Effect of Silymarin on Cathepsin Activity and Oxidative Stress in TNBS-induced Colitis in Rats

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Authors’ contributions

This work was carried out in collaboration among all authors. Authors GB and HS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors GB and MC managed the analyses of the study. Authors AB, ED and SE managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The purpose of this study was to defined protective effects of silymarin on the experimental colitis induced by intra-colonic application of 2,4,6-trinitrobenzene sulfonic acid (TNBS). The twenty eight Sprague-Dawley rats were randomly separated into four groups, each group containing seven rats represent as follows: group 1 determined as control; group 2 determined as colitis-untreated; group 3 determined as colitis rats administered silymarin (50 mg/kg) and group 4...
INTRODUCTION

Ulcerative colitis (UC) is an inflammatory disease of the colon tissue, which is generally defined by stages of ulceration, inflammation and losing blood of the colon mucosa [1]. The cause of the aberrant immune response is unknown, but mediators of inflammation such as eicosanoids, cytokines and much generation of free radicals by the inflamed mucosal surface has been suggested to subscribe considerable to the improving of tissue damage [2,3]. The inflamed colon is unprotected to oxidative stress generated by infiltrating macrophages and neutrophils with tissue of colon. It is reported that an over intestinal immune response and production of free radicals can play an major role in the pathophysiology of colitis [4]. Free radicals are effective inflammatory biomarkers probably to be related in tissue damage in inflammatory bowel disease (IBD). These biomarkers may act a major mission in the aspect of IBD and its complications. Free radicals can improve early-stage IBD and it could be a significant agent in the etiopathogenesis of colitis [5,6].

2,4,6- trinitrobenzene sulfonic acid (TNBS) is a nitroaryl oxidizing acid that has been used by researchers for years by being administered rectally in ethanol for the purpose of inducing experimental colitis [7]. This model can be preferred when it is desired to induce chronic colitis since ulceration and the thickening of the intestinal wall continue for approximately eight weeks [8,9].

The radical reaction chain, of which steps are known and studied at most, of biological media is lipid peroxidation. Malondialdehyde (MDA) is one of the recent products of lipid peroxidation with a toxic effect that emerges as a result of the decomposition of non-enzymatic oxidative lipid peroxides. The measurement of the amount of MDA is widely used in the determination of the levels of lipid peroxide [10,11].

Myeloperoxidase (MPO), which is among powerful oxidant sources in the living system, is an enzyme that is secreted by neutrophils at a high rate, and monocyte, macrophage and kupffer cells at a lower rate [12,13]. MPO supports oxidative stress in many inflammatory conditions and it utilizes hydrogen peroxide to catalyze the generation of potent oxidants containing chlorine bleach and free radicals [14].

Cathepsins make up an important proteolytic enzyme group in the body. The presence of the processes that include the controlled biosynthesis, maturation, function and destruction of proteins is very important in order for healthy organisms to survive. Proteolytic enzymes ensure the irreversible disconnection of the peptide bonds of proteins [15]. Cathepsins also play a significant role in physiological events such as the antigen presentation in the immune system, wound healing, bone remodelling, and reproduction, apart from the proteolytic activities [16,17]. Cathepsin B and cathepsin L that are the members of the cysteine cathepsin family act as a part of the proteolytic cascade and are present in an active and stable state in acidic cell structures such as lysosomes and endosomes [18,19].

The different novel therapeutic products have recently been proposed, of which a considerable number has been validated in basic studies and will now be verified in clinical studies [20]. Silymarin (SM), the active complex in milk thistle, is a lipophilic fruit extract and is formed of different isomer flavonolignans [21]. SM is one of the top ten most popular natural products consumed by western society, and has antioxidant, anti-inflammatory, anti-proliferative, immunomodulatory and hepatoprotective effects on human health [22,23]. The aim of the this study was to determine possible protective effects of silymarin on the reduction of colonic damage and inflammation in experimental acute colitis induced by intracolonic administration of TNBS. This study also was suggest to define the activity of MPO, levels of MDA, cathepsin B and cathepsin L in colon tissue.

Keywords: Cathepsin; colitis; myleperoxidase; oxidative stress; silymarin.
2. MATERIALS AND METHODS

2.1 Chemicals

2,4,6-trinitrobenzene sulfonic acid and silymarin were bought from Sigma Chemical Co. (St. Louis, MO).

2.2 Experimental Animal Groups

Twenty-eight adult male Sprague-Dawley rats (6-8 wks old, weighing between 230-250 g) were provided from Experimental Animal Research Centre. All groups were held in a light and temperature adjustable room with 12 h-light:12 h-dark cycles, where the temperature (22±2°C) and relative humidity (50-55%) was held permanent. The rats were fed a pellet and drinking water.

After acclimation for a period of one week, the rats were randomly divided into four groups, each group containing seven rats assigned as follows: group 1 determined as sham control rats (C); group 2 determined as colitis-untreated; group 3 determined as colitis rats administered silymarin (50 mg/kg b.w) and group 4 determined as colitis rats administered silymarin (100 mg/kg b.w).

2.3 Induction of Colitis and Treatments

After fasting the animals overnight and emptying the colons on the morning of experiment, inflammation was induced in the colon by the intrarectal administration of 0.8 ml of a 25 mg 2,4,6-trinitrobenzene sulfonic acid (TNBS) dissolved in 37% ethanol using an 8 cm-long cannula under anesthesia [24]. All rats divide into four experimental groups as sham control (n=7), TNBS (n=7), TNBS-SM (50 mg/kg b.w) (n=7), and TNBS-SM (100 mg/kg b.w) (n=7). Silymarin was dissolved with saline (2 ml/kg volume). Saline was given to the sham control group. TNBS was given to the colitis group with canula as intrarectal under anesthesia. Dose of 50 mg/kg SM was given to the seven days after creating colitis with intragastric. Dose of 100 mg/kg SM was given to the seven days after creating colitis with intragastric [25]. At the end of seventh-day, an overnight fasting all animals were sacrificed. The last 8 cm of the colon was interrupted, opened lengthwise, and shaken with saline solution. Colon tissue was removed from the distal colon area and all samples were held at −80°C for next analysis of the MDA, cathepsin B and cathepsin L and for the measurement of MPO activity.

2.4 Biochemical Analysis

The MDA levels were analysed using the method described by Okhawa et al. [26]. The absorbances were read spectrophotometrically at 532 nm. MDA levels were presented as nmol/mg protein. Colon tissue protein was assayed by the method of Bradford [27], using serum bovine albumin as standard.

The MPO activity was performed using the method defined by Bradley et al. [28]. Colon samples were homogenized in potassium phosphate buffer (pH 6.0) including 0.3% hexadecyltrimethyl ammonium bromide. Homogenize samples were separated by centrifugation (12,000 rpm, 10 min, 4°C) and supernatant was collected. Potassium phosphate buffer (pH 6.0) including 0.5 mM o-dianisidine dihydrochloride was added to 125 µl of supernatant and 0.05% H2O2. Alterations in optical density were evaluated at 460 nm. MPO activity was presented as U/mg protein.

Activities of cathepsin were analyzed with Z-Arg-Arg-MCA at pH 6.0 for cathepsin B and Z-Phe-Arg-MCA at pH 5.5 for cathepsin L as substrates by the method of Barrett and Kirschke [29]. Cathepsin activities were stated as ng/mg protein.

2.5 Statistical Analysis

The results were described as the mean ± S.D. One way analysis of variance (ANOVA) and TUKEY test were used for the analysis and comparison of data within and between groups (SPSS 12.0 for windows). Differences were considered significant at p<0.05.

3. RESULTS

Results of the experimental groups are presented in Table 1. When we compared our study groups, we observed that tissue MDA, cathepsin L and cathepsin B levels with MPO activity of the colitis group increased significantly in comparison to the control group (p<0.05). The administration of silymarin (50 mg/kg) decreased tissue levels of MDA (Fig 1), MPO (Fig 2), cathepsin B (Fig 3) and cathepsin L (Fig 4) with compared to the colitis group (p<0.05). Doses of 100 mg/kg silymarin decreased
tissue levels of MDA, cathepsin L and cathepsin B and activity of MPO enzyme with respect to the colitis group (p<0.05). However, the results revealed that post-treatment to colitis rats with silymarin especially at dos of 50 mg/kg, partially attenuated the colonic damage induced TNBS.

Table 1. Biochemical results of the groups

| Groups           | MDA (nmol/mg protein) | MPO (U/mg protein) | Cathepsin B (ng/mg protein) | Cathepsin L (ng/mg protein) |
|------------------|-----------------------|--------------------|-----------------------------|-----------------------------|
| Control          | 2.69±0.35             | 2.02±0.38          | 3.86±0.60                   | 10.02±1.94                  |
| Colitis          | 4.29±0.74\textsuperscript{a} | 18.48±7.19\textsuperscript{a} | 7.47±1.12\textsuperscript{a} | 13.70±1.94\textsuperscript{a} |
| Silymarin (50 mg/kg) | 2.51±0.63\textsuperscript{b} | 9.53±3.13\textsuperscript{b} | 3.50±1.46\textsuperscript{b} | 10.27±2.90\textsuperscript{b} |
| Silymarin (100 mg/kg) | 3.74±0.89\textsuperscript{b} | 11.89±7.16\textsuperscript{b} | 5.60±3.27\textsuperscript{b} | 13.02±4.96\textsuperscript{b} |

Results are expressed as mean±SD; \textsuperscript{a} Significantly different when compared with control group, (p<0.05); \textsuperscript{b} Significantly different when compared with colitis group, (p<0.05); MDA: Malondialdehyde; MPO: myeloperoxidase

Fig. 1. Tissue MDA levels in groups

*Significantly different when compared with control group, (p<0.05); ** Significantly different when compared with colitis group, (p<0.05)

Fig. 2. Tissue MPO levels in groups

*Significantly different when compared with control group, (p<0.05); ** Significantly different when compared with colitis group, (p<0.05)
Fig. 3. Tissue cathepsin B levels in groups
*Significantly different when compared with control group, (p<0.05); ** Significantly different when compared with colitis group, (p<0.05)

Fig. 4. Tissue cathepsin L levels in groups
*Significantly different when compared with control group, (p<0.05); ** Significantly different when compared with colitis group, (p<0.05)

4. DISCUSSION

Ulcerative colitis and Chron's disease are a systemic disease, which involves the gastrointestinal system, of which aetiology are not known completely and which affects the life quality of the patient significantly [30]. Prenatal events, lactation, childhood infectious diseases, microbial factors, smoking, oral contraceptives, nutrition, cleaning, business, education, environment terms, stress, psychological factors and physical activity may play a role in the development of ulcerative colitis [31,32]. It is designed to investigate the effects of SM through the antioxidant and apoptosis mechanisms in TNBS-induced colitis.

The colon is more sensitive to oxidative stress due to the comparatively a small quantity of antioxidants penetrable in the mucosa. The aggregation of ROS may induce injury to certain genes related in cell growth or differentiation or may produce alteration in antioxidant enzyme levels [33]. Free oxygen radicals form lipid peroxide radicals by causing lipid peroxidation. The resulting lipid peroxide radicals play a major role in the formation of the inflammatory process [34]. The MDA increase reflects the level of lipid peroxidation in the tissues and is taken into consideration as an indicator of the damaged tissue. Activated neutrophils enter the mucosa and submucosa of the intestine in acute inflammation by being separated from the
circulation and cause the excessive production of lipid mediators, reactive oxygen and nitrogen derivatives that may contribute to the damage of the intestine. The increase in reactive oxygen species causes the uncontrolled lipid peroxidation [35].

Menekse et al. [36] reported that TNBS rapidly increased the lipid peroxidation in the colon tissue and, consequently, the increased reactive oxygen species reduced the antioxidant enzyme levels. In another study, Lee et al. [37] suggested that TNBS also induced lipid peroxidation in the colon, which may be reason colitis and reactive oxygen species may be related in the pathogenesis of IBD. In our study, TNBS was observed to increase the MDA levels which was compatible with the literature. SM may be beneficial in treatment and protection of some inflammatory disease of the colon partially due to its antioxidative activity but at the same time, the other mechanisms are obscured. In our study, SM prevented from TNBS-induced lipid peroxidation in the colons of rat. These protective influence may be involved to various mechanisms: scavenger activity of free radicals that stimulation of lipid peroxidation, and furthermore inducing antioxidant improvement through decreased inflammation [38, 39].

MPO is a basic fragment of neutrophil azurophilic granules. It is an important biomarker of tissue damage and inflammation [40]. The basic function of neutrophils is phagocytosis and the digestion of microorganisms. Free radicals are released during these events, and they cause damage both in the tissues from which they are released and in far tissues [41]. MPO is an important enzyme that ensures the formation of free oxygen radicals. The accumulation of neutrophils in the area where oxidative damage occurs causes an increase in the tissue MPO activity [12]. Raab et al. [42] report the activity of MPO, a neutrophil granule constructive, in the perfusion fluid from sigmoid and rectal sections in patients with UC. The activity of MPO was rised different fold in the persons with ulerative colitis compared with healthy controls indicating to an improved neutrophil activity [43]. In a study conducted by Videla et al. [44] it was reported that the levels of MPO increased very rapidly in the colitis model induced by TNBS.

In colitis models induced by TNBS, the increase in the MPO enzyme in colon tissues is an indicator of neutrophil infiltration and the source of at least one oxygen metabolite in neutrophils [45]. While reactive oxygen species have a counterproductive influence on the tissues, on the one hand, they cause the accumulation of leukocytes in the damaged tissue, on the other hand. Therefore, they cause the worsening of the tissue damage by neutrophils [46]. Neutrophils mediate the formation of hypochlorous acid by releasing the MPO enzyme. They cause damage by exhibiting a cytotoxic effect by causing the oxidation of the resulting hypochlorous acid sulphides, inactivation of cytochrome and proteins, and the decomposition of amino acids and proteins [41]. Miroliaee et al. [47] suggested that among diverse parameters of oxidative stress, evaluation of MPO with lipid peroxidation and antioxidant power is sufficient in making correlation with seriousness of colitis and they showed to SM treatment decreases MPO activity in colon samples. Researchers demonstrated that SM can reduce MPO activity and this protective influence may be connected to the ability to weaken neutrophil recruitment by reducing chemoattractant cytokines and also degradation of neutrophil infiltration by sinking the expression of adhesive molecules [48]. In this study, MPO activity in the colitis group were statistically higher than in the control group. Activity of MPO decreased in colon mucosa of rats with SM. This leads to the total concept of attempting to repair the antioxidant defensive as a treatment for UC. Additionally, these findings could ensure proof for the protective effect of silymarin against tissue injury by reducing high MPO activity in inflammation.

In the literature, it has been reported that silymarin may be a powerful antioxidant and an anti-inflammatory agent. Its positive effects on inflammatory markers, free radicals and lipid peroxidation are attributed to the antioxidant effect of silymarin [49, 50]. Cathepsins can play a significant role in the regulation of apoptosis [51, 52]. The proteolytic and disruptive features of the lysosomal cathepsins have been shown to major role in degenerative, as well as chronic inflammatory diseases [53]. Cathepsin B and cathepsin L are probably to be related in various pathologies. These cathepsins might used as a prognostic mark during the disease direction. In normal physiological conditions, cathepsins are tightly regulated in a well-coordinated manner at multiple levels. The altered regulation of cathepsins can occur at various levels [54].

Menzel et al. [55] reported that the expressions of cathepsin B and cathepsin L increase in inflammatory bowel diseases and this increase
occurs especially in the areas of tissue damage and mucosal ulceration. The researchers also put forth in their study that the inhibition of cathepsins is related to the level of inflammation, and cathepsin inhibitors may be effective in the treatment of inflammatory bowel diseases. In this study, a significant increase occurred in cathepsin B and cathepsin L levels in colonic inflammation induced by TNBS when compared to the control group. Our data also now showed to a pathophysiological role of cathepsins in TNBS-induced colonic inflammation. Additionally, a significant decrease was observed in the levels of cathepsins in both doses of silymarin that we used for treatment purposes. Furthermore, we observed the most effective inhibition in the cathepsins levels at the dose of silymarin of 50 mg/kg just as we observed both in MDA levels and MPO activities. The proteolytic activity of cathepsins are limited to controlled protein disruption in cellular processes and metabolism. In colitis, proteolysis by cathepsins may also be initiated by bacterial infestation resulting in tissue extermination and inflammation [56,57]. Inflammatory bowel diseases are serious source of ROS production. Spite of the preventive barrier ensured by the epithelial layer, ingested materials and pathogens can induce inflammation by activating the neutrophils and macrophages to generate inflammatory mediators that conduce further to oxidative stress [57].

5. CONCLUSION

In conclusion, in this study investigating the effects of TNBS application on the bowel, as a result we determined that, silymarin can improve inflammation by regulating the cathepsin B/cathepsin L signaling pathway and by inhibiting oxidative stress. Our results put forward that silymarin protects the colonic tissue via its radical scavenging and antioxidant activities. At the same time, based on the results of the study, it can be said that silymarin can be used as an effective treatment agent in inflammatory bowel diseases.

ETHICAL APPROVAL

The experimental study was confirmed by the Eskisehir Osmangazi University Institutional Ethical Committee for Animal Care.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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