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From Cell Biology to Tissue Engineering

Histological and immunohistochemical effects of L-arginine and silymarin on TNBS-induced inflammatory bowel disease in rats

Abdul Majeed Al-Drees[1] and Mahmoud Salah Khalil[2]

[1]Department of Physiology, Prince Sultan Ben Abdulaziz College of EMS, and Department of Medical Education, College of Medicine, King Saud University, Riyadh, KSA and [2]College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia and Department of Histology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt

Summary. Inflammatory bowel disease (IBD) is a chronic disease that affects quality of life. Various mediators are involved in IBD pathogenesis including inducible nitric oxide synthase (iNOS), nuclear factor kappa B (NF-κB), cytochrome c, heat shock protein 70 (HSP70) and tumor necrosis factor (TNF)-α. L-Arginine (L-Arg) can be depleted in IBD, and silymarin inhibits neutrophil infiltration, NF-κB, and TNF-α, which have crucial roles in inducing IBD. This study aimed to investigate whether silymarin and L-Arg supplementation decreases IBD progression in trinitrobenzine-sulfonic acid (TNBS)-induced colitis. Fifty adult male albino rats were randomized into five groups (10 animals per group): Group I rats orally received 10 mg silymarin/100 g body weight once daily; Group II rats orally received 2 mg L-Arg/100 g body weight once daily; Group III rats rectally received 0.85 mL TNBS in 50% ethanol to induce colitis; Group IV rats were treated similar to group III and, on recovery from anesthesia, received silymarin as described for group I; and Group V rats were treated similar to group III and, on recovery from anesthesia, received L-Arg as described for group II. On day 7, the rats were anesthetized, and blood samples were collected to determine the serum concentrations of TNF-α. Laparotomy and total colectomy were performed for macroscopic, histological, and immunohistochemical investigations. The results showed that silymarin and L-Arg macroscopically and microscopically ameliorated TNBS-induced colitis; significantly decreased the serum levels of TNF-α; inhibited the colonic expression of iNOS, NF-κB, and cytochrome c; and increased expression of HSP70. Our results suggest that these complementary medicines could be used to supplement current treatments for IBD.

Key words: Inflammatory bowel disease, TNBS, Histology, Immunohistochemistry, L-Arginine, Silymarin

Offprint requests to: Mahmoud Salah Khalil, College of Medicine, King Saud University, Postal Code: 11451, Riyadh, Kingdom of Saudi Arabia. e-mail: msalah28@hotmail.com

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Introduction

Inflammatory bowel disease (IBD) comprises a group of chronic diseases including Crohn's disease and ulcerative colitis that severely affect quality of life (Baumgart and Carding, 2007; Baumgart and Sandborn, 2007). Epithelial wound repair deficiency resulting from dysregulation of the immune response is an important factor in the pathogenesis of IBD (Baumgart and Carding, 2007; Baumgart and Sandborn, 2007). However, the exact mechanism of the dysregulated immune response is still under investigation.

Medical treatment of IBD includes five major approaches: anti-inflammatory drugs, immune-suppressants, biologic agents, antibiotics, and drugs for symptomatic relief (Carter et al., 2004). Medical treatment can reduce periods of active disease and help to maintain remission. However, current treatments often have marginal results, leading IBD to become refractory, and side effects that can affect all body systems (Carter et al., 2004). Therefore, L-arginine (L-Arg) and silibinin

(silymarin) have been recognized as options for treating IBD with the potential to overcome the limitations of traditional pharmacological medical therapies (Esmaily et al., 2011; Becker et al., 2013).

L-Arg is a semi-essential amino acid that can be depleted under stressful conditions such as IBD (Castillo et al., 1994). In addition, L-Arg is a substrate for arginase, nitric oxide synthase (NOS), arginine, glycine amidinotransferase, and arginine decarboxylase (Coman et al., 2008). Arginase is an endogenous antagonist to inducible NOS (iNOS) as it competes for the same L-Arg substrate. Arginase metabolizes L-Arg to L-ornithine (L-Orn) and urea, whereas iNOS metabolizes L-Arg to nitric oxide (NO) and L-citrulline (Mori, 2007). L-Orn functions in attenuating the degree of intestinal tissue damage and promoting healing of the intestinal mucosa (Mori, 2007), whereas NO is a possible causative factor in the initiation and progression of IBD inflammation (Cross and Wilson, 2003). Under oxidative stress conditions, NO prevents cellular injury by scavenging free radicals (Cross and Wilson, 2003); however, increased iNOS activity inhibits cellular proliferation (Bani et al., 1995; Jenkins et al., 1995) and increases apoptosis (Messmer et al., 1994) and cytotoxicity (Fukuo et al., 1995).

Silibinin (silymarin) has two diastereomers, silibinin A and silibinin B (Gazák et al., 2007), and inhibits neutrophil infiltration, cyclo-oxygenase-2 (COX-2), Tumor necrosis factor alpha (TNF-α), interleukin (IL)-1, tissue metalloproteinase, adhesion molecules, and leukotrienes but has a stabilizing effect on mast cells (Malihi et al., 2009). In addition, silibinin inhibits nuclear factor kappa B (NF-ϰB) activation secondary to its antioxidant effect and has a positive effect in experimental colitis (Esmaily et al., 2011). In IBD, NF-ϰB is activated by increased levels of TNF-α, a proinflammatory cytokine, leading to its translocation into the nucleus (Ghosh et al., 1998) and downregulation of trefoil factor (TFF3) peptides. TFF3 downregulation induces apoptosis, reduces inflammatory cell migration, and reduces the barrier function, resulting in induction of colonic ulceration (Loncar et al., 2003).

L-Arg and silymarin have fewer side effects than the traditional pharmacological therapies and might be effective for the treatment of IBD. However, because the few reported studies have employed heterogeneous methodologies, further in-depth research is necessary to evaluate the potential of L-Arg and silymarin for treatment of IBD (Langhorst et al., 2015). Therefore, this study aims to investigate whether silymarin and L-Arg supplementation affect IBD progression in a trinitrobenzine-sulfonic acid (TNBS)-induced rat model of colitis toward understanding their potential for therapeutic application in human IBD.

Materials and methods

A total of 50 adult Wistar male albino rats (200-250 g) were allowed water and food ad libitum at constant humidity (55±10%) and temperature (25±2°C) on a 12/12 h light/dark cycle. The animals were acclimatized to the laboratory conditions for one week before conducting the experiment.

The animals were randomized into five groups (10 animals per group).

Group I (control 1): Rats received 10 mg silymarin/100 g body weight (Sigma-Aldrich, St. Louis, MO, USA) suspended in 2 mL saline through oral gavage once daily for 7 days (Esmaily et al., 2011).

Group II (control 2): Rats received 2 mg L-Arg/100 g body weight (Sigma-Aldrich) suspended in 5 mL distilled water through oral gavage once daily for 7 days (Fotiadis et al., 2004).

Group III (TNBS-induced colitis): Animals were fasted overnight and allowed to drink only water before induction of colitis. The animals were anesthetized by intramuscular injection of 0.3 mL ketamine (Koçak et al., 2011). Initially, each rat received a 1-mL saline (0.9%) flush followed by manual palpation of the abdomen to remove any feces. Thereafter, 0.85 mL TNBS in 50% ethanol (10 mL TNBS in 4.15 mL 50% ethanol) (SIGMA pharmaceuticals-P2297) was instilled through a 6F plastic catheter that was rectally inserted until the tip was 5 cm proximal to the anus. The animals were handled in the Trendelenburg position during the procedure to prevent immediate anal leakage of the TNBS solution. During the instillation, the catheter was withdrawn slowly to allow distribution of the TNBS over an area of the colon (Fotiadis et al., 2004). Thereafter, all rats were maintained in a head-down position for 60 s to limit expulsion of the solution.

Group IV (TNBS + silymarin): Animals were treated similar to group III, then, on recovery from anesthesia, the rats received silymarin as described for group I once daily for 7 days.

Group V (TNBS + L-Arg): Animals in this group were treated similar to group III, then, on recovery from anesthesia, rats received L-Arg as described for group II once daily for 7 days.

Disease manifestations in the form of diarrhea, hematochezia, and weight loss were recorded daily (Latella et al., 2008). On the morning of the seventh day, the rats were anesthetized by ether inhalation and euthanized by heart puncture.

Blood samples were collected during the heart puncture and used to determine serum concentrations of TNF-α via ELISA (Euroclone, Devon, UK) according to the manufacturer's instructions.

Laparotomy and total colectomy were performed. The presence of structures, dilatation of the colon, and adhesions between the colon and the adjacent organs were recorded (Latella et al., 2008). The lumen of the resected colon was irrigated with 0.9% NaCl. The distal colon segment was then split longitudinally into two pieces for macroscopic, histological, and immunohisto-chemical investigations. The animal experiments were performed in the Animal Laboratory Center of College of Medicine, King Saud University (Riyadh, KSA), in

accordance with the institutional and national guidelines for the care and use of laboratory animals. The experiment was approved by The Ethical Committee of College of Medicine, King Saud University.

Macroscopic studies

Macroscopic lesions of colon injury were investigated by stereomicroscope (LEICA, L2, Germany) and recorded by two independent observers to determine the extent of gross morphologic damage. The lesion score scale which ranged from 0 to 5: 0, no damage; 1, localized hyperemia without ulcers or erosions; 2, ulcers or erosions with no significant inflammation; 3, ulcers or erosions with inflammation at one site; 4, two or more sites of ulceration and/or inflammation; and 5, two or more major sites of inflammation and ulceration or one major site of inflammation and ulceration extending >1 cm along the length of the colon (Koçak et al., 2011).

The colon specimens for each rat were fixed in 10% neutral buffered formalin solution (Sigma Chemical Co.) for 24 h. The colon specimens were processed to prepare 4-μm thick paraffin sections. The sections were stained for histological and immunohistochemical studies.

Histological studies: Hematoxylin & eosin (H & E) (Drury and Wallington, 1983) and Masson's trichrome (Drury et al., 1976) were used to visualize the histological changes and collagen fibers, respectively. The sections were evaluated by light microscopy and scored on a scale of 0-10 (Table 1) (Wang et al., 2002) by two blinded observers.

Immunohistochemical studies

Paraffin sections were processed for iNOS (sc-7271), NF-κB p50 (C-19; sc-1190), cytochrome c (A-8; sc-13156), and heat shock protein 70 (HSP70, sc-24) antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After deparaffinization in xylene, immunohistochemistry was performed using a three-step indirect process based on the labeled avidin-biotin-peroxidase complex (ABC) method. The sections were rehydrated in descending grades of alcohol followed by blocking endogenous peroxidase activity with 3% H_2O_2 in methanol and non-specific binding sites with a protein blocker. The sections were incubated for 32 min with a 1:100 dilution of the primary antibodies (rabbit anti-rat iNOS, NF-κB, cytochrome c, and HSP70 polyclonal antibodies, Santa Cruz Biotechnology). The biotinylated secondary antibody was added at a concentration of 2% for 30 min (37°C), and then the ABC was added. Visualization of the reaction was performed using 3,3-diaminobenzidine (DAB), which produces a dark brown precipitate that is readily detected by light microscopy. The sections were then counterstained with Mayer's hematoxylin, dehydrated in ascending grades of alcohol, cleared in xylene, and mounted with DPX (iNOS, NF-κB, cytochrome c, and HSP70, nucleus and cytoplasm, were stained brown). The negative control included sections incubated without primary antibody. The sections were independently examined by one of the authors and a pathologist.

Table 1. Criteria for the scoring of microscopic damage histological lesion Score.

Ulcer	None	0
	<3mm	1
	>3mm	2
Inflammation	None	0
	Mild	1
	Severe	2
Granuloma	None	0
	Present	1
Depth of the disease	None	0
	Submucosal layer	1
	Muscular layer	2
	Serosal layer	3
Fibrosis	None	0
	Mild	1
	Severe	2

Quantitative analysis

Quantitative measurements were carried out using an image analyzer (Super eye-Heidi soft; Anatomy Department, College of Medicine, King Saud University, KSA). Fifteen high-power fields (Objective, x400) were captured for each animal. The mean optical density (OD) for reactions with antibodies targeting iNOS, NF-κB, cytochrome c, HSP70, cytoplasm and white blood cells were measured. The image analyzer was calibrated for color measurement before use.

Statistical analysis

The data were analyzed using SPSS statistical software version 19. The values were expressed as mean ± SEM. The Mann-Whitney U-test was used to compare the macroscopic and microscopic values. ANOVA was used to compare the ODs of the immune-positive immunohistochemical reactions among the studied groups. The significance level was set at $p \leq 0.05$.

Results

The mean sera levels of TNF-α were significantly higher ($p=0.01$) in group III (161±7 pg/L) than in groups I (15±2 pg/L), II (18±2 pg/L), IV (80±6 pg/L), or V (75±5 pg/L).

Group III exhibited the highest mortality rate, whereas groups I and II had no mortality; the mortalities of groups IV and V were intermediate.

Disease activities in the form of diarrhea, body weight loss, hematochezia, colonic stricture, dilatation, and adhesions to the surrounding organs were not noted in either control groups (groups I and II), but were

observed in groups III-V, with the highest levels in group III (Table 2). The mean macroscopic ulcer score was significantly higher (p=0.03) in group III (3.8±0.85) than in groups I (0±0), II (0±0), and V (1.5±0.54), but the difference was not significant compared to group IV (2.1±0.85) (Table 3). H&E staining of the controls (groups I and II) showed normal colonic structure with intact mucosal architecture (Fig. 1A,B). In contrast, group III showed mucosal necrosis, numerous dilated mucosal vessels, hemorrhage, edematous mucosa and submucosa, infiltration of mucosa and submucosa with massive acute inflammatory cells, the presence of several submucosal lymphoid follicles, and decreased sizes and numbers of goblet cells. In addition, the non-intact muscularis mucosa was noticed (Fig. 2A-C). Group IV showed some areas of desquamated mucosal epithelium and mild submucosal inflammatory cell infiltration (Fig. 3A,B), and group V showed small areas of non-intact mucosa without acute inflammatory cell infiltration (Fig. 4A,B).

The mean score of microscopic damage of histological lesion record was significantly higher (p=0.01) in group III (6.5±0.9) than in groups I (0±0), II (0±0), IV (3.1±0.43), or V (1.9±0.23).

Masson's trichrome sections staining showed fine collagen fibers in the lamina propria between the crypts of control groups I and II (Fig. 1C). Coarse collagen fibers in the mucosa and submucosa of group III (Fig. 2D) were also seen. Collagen fibers were moderate and fine in groups IV (Fig. 3C) and V (Fig. 4C), respectively.

Immunohistochemical staining showed very mild expression of iNOS (Fig. 1D) in the mucosa and lamina propria of control groups (I and II). However, marked expression of iNOS was noted in the mucosal epithelial cells, lamina propria, and submucosa of the colon in group III (Fig. 2E). Moderate iNOS expression was noted in the lamina propria and submucosa of group IV (Fig. 3D), while mild expression was noted in the lamina propria of group V (Fig. 4D).

NF-κB was mildly expressed in some parts of the surface epithelium and lamina propria of control groups I and II (Fig. 1E). However, group III showed marked NF-κB expression in almost all regions of the mucosa (Fig. 2F). Moderate expression was noted in parts of the lamina propria and submucosa of group IV (Fig. 3E). In addition, group V showed mild expression limited to the epithelial cells and lamina propria of the mucosa (Fig. 4E).

Cytochrome c (Fig. 1F) was mildly expressed in the mucosal surface epithelium and lamina propria as well as the submucosa of control groups (I and II). However, group III exhibited marked and moderate cytochrome c expression in the mucosa and submucosa, respectively (Fig. 2G). Moderate and mild expression was noted in the mucosa and submucosa of group IV, respectively (Fig. 3F). In addition, group V showed mild expression in the mucosa and submucosa (Fig. 4F).

HSP70 was moderately expressed in the mucosa and submucosa of group III (Fig. 2H). However, it was markedly expressed in the colonic mucosa and submucosa of groups I, II (Fig. 1G), IV (Fig. 3G), and V (Fig. 4G).

Quantitative immunohistochemical analysis (Table 3) showed that iNOS, NF-κB, and cytochrome c expressions were significantly higher in group III (0.25±0.04AU, 0.29±0.03AU, and 0.21±0.01AU, respectively) than in groups I (0.0083±0.0007AU, 0.01±0.0003AU, and 0.07±0.002AU, respectively), II (0.009±0.0006AU, 0.009±0.0008AU, and 0.09±0.001 AU, respectively), IV (0.055±0.004AU, 0.23±0.09AU, and 0.18±0.05AU, respectively), and V (0.043±0.003 AU, 0.12±0.002AU, and 0.12±0.002AU, respectively). HSP70 expression, however, was significantly lower in group III (0.12±0.006AU) than in groups I (0.17±0.07AU), II (0.15±0.08AU), VI (0.22±0.06AU), and V (0.31±0.08AU).

Table 2. the disease activity and numbers of affected animals.

	Group I	Group II	Group III	Group IV	Group V
Number of dead animals	0	0	3	1	1
Diarrhea	0	0	6	3	3
Body weight (Mean±SEM)	220±40	230±43	160±33	190±30	185±35
Haematochezia	0	0	5	2	2
Presence of strictures	0	0	2	1	1
Presence of dilatation	0	0	5	1	1
Presence of adhesions	0	0	4	1	1

Table 3. Comparison of the macroscopic, microscopic ulcer and optical density (OD) scores of the groups.

		Group I	Group II	Group III	Group IV	Group V	p-value
Macroscopic		0±0	0±0	3.8±0.85*	2.1±0.85	2.5±0.54	0.03
Microscopic		0±0	0±0	6.5±0.9*	3.1±0.43	1.9±0.23	0.01
OD	iNOS	0.0083±0.0007	0.009±0.0006	0.25±0.04*	0.055±0.004	0.043±0.003	0.005
	NFκB	0.01±0.0003	0.009±0.0008	0.29±0.03*	0.23±0.09	0.12±0.002	0.001
	Cytochrome C	0.07±0.002	0.09±0.001	0.21±0.01*	0.18±0.05	0.12±0.002	0.0008
	HSP70	0.17±0.07	0.15±0.08	0.12±0.006*	0.22±0.06	0.31±0.08	0.0005

*: Significant p value comparing to other groups.

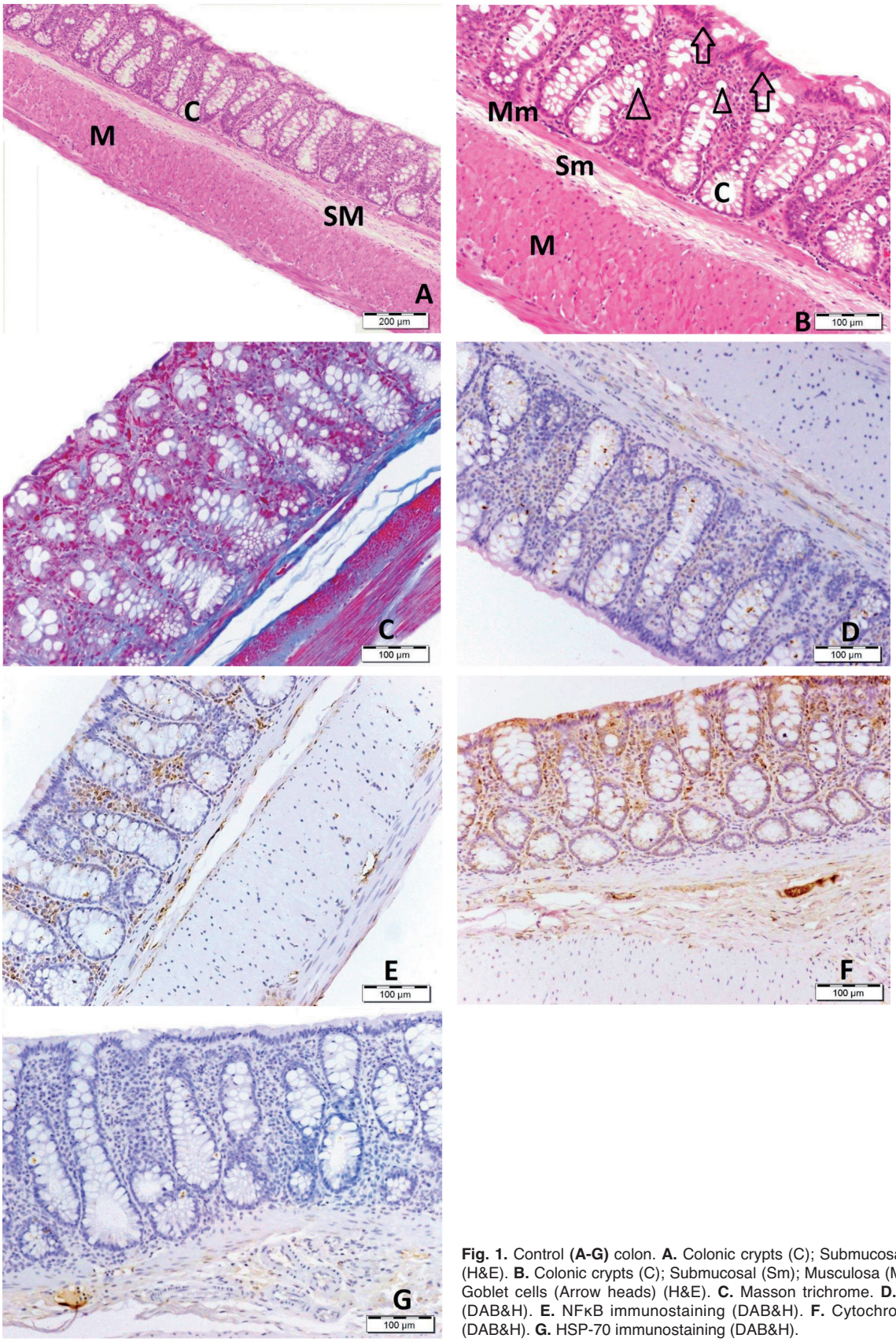

Fig. 1. Control **(A-G)** colon. **A.** Colonic crypts (C); Submucosal (Sm); Musculosa (M) (H&E). **B.** Colonic crypts (C); Submucosal (Sm); Musculosa (M); Epithelium (Arrows); Goblet cells (Arrow heads) (H&E). **C.** Masson trichrome. **D.** iNOS immunostaining (DAB&H). **E.** NFκB immunostaining (DAB&H). **F.** Cytochrome C immunostaining (DAB&H). **G.** HSP-70 immunostaining (DAB&H).

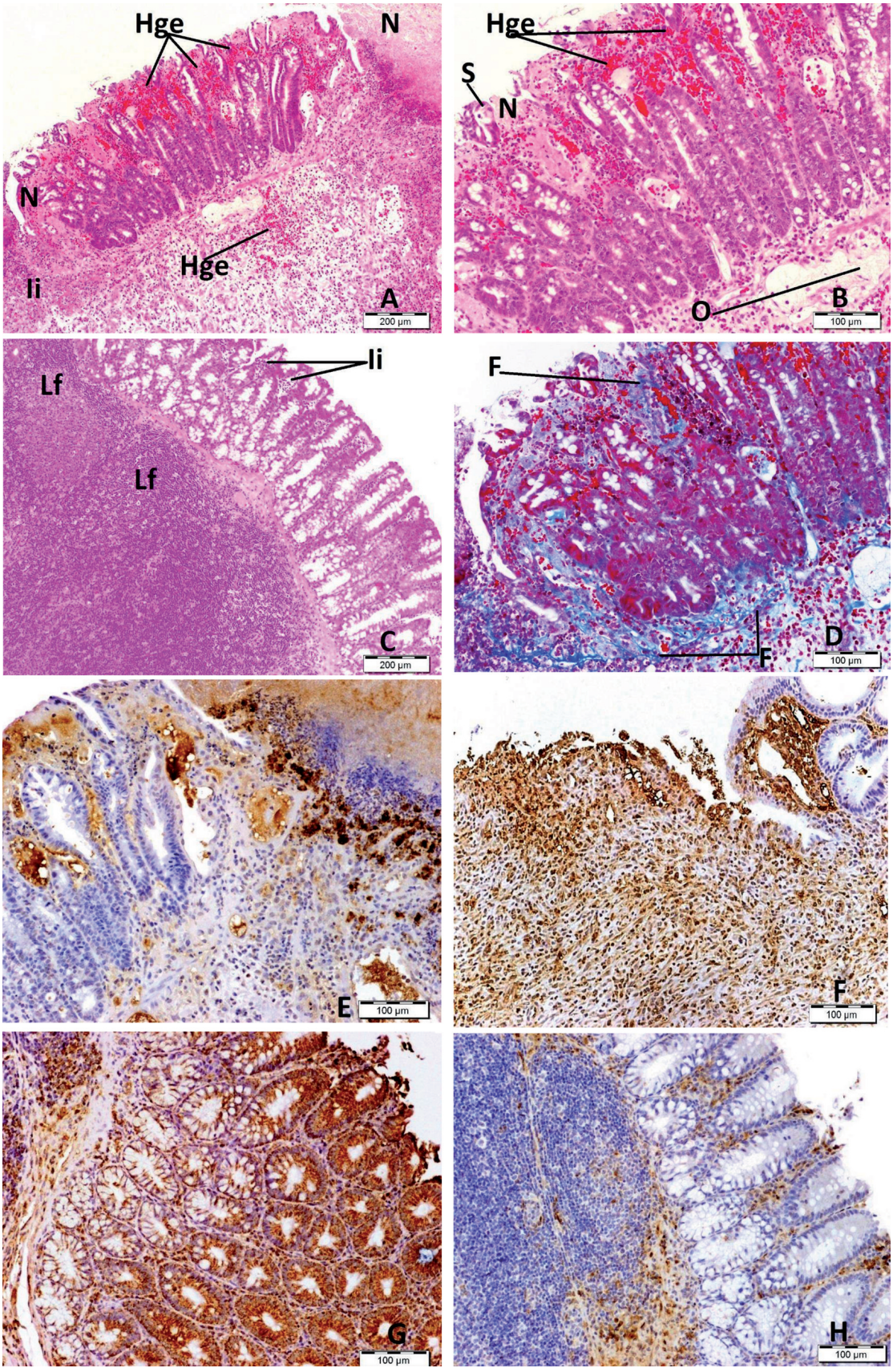

Fig. 2. (TNBS induced Colitis).
A. Hemorrhage (Hge); Necrosis (N); Leukocyte infiltration (Li) (H&E).
B. Hemorrhage (Hge); Necrosis (N); Desquamated epithelium (S); Edema (O) (H&E).
C. Leukocyte infiltration (Li); Submucosal lymphoid follicles (Lf) (H&E).
D. Connective tissue fibers (F) (Masson trichrome).
E. iNOS immunostaining (DAB&H).
F. NFκB immunostaining (DAB&H).
G. Cytochrome C immunostaining (DAB&H).
H. HSP-70 immunostaining (DAB&H).

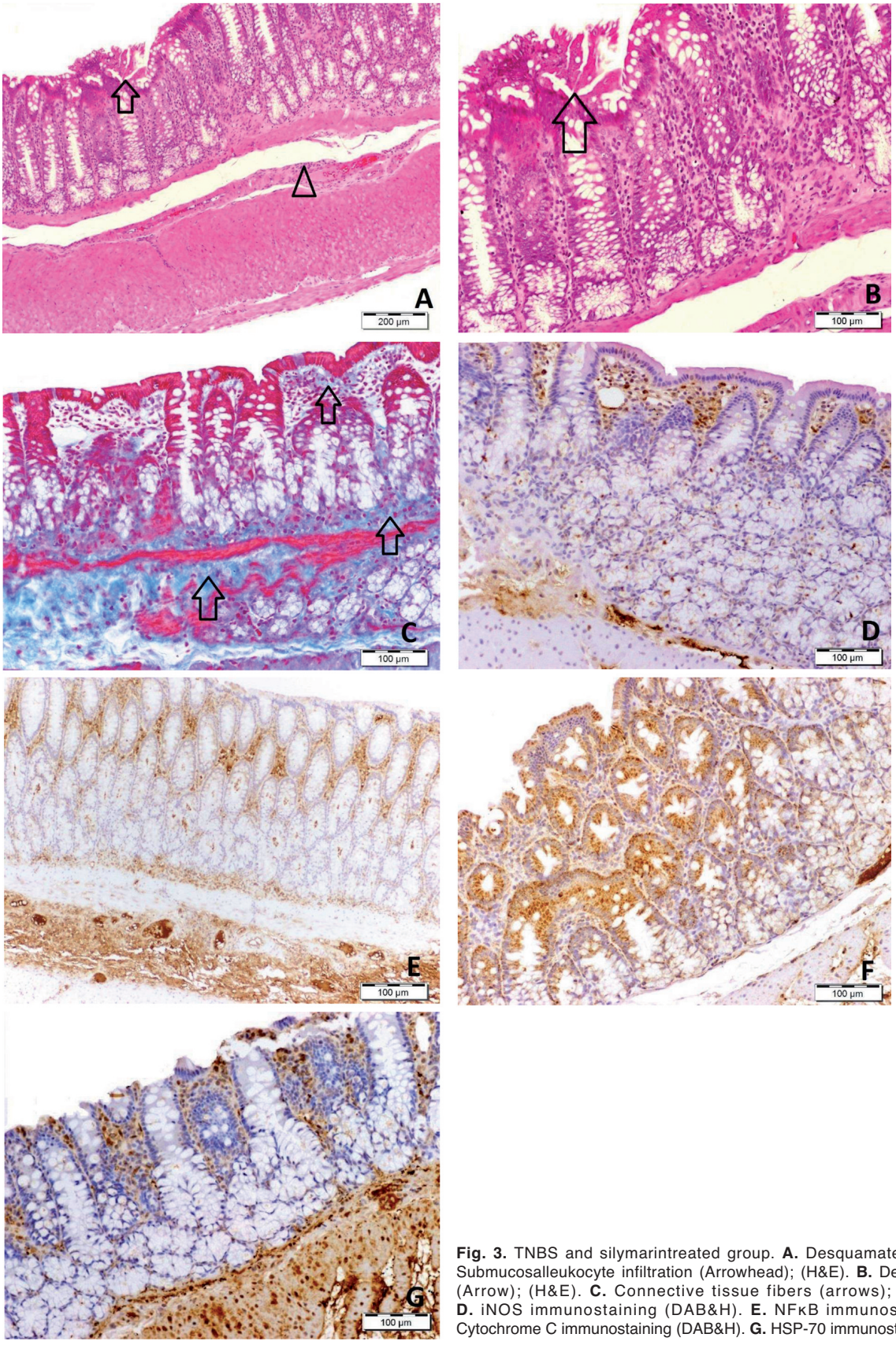

Fig. 3. TNBS and silymarintreated group. **A.** Desquamated epithelium (Arrow); Submucosalleukocyte infiltration (Arrowhead); (H&E). **B.** Desquamated epithelium (Arrow); (H&E). **C.** Connective tissue fibers (arrows); (Masson trichrome). **D.** iNOS immunostaining (DAB&H). **E.** NFκB immunostaining (DAB&H). **F.** Cytochrome C immunostaining (DAB&H). **G.** HSP-70 immunostaining (DAB&H).

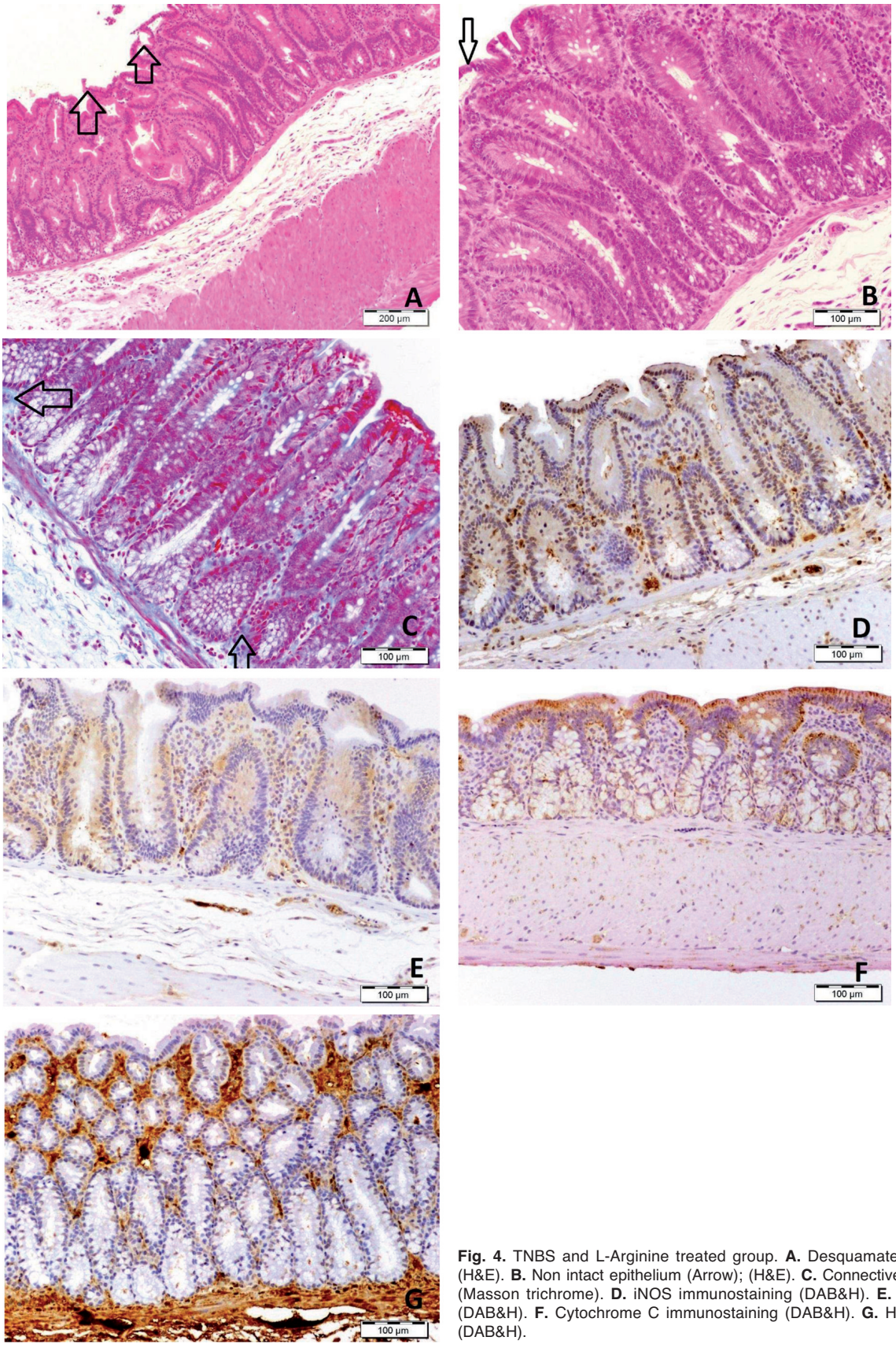

Fig. 4. TNBS and L-Arginine treated group. **A.** Desquamated epithelium (Arrows); (H&E). **B.** Non intact epithelium (Arrow); (H&E). **C.** Connective tissue fibers (arrows); (Masson trichrome). **D.** iNOS immunostaining (DAB&H). **E.** NFκB immunostaining (DAB&H). **F.** Cytochrome C immunostaining (DAB&H). **G.** HSP-70 immunostaining (DAB&H).

Discussion

The objective of this study was to determine whether silymarin and L-Arg supplementation could inhibit the progression of IBD in a rat model of colitis and, thus, have a potential for therapeutic application in human IBD. TNBS-induced colitis has been widely used as an experimental model because of its similarities with human IBD (Latella et al., 2008; Yang et al., 2011; Szalai et al., 2014). Our data indicate clearly that rectal administration of TNBS increases the serum levels of TNF-α and induces diarrhea, body weight loss, hematochezia strictures, dilatation of the colon, and adhesions between the colon and adjacent organs. Moreover, we found that TNBS induced significant macroscopic changes in the colon in terms of mucosal hyperemia, erosion, and ulcer. Similar results from other studies showed that TNBS induced Crohn's disease rather than ulcerative colitis in an animal model (Latella et al., 2008), and it is an established experimental model for studying the pathogenesis of Crohn's disease and evaluating experimental treatment effects because of its similarities with human IBD (Yang et al., 2011; Szalai et al., 2014). Microscopically, TNBS induced necrosis, mucosal vessel dilation and hemorrhage, and mucosal and submucosal edema in addition to massive mucosal and submucosal inflammatory cell infiltration. Submucosal lymphoid follicles, non-intact muscularis mucosa, and massive fibrosis, especially in the mucosa and submucosa, were also noted.

Inflammatory cell infiltration, accumulation of collagen, edematous areas, the appearance of epithelial ulcers, and desquamated areas suggest a specific autoimmune response to TNBS (Fotiadis et al., 2004). Moreover, TNBS induces microscopic changes similar to those seen in IBD, including the presence of submucosal lymphoid follicles and fibrosis, which are characteristic features of Crohn's disease (Krauss et al., 2012).

In the current study, iNOS was highly expressed in the colonic mucosal epithelial cells, lamina propria, and submucosa of the TNBS-induced colitis group. This finding is consistent with a previous study, which showed that iNOS was upregulated in inflamed areas of the colon epithelial cells; the lamina propria, especially in the presence of infiltrations with mononuclear cells; and neutrophils (Hofseth et al., 2003).

Inflammatory cell infiltration produces pro-inflammatory cytokines such as interleukins, TNF-α, and interferon-γ, which induce the expression of inflammation-associated genes such as iNOS and NF-κB (Sandborn and Targan, 2002). Thus, inflammatory cell infiltration in the colons of group III (TNBS-induced colitis) rats could account for the upregulation of iNOS (Miller et al., 1995). The stimulation of iNOS activity increases the production of the free radical NO, which is a marker of inflammation that induces colonic cytotoxicity and undermines the integrity of the colon (Aktan, 2004). Furthermore, NO has been previously identified in infiltrating neutrophils and macrophages in the colon (Miller et al., 1995). iNOS is upregulated in the mucosa and submucosa of the colon in several animal models of IBD (Miller et al., 1995) and in patients with IBD (Palatka et al., 2005). In addition, the increased serum levels of TNF-α could be attributed to the presence of the inflammatory cell infiltrates. TNF-α disrupts the colon epithelial barrier (Schulzke et al., 2006) by inducing apoptosis (Abreu et al., 2000).

The current study showed marked expression of NF-κB in almost all parts of the mucosa in rats with TNBS-induced colitis, which is consistent with previous demonstrations of increased NF-κB expression in the acute phase of TNBS-induced colitis (Loncar et al., 2003; Arab et al., 2014). NF-κB activates the expression of many cytokines, chemokines, and adhesion molecules, which recruit and activate the immune cells (Karin et al., 2002). Activation of NF-κB plays a major role in the pathogenesis of IBD through downregulation of intestinal TFF3 (Loncar et al., 2003). TFF3 confers colonic epithelial resistance to apoptosis and stimulates inflammatory cell migration (Xian et al., 1999). TFF3 is also associated with the commitment of the epithelial cells to transform into goblet cells (Kinoshita et al., 2000). Downregulation of TFF3 could explain the decreased numbers and size of goblet cells we observed in group III. Moreover, NF-κB regulates the expression of proinflammatory genes, including TNF-α, COX-2, and iNOS, that play key roles in TNBS induction of IBD (Xie et al., 1994; Hume et al., 2008). NF-κB is retained in an inactive state by binding with the inhibitory protein IκBα in the cytoplasm. Upon exposure to stress conditions such as TNBS, NF-κB is activated by phosphorylation and proteasomal degradation of IκBα, which allows it to translocate into the nucleus and control the expression of target genes (Atreya et al., 2008).

There are inconsistencies in the data linking NF-κB and apoptosis. One study found that NF-κB blocks apoptosis by positively regulating the expression of anti-apoptosis proteins; others found that NF-κB upregulates cytochrome c and consequently activate apoptosis (Shou et al., 2002).

The results of the current study showed increased mucosal and submucosal expressions of cytochrome c in the TNBS-induced colitis group, which indicated the activation of apoptosis in colonic tissues (Arab et al., 2014). This result is consistent with another study that demonstrated TNBS-induced apoptosis activation as evidenced by concurrent upregulation of cytochrome c, Bax, and caspase-3 pro-apoptotic genes and downregulation of the anti-apoptotic Bcl-2 (Liu and Wang, 2011; Crespo et al., 2012; Arab et al., 2014). The increased apoptosis of colonic epithelial cells likely results in alteration of the epithelial barrier, thereby inducing intestinal injury (Becker et al., 2013).

Oxidative stress triggers the expression of several genes responsible for cellular death by apoptosis (Crespo et al., 2012). Apoptosis is regulated by the Bcl-2 family, including Bcl-2 and Bax. An increased Bax/Bcl-2 ratio enhances the release of cytochrome c from mitochondria

to the cytosol, which activates caspase-9 and ultimately caspase-3, thereby inducing apoptosis (Crespo et al., 2012; Becker et al., 2013).

HSP70 suppresses the necrotic and apoptotic cell death that are independent of caspases and not blocked by Bcl-2 (Beere et al., 2000). Our results revealed significantly lower expression of HSP70 in the mucosa and submucosa of the TNBS-induced colitis group compared to other groups, including the controls. This is consistent with a previous demonstration that normal rat intestinal epithelial cells express HSP70 (Venkatraman et al., 2003). Stress can decrease the level of HSP70 in intestinal epithelial cells, and pretreatment with HSP70 inhibits chronic stress and induces intestinal epithelial layer hyperpermeability (Venkatraman et al., 2003).

This study showed that silymarin and L-Arg decreased the serum levels of TNF-α and ameliorated disease activities and macroscopic and microscopic pathological changes of TNBS-induced colitis to variable degrees. We also showed that silymarin and L-Arg decreased the acute inflammatory infiltration and collagen density seen in TNBS-induced colitis. Furthermore, our results revealed that silymarin and L-Arg decreased the expressions of colonic iNOS, NF-ϰB, and cytochrome c but increased that of HSP70.

The decreased serum levels of TNF-α in groups IV and V compared to group III could be attributed to the fact that silymarin and L-Arg attenuate the inflammatory cell infiltration induced by TNBS (Esmaily et al., 2011). Silymarin and L-Arg were previously shown to attenuate tissue damage, promote healing of the intestinal mucosa, decrease pro-inflammatory cytokine and chemokine responses, decrease inflammatory cell infiltration, improve mucosal integrity, enhance epithelial cell migration, and improve clinical parameters in colitis-induced rats (De La Puerta et al., 1996; Monna et al., 1999; Fotiadis et al., 2007; Juma'a et al., 2009; Esmaily et al., 2011; Becker et al., 2013). Furthermore, silymarin and L-Arg diminish NF-ϰB activity and neutrophil infiltration/activation in the inflamed colon (De La Puerta et al., 1996; Monna et al., 1999; Fotiadis et al., 2007; Juma'a et al., 2009; Esmaily et al., 2011; Becker et al., 2013; Ren et al., 2014; Rastegarpanah et al., 2015). NF-ϰB regulates and coordinates the expression of various genes involved in the inflammatory process such as IL-1 and IL-6, TNF-α, lymphotoxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon gamma (Polyak et al., 2007). Silymarin and L-Arg also significantly increase the expression of HSP70 in mesenteric ischemia-reperfusion injury, thereby protecting cells against stress and apoptosis in response to cellular insults (Wu et al., 2010; Demir et al., 2014).

Increased expression of HSP70 indicates that silymarin and L-Arg protect colonic cells against TNF-α-mediated cytotoxicity. Studies have demonstrated that HSP70 can block the production of proinflammatory cytokines through inhibition of NF-ϰB and mitogen-activated protein kinase pathways or activate anti-inflammatory cytokines to control the magnitude of the immune response (Barbatis and Tsopanomichalou, 2009; Wu et al., 2010). The increased expression of HSP70 mediates resistance to apoptotic cell death by inhibiting mitochondrial apoptosis (Wu et al., 2010), which could explain the decreased expression of cytochrome c in groups IV and V herein. Alternatively, the decreased expression of cytochrome c in groups IV and V could be attributed to decreased expression of NF-ϰB (Shou et al., 2002), as it is an indication of anti-apoptotic activities and could consequently reduce the TNBS-induced colonic epithelial injury (Li et al., 2007; Becker et al., 2013).

In addition, silymarin and L-Arg inhibit iNOS activity, which is associated with perpetuation of the inflammatory process (Barrachina et al., 2001; Kang et al., 2002). The induction of iNOS is mediated by NF-ϰB (Xie et al., 1994); therefore, it is not surprising that iNOS and NF-ϰB were concomitantly decreased in groups IV and V herein. Moreover, the decreased expression of iNOS could be linked to TNBS-induced depletion of endogenous L-Arg (Castillo et al., 1994). Arginase, an endogenous iNOS antagonist, metabolizes L-Arg to L-Orn and urea, whereas iNOS produces NO and L-citrulline (Mori, 2007). L-Orn is used by ornithine decarboxylase (ODC) to produce the polyamine putrescine, which is then converted into the polyamines spermidine and spermine by the constitutively expressed spermidine and spermine synthases, respectively. Polyamines have been associated with mucosal protection in the gastrointestinal tract (Fritz, 2013).

Further studies are needed to corroborate our results and further clarify the mechanism by which silymarin and L-Arg ameliorate the histological changes of TNBS-induced colitis. In addition, further investigation of the potential for silymarin and L-Arg alone or in combination to supplement the current conventional medical regimens for treating IBD is needed.

Conclusion

In this study, the prophylactic oral administration of both silymarin and L-Arg ameliorated the development of TNBS-induced colitis in rats, apparently by inhibiting the colonic expression of iNOS, NF-ϰB, and cytochrome c and increasing the expression of HSP70. Thus, they show potential for therapeutic application in human IBD.

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Authors' contributions: AMD conceived the study, designed the experiments, performed statistical analyses, and finalized the paper. MSK conducted the experiments, analyzed the histological and immunohistochemical data, and wrote the first draft of the manuscript.

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