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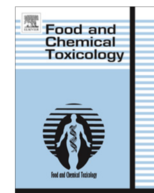

Camel's milk ameliorates TNBS-induced colitis in rats via downregulation of inflammatory cytokines and oxidative stress

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ABSTRACT

Current treatment strategies for inflammatory bowel diseases (IBD) are associated with several adverse effects, and thus, the search for effective agents with minimal side effects merits attention. Camel's milk (CM) is endowed with antioxidant/anti-inflammatory features and has been reported to protect against diabetes and hepatic injury, however, its effects on IBD have not been previously explored. In the current study, we aimed to investigate the potential alleviating effects of CM against TNBS-induced colitis in rats. CM (10 ml/kg b.i.d. by oral gavage) effectively suppressed the severity of colon injury as evidenced by amelioration of macroscopic damage, colon weight/length ratio, histopathological alterations, leukocyte influx and myeloperoxidase activity. Administration of CM mitigated the colonic levels of TNF-α and IL-10 cytokines. The attenuation of CM to colon injury was also associated with suppression of oxidative stress via reduction of lipid peroxides and nitric oxide along with boosting the antioxidant defenses through restoration of colon glutathione and total anti-oxidant capacity. In addition, caspases-3 activity, an apoptotic marker, was inhibited. Together, our study highlights evidences for the promising alleviating effects of CM in colitis. Thus, CM may be an interesting complementary approach for the management of IBD.

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1. Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, are chronic, relapsing, immunologically mediated inflammation of the gastrointestinal tract that distress the quality of life of patients suffering from these disorders (Fiocchi, 1998). The pathogenesis of IBD is versatile, involving derangement of the intestinal epithelial barrier as the key event, which is followed by uncontrolled immune and inflammatory responses toward intestinal flora in a context of genetic predisposition (Ohman and Simren, 2010; Salim and Soderholm, 2011). As a result, various cytokines, inflammatory mediators which play a central role in IBD pathogenesis, are generated with consequent recruitment of neutrophils and macrophages to the intestinal mucosa (Ohman and Simren, 2010; Salim and Soderholm, 2011). Among them, TNF-α, is a key immunoregulatory cytokine that amplifies the inflammatory response by stimulating the production of other cytokines, ROS, arachidonic acid metabolites, and proteases. The elevated serum levels of TNF-α correlate well with the clinical status and the intestinal disease activity of patients with IBD (Sanchez-Munoz et al., 2008). Besides, elevated levels of IL-10 have been reported in IBD patients (Kucharzik et al., 1995) and experimental models (Barada et al., 2007; Tomoyose et al., 1998) where they attenuate mucosal inflammation through inhibition of pro-inflammatory cytokines (Schreiber et al., 1995).

Activation of inflammatory cells including neutrophils, macrophages and lymphocytes provokes oxidative stress via generation

Abbreviations: CM, Camel's milk; DTNB, 5,5′-dithiobis 2-nitrobenzoic acid; GSH, glutathione; H&E, hematoxylin and eosin; IBD, inflammatory bowel diseases; IL-10, interleukin-10; MDA, malondialdehyde; MPO, myeloperoxidase; NO, nitric oxide; ROS, reactive oxygen species; RNS, reactive nitrogen species; TAC, total antioxidant capacity; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF-α, tumor necrosis factor-α.

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of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radicals (Fiocchi, 1998). These species induce destructive oxidation of membrane lipids in a process known as lipid peroxidation (Rangan and Bulkley, 1993). Besides, reactive nitrogen species (RNS), such as nitric oxide (NO) have been implicated in the development of IBD (Cross and Wilson, 2003). The pathogenesis of IBD also involves increased apoptosis of intestinal epithelial cells (Becker et al., 2013) associated with elevated caspase-3 activity as observed in experimental colitis (Akcan et al., 2008).

Current therapy of IBD relies on the use of sulfasalazine, corticosteroids, and immunosuppressive agents as the mainstay treatment for downregulating aberrant immune response and inflammatory cascades. However, the adverse effects associated with these drugs during prolonged treatment and the high relapse rate limit their use (Yamamoto-Furusho, 2007). Thus, the search for agents that possess pharmacological efficacy with minimal adverse reactions merits attention. Camel's milk (CM) might represent such a potential candidate. CM has been consumed as an essential nutritional supplement with high energy and vitamin content to help immune-deficient patients (Yateem et al., 2008). It has been regarded as superior to other types of milk since it is well tolerated by lactase-deficient children with hypersensitivity to cow milk (El Agamy et al., 2009). CM has unique composition that differs from other ruminants' milk with low content of fat, cholesterol and lactose along with higher minerals including calcium, iron, magnesium, copper and zinc and vitamins as vitamin A, B_2, C and E (Zhang et al., 2005). Besides its high content of secretory IgA and IgM, CM contains several nanoantibodies with marked antibacterial and antiviral activities (El Agamy et al., 1992). It also contains various bioactive proteins with immunomodulatory properties including lysozymes, lactoperoxidase and N-acetyl glucosaminidase (Al-Ayadhi and Elamin, 2013). Several oligosaccharides have been characterized in CM and they are thought to serve as inhibitors for the attachment of pathogenic microorganisms to colonic mucosa and as prebiotics which favor the growth of colonic bifidobacteria (Alhaj et al., 2013; Yateem et al., 2008). CM has displayed distinctive antioxidant properties (Ozdemir and Inanc, 2005; Ozturk et al., 2003). Besides, CM is rich in lactoferrin, a protein with marked antioxidant and anti-inflammatory properties (Haversen et al., 2002; Legrand et al., 2005). CM has been reported to exhibit protective and alleviating properties against diabetes (Agrawal et al., 2011a,b), diabetic nephropathy (Agrawal et al., 2009) and alcohol-induced hepatic injury (Darwish et al., 2012). Thus, we aimed to investigate the potential alleviating effects of CM, a natural agent with antioxidant/anti-inflammatory properties, in TNBS-induced colitis.

In the current study, colon inflammation was assessed by change in body weight, colon weight/length ratio, colon macroscopic damage, area of colonic lesions, histopathological assessment and leukocyte invasion as indicated by myeloperoxidase (MPO) activity. Besides, we investigated the effects of CM on the inflammatory status by measuring the levels of TNF-α and IL-10 cytokines. Additionally, the redox status was monitored by assessing the levels of lipid peroxides, nitric oxide, glutathione and total antioxidant capacity besides caspases-3 activity as an apoptotic marker. To the best of our knowledge, this may be the first report describing the ameliorating effect of CM on TNBS-induced colitis via its anti-inflammatory, anti-oxidant and anti-apoptotic features.

2. Materials and methods

2.1. Animals

Adult Wistar rats (200 ± 20 g), supplied by the animal house of King Fahd medical research center, King Abdul Aziz University, Jeddah, KSA, were kept at controlled environment conditions at a constant temperature (23 ± 2 °C), humidity (60 ± 10%), and a 12/12 h light/dark cycle. Animals were acclimatized for one week before any experimental procedures and were allowed standard rat chow and water *ad libitum*. All procedures relating to animal care and treatments were according to the guidelines of the Research Ethical Committee of College of Pharmacy, Taif University, Taif, Saudi Arabia.

2.2. Chemicals and kits

TNBS was purchased from Sigma–Aldrich (St. Louis, MD, USA). CM was obtained from Al-Turath Al-Saudia Company, Jeddah, KSA. All Other chemicals were of highest purity and analytical grade. Total antioxidant capacity (TAC) kit was purchased from Cayman Chemical Company, Ann arbor, MI, USA. Caspase-3 colorimetric kit and TNF-α and IL-10 ELISA kits were provided by R&D systems incorporation, USA.

2.3. Experimental design and treatment protocol

In the current study, 32 rats were randomly divided into four groups ($n = 8$ per group). Group I (Control gp): received physiological saline rectally. Group II (Control + CM gp): received saline rectally + CM (10 ml/kg) by oral gavage twice per day. Group III (TNBS gp): received rectal TNBS instillation (50 mg/kg). Group IV (TNBS + CM): received TNBS rectally + oral CM (10 ml/kg b.i.d.). The selected dose of CM has been proven to be effective in experimental models of alcohol-induced hepatic injury (Darwish et al., 2012) and cadmium-induced anemia and oxidative stress in RBCs (Dallak, 2009). The administration of CM started 1 week before the induction of TNBS colitis and was continued till the 4th day post TNBS instillation. The animals were euthanized using an overdose of anesthesia on the 5th day of TNBS-induction.

2.4. Induction of colitis

TNBS colitis was induced according to the procedures described by Morris et al. (1989) with modification (Qin et al., 2012). Briefly, animals were fasted for 24 h with free access to water and then anaesthetized with chloral hydrate (300 mg/kg, i.p.). A medial grade polyurethane catheter with 2 mm external diameter was inserted into the anus and the tip was advanced in the descending colon to 8 cm from the anus verge. Rats were kept in a vertical head-down position and TNBS (50 mg/kg) in 50% ethanol was rectally instilled slowly within 1 min and the catheter was kept in place for another min, and then gently removed. TNBS-treated rats were left in the head-down position for 2 min to avoid leakage of the intracolonic instillate and then kept on warm bedding till regain of consciousness. The control group received physiological saline instead of TNBS solution.

2.5. Tissue collection and preparation

On the 5th day post TNBS-instillation, rats were euthanized under deep ether anesthesia and laparatomy was immediately performed. The distal 8 cm portion of the colon was excised, freed of adherent adipose tissue, longitudinally split, washed with saline to remove fecal residues and weighed. The colons were photographed and the digital photos were used for assessment of macroscopic damage and for determination of the area of colonic lesions using imageJ 1.48d software (National Institute of Health, USA). One segment of the colon was fixed in 10% buffered formol saline for the histopathological assessment while the remaining tissue was divided into 2 parts and stored at −20 °C for the determination of biochemical parameters. One part (100 mg) of the colon was homogenized in 10 volumes of lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton × 100, 5 mM DTT, 1 mM pefablock, pH 7.4). The resultant lysate was centrifuged at 10,000×g for 20 min and the supernatant was used for estimation of TNF-α, IL-10 and caspases-3.

The second part of colon was homogenized in 10 volumes of ice-cold double distilled water. Aliquots of the aqueous homogenate were used for the determination of GSH, MDA and NO. A fourth aliquot (1 ml) of the 10% homogenate was centrifuged at 600×g for 10 min at 4 °C. The pellet was homogenized in 1 ml of phosphate buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose) and subsequently centrifuged at 10,000×g for 15 min at 4 °C and the obtained supernatant was used for the estimation of TAC. Finally, 0.5 ml of 10% aqueous homogenate was mixed with equal volume of ice-cold 100 mM potassium phosphate buffer (pH 7.4), and centrifuged at 10,000×g for 15 min at 4 °C and the pellet was homogenized in 50 mM potassium phosphate buffer (pH 6), containing 0.5% hexadecyltrimethylammonium bromide (HETAB) and the resultant homogenate was used for the estimation of MPO activity.

2.6. Measured parameters

2.6.1. Change in body weight and colon weight/length ratio

Individual body weights of animals were recorded at the beginning of the experiments and on the termination day and the difference was calculated as the change in body weight. Weights and lengths of excised colons were listed and the colon weight/length ratio was calculated to indicate colon inflammation (Martin et al., 2006).

2.6.2. Colon macroscopic damage and area of colonic lesions

The severity of colitis was evaluated by an independent observer blind to the identity of treatments according to the criteria of Millar et al. (1996) as follows: 0 = No macroscopic changes; 1 = Mucosal erythema only; 2 = Mild mucosal edema, slight bleeding or small erosions; 3 = Moderate edema, bleeding ulcers or erosions; 4 = Severe ulceration, erosions, edema and tissue necrosis. Besides, the extent of colon injury was indicated by the area of colonic lesions.

2.6.3. Histopathological examination

Full thickness colon biopsy specimens were fixed in 10% buffered formol saline for 24 h. The specimens were washed, dehydrated by alcohol, cleared in xylene and embedded in paraffin at 56 °C in hot air oven for another 24 h. Sections of 5 μm thickness were stained with hematoxylin and eosin (H&E) and examined under the light microscope (Leica Microsystems, Germany). All histopathologic processing and assessment of specimens were performed by an experienced technician/observer blinded to the identity of the sample being examined to avoid any bias.

2.6.4. Biochemical parameters

2.6.4.1. Determination of colon myeloperoxidase (MPO) activity. The MPO activity, a marker for neutrophil infiltration, was estimated according to the method of Krawisz et al. (1984) with modifications. One unit of MPO activity is defined as the amount of enzyme converting 1 μmol of H_2O_2 to water in 1 min at 25 °C. The 10% homogenate in potassium phosphate buffer (pH 6), containing HETAB was subjected to 3 cycles of freezing/thawing, 30 s of sonication and centrifuged at 20,000×*g* for 20 min at 4 °C. O-dianisidine hydrochloride (0.167%) and H_2O_2 (0.0005%) in potassium phosphate buffer (50 mmol/L, pH 6) were added to the supernatant and the absorbance rate was monitored at 460 nm for 4 min using Optima SP-3000 spectrophotometer, Japan.

2.6.4.2. Estimation of inflammatory cytokines (TNF-α and IL-10). The levels of TNF-α and IL-10 in colon homogenate supernatants were measured using ELISA kits (R&D systems incorporation, USA). All the procedures were performed according to the manufacturer's instructions. The assays of these cytokines employ the quantitative sandwich enzyme immunoassay technique. Specific antibodies are pre-coated onto the microplate. The standards, control, and samples were pipetted into the wells and the rat cytokines were bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked secondary antibody specific for rat TNF-α and IL-10 were added to the wells. Following color development, the assay was stopped, and the absorbance was read at 450 nm using BioTek ELx800 microplate reader. The intensity of the color was proportional to the amount of the corresponding cytokine bound in the initial step.

2.6.4.3. Determination of caspase-3 activity. Caspase-3 activity was colorimetrically assayed using R&D systems kit according to the manufacturer's instructions based on the method of Fernandes-Alnemri et al. (1994). Briefly, an aliquot of the homogenate supernatant (containing approximately 50–200 μg protein) was incubated with the labeled substrate DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide). The cleavage of the peptide by the caspase releases the chromophore pNA, which was read at 405 nm using BioTek ELx800 microplate reader.

2.6.4.4. Estimation of lipid peroxides. The determination of the levels of lipid peroxides, expressed as malondialdehyde (MDA), was carried out according to the thiobarbituric acid assay of Buege and Aust (1978). One part of the sample was mixed with double volume of a reagent consisting of 15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid and 0.25 N HCl. The mixture was heated at 90 °C in a water bath for 30 min. After cooling, the precipitate was removed by centrifugation at 1000×*g* for 10 min. The absorbance of the clear supernatant was recorded at 535 nm against a blank prepared by using bidistilled water instead of the sample using Optima SP-3000 spectrophotometer (Japan).

2.6.4.5. Nitric oxide (NO) estimation. Total NO was determined by measuring its stable metabolites, particularly, nitrite (NO_2^-) and nitrate (NO_3^-) based on the method of Miranda et al. (2001) with the modification of replacing zinc sulfate instead of ethanol for the precipitation of proteins in the supernatant of colon homogenates. Colonic NO was extracted through centrifugation of 1 ml of 10% aqueous homogenate at 21,000×*g* for 15 min. To an aliquot of the resultant supernatant, vanadium trichloride (0.8% in 1 M HCl) was added for reduction of nitrate to nitrite, followed by rapid addition of Griess reagent consisting of N-(1-naphthyl) ethylenediamine dihydrochloride (0.1%) and sulfanilamide (2% in 5% HCl). The mixture was incubated for 30 min at 37 °C, allowed to cool and then the absorbance was measured at 540 nm (Optima SP-3000 spectrophotometer, Japan).

2.6.4.6. Reduced glutathione (GSH) estimation. Colon reduced glutathione level was determined as previously described by Beutler et al. (1963), where colon homogenates were deproteinated with 10% trichloroacetic acid for 30 min at 4 °C, and then centrifuged at 10,000×*g* for 15 min at 4 °C. An aliquot of the supernatant was diluted with 0.3 M phosphate solution and 10 mM DTNB (5,5′-dithiobis 2-nitrobenzoic acid) was added. The colored product optical density was read at 412 nm.

2.6.4.7. Determination of total antioxidant capacity (TAC). The TAC was determined using Cayman total antioxidant assay kit according to the manufacturer's instructions. The assay relies on the ability of antioxidants in the supernatants of colon homogenates to inhibit the oxidation of ABTS (2,2-azino-di-[3-ethylbenzthiazoline sulfonate]) by metmyoglobin. The amount of the oxidized product was estimated by reading absorbance at 405 nm. The capacity of the antioxidants in the sample to prevent ABTS oxidation was compared to that of Trolox, a water-soluble tocopherol analogue and was quantified as μmol of Trolox equivalent/g tissue.

2.7. Statistical analysis

The parametric data were expressed as mean ± SEM, and statistical comparisons were carried out using one-way analysis of variance (ANOVA). When differences were significant, Tukey–Kramer post hoc test was used for multiple comparisons between groups. The non-parametric values were expressed as median (maximum – minimum) and the statistical differences among groups were identified using Kruskal–Wallis analysis of variance followed by the rank-based Mann–Whitney *U*-test for the group comparisons. Statistical analysis was performed using SPSS program, version 17. The minimal level of significance was identified at $p < 0.05$.

3. Results

3.1. Camel's milk alleviates the severity of TNBS-induced colitis in rats

To investigate the potential of CM to alleviate colon inflammation, we assessed its efficacy in TNBS-induced colitis in rats as an experimental model of IBD. Five days post intrarectal instillation of TNBS, rats suffered a significant loss of body weight (Table 1) along with elevation of the colon weight/length ratio, an indicator of the ongoing colon inflammation. The animals also suffered a marked colonic mucosal damage, with edema, deep ulcerations and hemorrhage. The severity of the lesions in the distal colon were quantified using a macroscopic damage score (range 0–4 as indicated in Materials and Methods) whereas the extent of damage was indicated by the area of colonic lesions. Oral administration of CM effectively inhibited the loss of body weight and attenuated the

Table 1

Effect of Camel's milk administration on change in body weight, colon weight/length ratio, colon macroscopic damage and area of colonic lesions in rats with TNBS-induced colitis.

Groups	Change in body weight (g)[a]	Colon weight/length ratio (mg/cm)	Colon macroscopic damage (score 0–4)	Area of colonic lesions (cm^2)
Control	9.08 ± 0.22	101.6 ± 2.32	0 (0–0)	0 ± 0
Control + CM	9.51 ± 1.38	98.2 ± 3.62	0 (0–0)	0 ± 0
TNBS	−19.18 ± 3.30*	279.4 ± 8.01*	4 (3–4)*	5.34 ± 0.34*
TNBS + CM	2.78 ± 1.02#	183.8 ± 5.23*,#	2 (1–2)*,#	2.59 ± 0.15*,#

Colon injury was induced by a single intrarectal instillation of TNBS (50 mg/kg) in 50% ethanol solution. The control group received saline solution rectally. CM (10 ml/kg) was orally administered twice daily, starting 1 week before TNBS instillation and was continued till day 4. On the 5th day post TNBS instillation, rats were euthanized and colons were immediately excised. Values of change in body weight, colon weight/length ratio and area of colonic lesions (parametric data) are expressed as mean ± SEM ($n = 8$) while macroscopic damage scores (non-parametric) are expressed as median (minimum – maximum), ($n = 8$). CM; Camel's milk, TNBS; tri-nitrobenzene sulfonic acid.

* Significant difference from control gp at $p < 0.05$.

\# Significant difference from TNBS colitis gp at $p < 0.05$.

[a] Change in body weight = final body weight – initial body weight.

colon weight/length ratio along with the severity and the extent of colonic injury as compared to rats with TNBS colitis. It is worthy to mention that throughout our experiments, there were no significant differences between control rats receiving CM (group II) and control rats (group I).

3.2. Camel's milk attenuates colonic histopathological alterations and leukocyte invasion

The histopathological assessment of sham-treated control group and control group receiving CM indicated a typical normal architecture of the colon tissues (Fig. 1A and B). On the other hand, microscopic examination of the colons on the 5th day post TNBS instillation revealed focal necrosis of the mucosa with ulceration and diffuse immuno-inflammatory cell infiltration (Fig. 1C). These morphological signs of cell damage and inflammatory changes were attenuated by CM as evidenced by mitigated inflammatory cell influx and intact epithelium in the colon mucosa (Fig. 1D) suggesting the commencement of re-epithalization in CM-treated colitic rats.

In colitis, the massive leukocyte infiltration was further evidenced by a 7-fold increase of colonic MPO activity, a biochemical marker for neutrophil invasion, as compared to control group (Fig. 2). Consistent with the attenuation of histopathological alterations, CM intake suppressed the MPO activity by 47% as compared to TNBS-induced colitis group.

3.3. Camel's milk diminishes colonic inflammatory cytokine levels

To gain an insight into the effect of CM on the inflammatory status of animals with TNBS colitis, we assessed the levels of inflammatory cytokines such as TNF-α and IL-10. There was a remarkable increase in the level of TNF-α (400%) and IL-10 (360%), as compared to the control group (Fig. 3). Administration of CM effectively lowered the content of TNF-α and IL-10 by 53% and 55%, respectively, as compared to TNBS colitis group.

3.4. Camel's milk ameliorates the oxidative stress and enhances the anti-oxidant defenses

In IBD, the process of colon inflammation and oxidative stress are tightly linked (Kretzmann et al., 2008). Thus, we investigated the oxidative stress provoked by TNBS instillation by measuring the levels of lipid peroxides expressed as MDA and NO in colon tissues of animals with colitis. As shown in Fig. 4, TNBS instillation afforded 3.1 and 2.4-fold increases in the content of MDA and NO, respectively, as compared to control group. Meanwhile, the levels of antioxidant defenses such as GSH and TAC were decreased by 77% and 46%, respectively, as compared to control group (Fig. 5). CM effectively counteracted the above changes as evidenced by a decrease of MDA and NO by 50% and 42%, respectively, in addition to normalization of GSH and TAC as compared to TNBS colitis group. Besides its involvement in leukocyte invasion, MPO activity contributes to the colonic oxidative stress through generation of hypochlorous acid (Eiserich et al., 1998). As described in Fig. 2, TNBS provoked a marked increase in MPO activity, which was effectively inhibited by CM administration. Together, these effects indicated the efficacy of CM in mitigation of oxidative stress and boosting the antioxidant defenses in IBD.

3.5. Camel's milk inhibits caspases-3 activity

We also assessed the activity of caspase-3 as a marker for colonic apoptosis following the instillation of TNBS. Tissues from inflamed colons displayed a 2.5-fold increase of caspase-3 activity as compared to control group suggesting a marked activation of apoptosis in colon cells subjected to TNBS (Fig. 6). CM inhibited the activity of caspase-3 activity by 46% as compared to TNBS

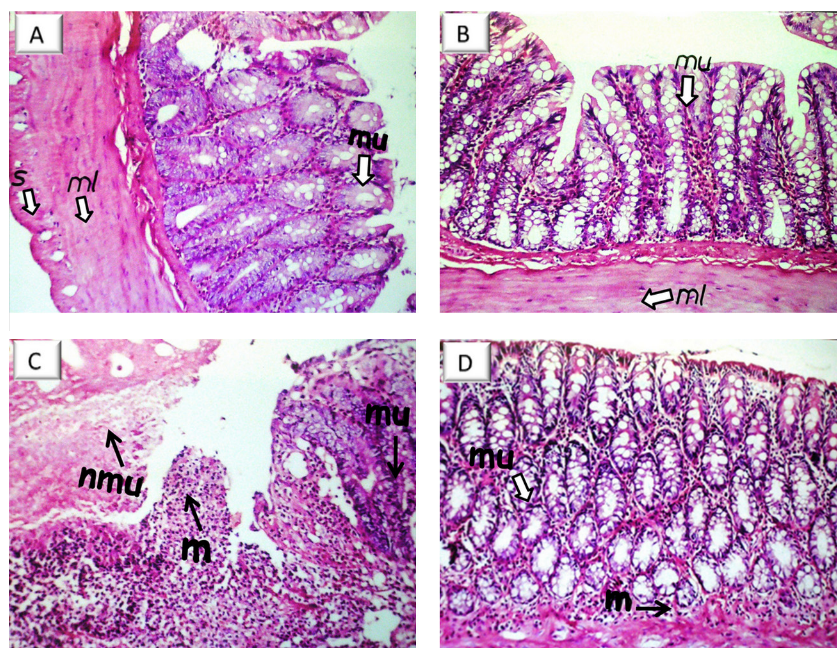

Fig. 1. Camel's milk mitigates the histopathological damage in colon tissues of rats subjected to TNBS colitis. Representative photomicrographs of sections from colonic samples taken on the 5th day post TNBS-rectal instillation. (A) Control group receiving saline rectally displayed normal intact mucosa (mu), muscularis (ml) and outer serosa (s) (indicated by white arrow). (B) Control group receiving saline rectally + oral CM (10 ml/kg b.i.d) exhibit no histological modifications. (C) TNBS-treated group (50 mg/kg) was characterized by focal necrosis of the mucosa (nmu) with ulceration and massive diffuse infiltration of inflammatory cells (m). (D) TNBS + CM (10 ml/kg b.i.d.) revealed mucosal preservation and attenuated inflammatory cell invasion (m). Hematoxylin and eosin staining, original magnification: 40×.

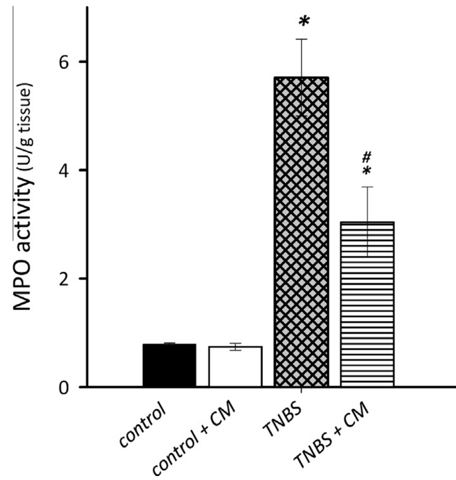

Fig. 2. Inhibitory effect of Camel's milk on colon myeloperoxidase activity in TNBS colitis. Colitis was induced by rectal instillation of TNBS (50 mg/kg) and CM was administered at 10 ml/kg b.i.d by oral gavage. The colon tissues were harvested on the 5th day post TNBS instillation. Values represent mean ± SEM ($n = 8$). *Significant difference from control gp at $p < 0.05$. #Significant difference from TNBS colitis gp at $p < 0.05$. CM; Camel's milk, TNBS; tri-nitrobenzene sulfonic acid.

colitis group, implying a role of CM for combating apoptosis and consequent loss of colon cells in IBD.

4. Discussion

Throughout centuries, CM has been regarded as an important dietary supplement consumed as fresh or curdled for maintaining good health and for the management of various diseases (Yateem et al., 2008). The current study may be the first to demonstrate the beneficial effects of CM in TNBS-induced colitis as an experimental model of human IBD. This was evidenced by effective amelioration of the severity and extent of colon injury along with attenuation of the histopathological alterations provoked by TNBS. The promising alleviating effects of CM are likely mediated via abrogation of leukocyte migration, attenuation of TNF-α and IL-10 cytokines. The mitigation of CM to colonic injury was accompanied with suppression of oxidative stress via inhibition of elevated lipid peroxides and NO and restoration of GSH and TAC in colon tissues besides inhibition of colon cell apoptosis as indicated by caspase-3 activity. Overall, the observed protective actions of CM can be ascribed to its antioxidant and anti-inflammatory features. In addition, the nutritional value of CM cannot be ignored.

TNBS-induced colitis resembles most features of human IBD with respect to several histological alterations including mucosal invasion of polymorphonuclear cells with excessive generation of inflammatory mediators that inflict injury to colon tissues (Neurath et al., 2000). A well characterized marker for neutrophil infiltration is the MPO enzyme which is stored in azurophilic granules and is subsequently released upon neutrophil activation and degranulation (Eiserich et al., 1998).

In the current study, oral administration of CM effectively abrogated leukocyte invasion to inflamed colon following TNBS instillation as revealed by the histopathological assessment. This observation was further corroborated by the inhibition of MPO by CM in rats with TNBS-induced colitis. These observations are in agreement with a previous study with similar findings in ethanol-induced hepatic injury (Darwish et al., 2012). The mitigation of leukocyte influx may account for the beneficial effects of CM against colon injury, most likely through the observed inhibition of TNF-α and oxidative stress by CM since TNF-α and ROS trigger leukocyte recruitment mainly via increasing the expression of P-selectin, ICAM and MAdCAM-1 adhesion molecules in colon mucosa in IBD (Martin et al., 2006; Tanida et al., 2011).

Our data described a marked upregulation of the inflammatory status with increases in the levels of the proinflammatory TNF-α in rats with TNBS-induced colitis. These findings are consistent with previous literature (Kretzmann et al., 2008; Martin et al., 2006; Witaicenis et al., 2012). TNF-α is a pleiotropic cytokine which is involved in many aspects of inflammation relevant to the pathogenesis of IBD, including expression of adhesion molecules, leukocyte recruitment, enhanced intestinal permeability, activation of immune cells with subsequent release of other proinflammatory cytokines e.g., IL-1β and IL-6 along with initiation of cytotoxic, apoptotic and acute phase responses. Meanwhile, our data revealed increased colonic levels of IL-10, a cytokine with anti-inflammatory features, on the 5th day post TNBS instillation. While previous literature has described diminished IL-10 levels in experimental colitis (Borrelli et al., 2013), the upregulation of IL-10 has been reported in several studies including patients with IBD (Kucharzik et al., 1995) and rats with TNBS- and dextran sulfate-induced colitis (Barada et al., 2007; Tomoyose et al., 1998). A temporal description of the increase of colonic IL-10 in TNBS-treated rats has been highlighted by Barada et al. (2007). A significant

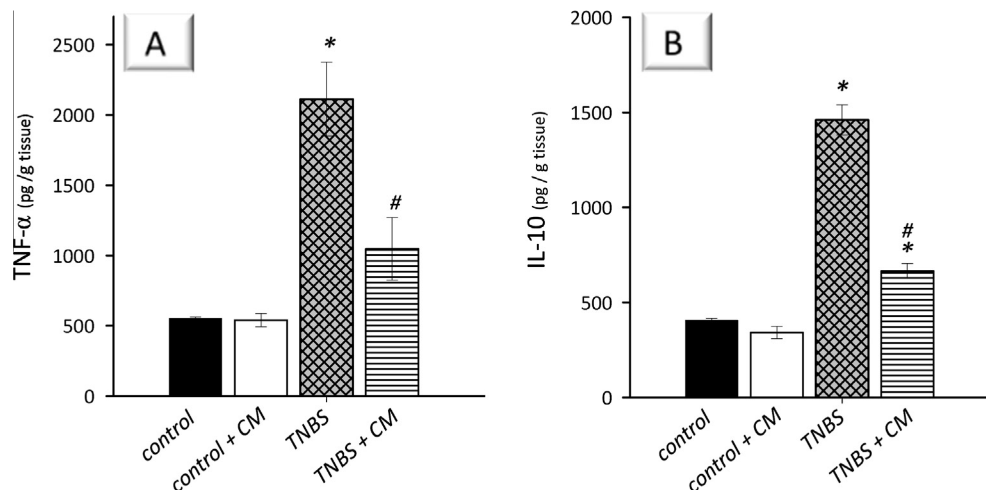

Fig. 3. Camel's milk lowers the inflammatory cytokine levels in the colon of rats subjected to TNBS colitis. (A) Levels of tumor necrosis factor-α (TNF-α). (B) Levels of interleukin-10 (IL-10). The induction of colon injury and treatment protocols were described in Table 1. Values represent mean ± SEM ($n = 8$). *Significant difference from control gp at $p < 0.05$. #Significant difference from TNBS colitis gp at $p < 0.05$. CM; Camel's milk, TNBS; tri-nitrobenzene sulfonic acid.

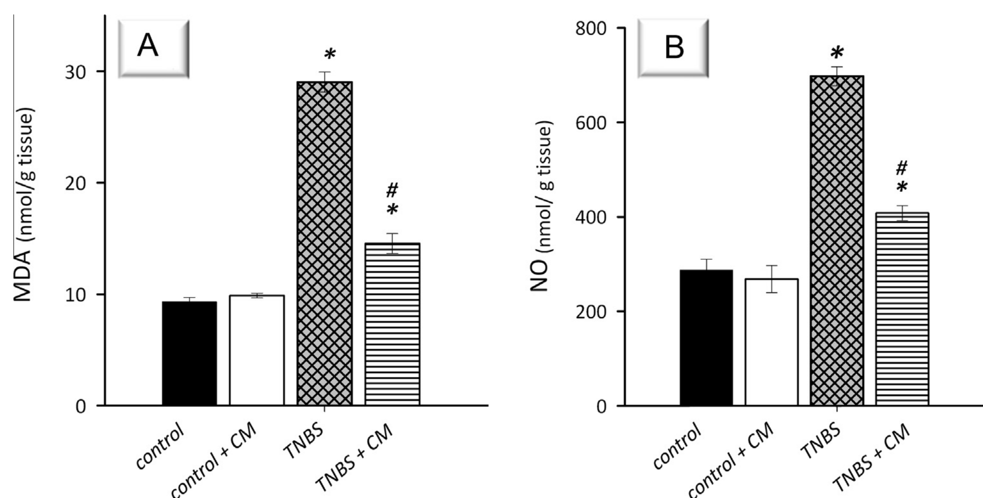

Fig. 4. Camel's milk alleviates colon oxidative stress in rats subjected to TNBS-induced colitis. (A) Levels of lipid peroxides expressed as malondialdehyde (MDA). (B) Levels of nitric oxide (NO). The induction of colon injury and treatment protocols were described in Table 1. Values represent mean ± SEM ($n = 8$). *Significant difference from control gp at $p < 0.05$. #Significant difference from TNBS colitis gp at $p < 0.05$. CM; Camel's milk, TNBS; tri-nitrobenzene sulfonic acid.

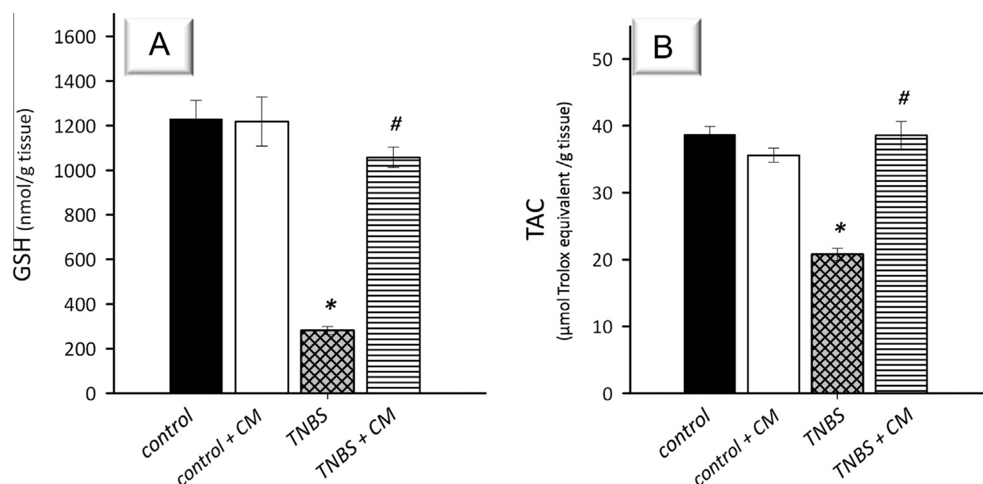

Fig. 5. Camel's milk enhances the antioxidant defenses in the colon of rats with TNBS colitis. (A) Levels of reduced glutathione (GSH). (B) Levels of the total antioxidant capacity (TAC). The induction of colon injury and treatment protocols were described in Table 1. Values represent mean ± SEM ($n = 8$). *Significant difference from control gp at $p < 0.05$. #Significant difference from TNBS colitis gp at $p < 0.05$. CM; Camel's milk, TNBS; tri-nitrobenzene sulfonic acid.

elevation of IL-10 started as early as 3 h post colitis induction, peaked at 1 week (6-fold increase) and persisted till 3 weeks post TNBS administration with a 4.5-fold elevation of IL-10. The elevation of colonic IL-10 can be regarded as a compensatory mechanism against the colonic injury and is thought to play a role in curbing mucosal inflammation and preventing it from becoming uncontrolled since IL-10 downregulates MHC class II antigen presentation and subsequent release of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (Schreiber et al., 1995). However, such upregulation of IL-10 might not be sufficient to fully control the ongoing intestinal inflammation (Autschbach et al., 1998).

Feeding with CM decreased the levels of TNF-α along with IL-10 in the colons of animals with TNBS colitis. These results imply that CM partly exerted its beneficial effects on colon inflammation by lowering the colonic content of proinflammatory cytokines such as TNF-α. In fact, CM has been reported to suppress inflammation and elevated levels of TNF-α in ethanol-induced hepatic injury (Darwish et al., 2012). Likewise, lactoferrin, an anti-inflammatory protein component of CM, has been reported to inhibit the production of proinflammatory cytokines e.g., TNF-α, IL-1 and IL-6 in mononuclear cells *in vitro* and *in vivo*, in response to lipopolysaccharide activation. Mechanistically, the inhibition of these pro-inflammatory cytokines production could result from inhibition of NF-κB activation following internalization of lactoferrin into monocytes (Haversen et al., 2002). The restoration of IL-10 in colon tissues probably reflects mitigation of colon inflammation which was evidenced by suppression of neutrophil infiltration, MPO activity and TNF-α surge. Virtually, the observed CM lowering of TNF-α may be implicated in the restoration of colonic IL-10 levels since the release of IL-10 is triggered by increased levels of pro-inflammatory cytokines (Schreiber et al., 1995).

Following the disruption of intestinal epithelial barrier, immune cells are exposed to bacterial products which trigger a cascade of events leading to mucosal immune activation and robust inflammatory responses. As a result, various cytokines are generated which recruit neutrophils and macrophages to the mucosa and create self-perpetuating inflammatory responses (Ohman and Simren, 2010; Salim and Soderholm, 2011). Activation of inflammatory cells provokes oxidative stress via release of ROS and RNS which inflict intestinal injury (Fiocchi, 1998). Conversely, oxidative stress and redox signaling are implicated in the upregulation of inflammatory cytokines and recruitment of inflammatory cells through various signaling pathways including NF-κB. Thus, inflammation and oxidative stress are regarded as two entangled molecular

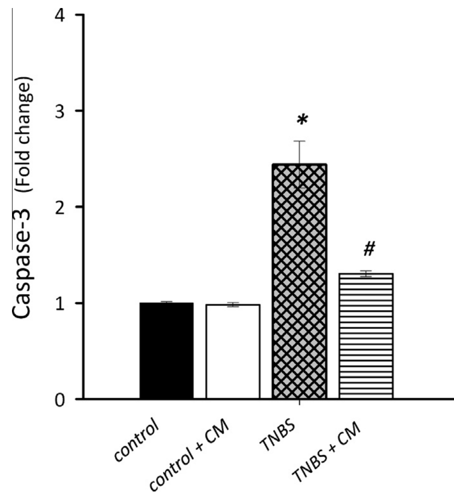

Fig. 6. Camel's milk inhibits caspases-3 activity in the colon of rats subjected to TNBS colitis. The induction of colon injury and treatment protocols were described in Table 1. Values represent mean ± SEM ($n = 8$). *Significant difference from control gp at $p < 0.05$, #Significant difference from TNBS colitis gp at $p < 0.05$. CM; Camel's milk, TNBS; tri-nitrobenzene sulfonic acid.

pathways that contribute to IBD pathogenesis (Zhu and Li, 2012). In the current study, enhanced oxidative stress was verified by marked increases in lipid peroxides, NO and MPO activity besides decreases of GSH and TAC in TNBS-induced colitis. These observations are in agreement with previous studies (Huang et al., 2011; Southey et al., 1997; Witaicenis et al., 2012; Zhou et al., 2006). Oxidative stress and its consequent lipid peroxidation could exacerbate free radical chain reactions and activate the release of proinflammatory mediators (Huang et al., 2011; Witaicenis et al., 2012). Besides, sustained high NO production in the colon, mainly generated by iNOS, plays a central role in the pathogenesis of IBD and experimental colitis (Cross and Wilson, 2003). Nitric oxide in conjunction with superoxide anion generates significant amounts of peroxynitrite anion, a potent oxidizing agent, which inflicts cellular injury and necrosis probably via mechanisms including DNA fragmentation and lipid oxidation (Valko et al., 2007). The MPO enzyme plays a central role in colonic oxidative stress via generation of a surplus of hypochlorous acid, a potent cytotoxic oxidant that contributes to the clinical features of IBD (Eiserich et al., 1998). On the other hand, evidence has indicated that colonic GSH levels are lowered both in human and experimental colitis (Giris et al., 2007). In fact, GSH is regarded as a primary defense line which combats oxidative stress and inflammatory cascades (Zhu and Li, 2012). Thus, the observed decrease in colonic GSH level is due to its consumption during detoxification of peroxides e.g., H_2O_2 via the action of glutathione peroxidase (Yao et al., 2010). Additionally, TAC depicts the synergy among different antioxidants, thus providing an insight into the delicate *in vivo* balance between oxidants and non enzymatic antioxidants (Ghiselli et al., 2000).

In the current study, CM effectively enhanced the antioxidant status in animals with TNBS-induced colitis as evidenced by reduction of MDA and NO levels and MPO activity together with reinstatement of GSH and TAC. These findings are in agreement with previous studies (Darwish et al., 2012; Salami et al., 2011) and they reinforce the premise that the antioxidant properties of CM are implicated in the alleviation of TNBS-induced colitis. There is mounting evidence that CM is rich in vitamins A, B_2, C and E (Zhang et al., 2005) proven to prevent toxicant-induced tissue injury (Yousef, 2004). CM also contains a high concentration of zinc, selenium and other trace elements that further contribute to the antioxidant activity of CM (Zhang et al., 2005). Zinc has been reported to prevent lipid peroxidation in various rat tissues via boosting of the anti-oxidant system (Ozdemir and Inanc, 2005) whereas selenium, an essential component of selenioproteins and glutathione peroxidase, is responsible for ridding the cells of peroxide radicals (Cabrera et al., 2003). Among ruminants' milk, CM has been shown to possess the greatest level of lactoferrin, a protein with remarkable anti-inflammatory and antioxidant features, as compared to other types of milk. A major anti-oxidant activity of lactoferrin is linked to the scavenging of free iron, which accumulates in inflamed tissues and catalyzes the production of tissue-toxic hydroxyl radicals (Legrand et al., 2005). Equally important, chymotrypsin-induced digestion of camel milk β-casein generates peptides with high antioxidant activity (Salami et al., 2011). These antioxidant features of CM could explain its efficacy in lowering lipid peroxides besides enhancing GSH and TAC levels as a result of boosted antioxidant status in rats with TNBS colitis. CM has also been reported to preserve the endogenous antioxidant defenses which are consumed when tissues or cells are subjected to oxidative stress (Darwish et al., 2012). On the other hand, the observed decrease of NO levels in response to CM could be due to the lowering effect of CM on TNF-α, since TNF-α upregulates the expression of iNOS, the major source of NO generation during inflammation (Cross and Wilson, 2003).

Our results also described an increased activity of caspase-3, an apoptotic marker, in colon of animals with TNBS colitis. This observation is consistent with previous reports (Akcan et al., 2008; Crespo et al., 2012). In fact, considerable elevation in the frequency of apoptosis and subsequent loss of colon epithelial cells has been described is patients with IBD (Becker et al., 2013). While fourteen caspases have been implicated in the apoptotic pathway cascade, caspase-3 is considered to be a major execution protease (Nicholson, 1999). The administration of CM inhibited the activity of caspase-3 in colon tissues of colitic animals. This finding is consistent with a previous work that highlighted the inhibition of caspase-3 surge by CM in ethanol-induced hepatic injury (Darwish et al., 2012). The observed inhibition of caspases-3 could be attributed to the alleviation of CM to colonic oxidative stress since excessive exposure of intestinal mucosa to oxidative stress under inflammatory stimuli might enhance epithelial apoptosis (Kruidenier et al., 2003). Besides, the lowering of TNF-α levels by CM also contributes to the prevention of colon apoptosis since high levels of TNF-α trigger epithelial cell apoptosis in the gut, possibly, through caspase-8 activation, resulting in oxidative stress and loss of membrane permeability (Ruemmele et al., 2002; Salim and Soderholm, 2011).

5. Conclusions

Taken together, the current study provides evidences for the promising ameliorating effects of CM on TNBS-induced colitis that could be mediated through mitigation of leukocyte influx to colonic tissues, suppression of inflammatory cytokines and colon apoptosis together with the alleviation of oxidative stress. Thus, our data represent a valid rationale for the use of CM as a complementary approach, with lower incidence of side effects, during the management of IBD. In fact, further studies are warranted to elucidate the exact mechanisms of CM actions in TNBS colitis including the implicated signaling pathways. In addition, the efficacy of CM in clinical trials needs to be established.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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