in our study, but it was 10mg TNBS dissolved in 50% ethanol in Martin *et al.* pretreatment study. By using the same the RSV dose, we observed again a marked efficiency in TNBS-induced colitis despite 2, 5 times more TNBS. Moreover, despite allowing the RSV to show its efficiency on the tissue with the colitis almost half time compared to that of Martin *et al.* pretreatment study, the RSV pretreatment showed again significant efficiency. Furthermore, as known, if there is no specific reason, generally the male rats are first preferred in the research to edge out the effects of the estrogen and other factors belongs to female body and female immune system. This study demonstrated that the RSV pretreatment has the preventive effects on TNBS colitis in female rats.

In terms of showing that the RSV is also effective in a different way, in a higher dose of TNBS, in a shorter time and in a different gender of animal, the present study has the value.

5. CONCLUSIONS

Intraperitoneal the RSV pretreatment is also effective on prevention of TNBS induced colitis like intragastric the RSV pretreatment. By these features of the RSV, it can be considered as adjuvant agent in IBD treatments.

CURRENT & FUTURE DEVELOPMENTS

It has been understood that the basic mechanisms of IBD was much more complicated than previously predicted via data from animal models. Animal model studies have also detected potential candidate molecules for IBD therapy for instance 6-mercaptopurine [38], trappin-2 protein [39], tolerogenic dendritic cells [40], inhibitors of NF- κ B activation like chitosan nanofibers [41], propionic acid and benzoic acid derivatives [42]. Some of them such as IL-12p40 and CD3 were targeted in human clinical trials [43, 44]. Naturally occurring compounds proven to have antioxidant and anti-inflammatory effects such as resveratrol, caffeic acid phenethyl ester (CAPE) [45] and any other potential candidate drug or extract especially extracts of *Atractylodes* spp. and *Poncirus trifoliata* [46] can be used as adjuvant agent with new potential candidate molecules for IBD therapy. Even, it could be in the form of single or double combinations.

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

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