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Comparative efficacies of 2 cysteine prodrugs and a glutathione delivery agent in a colitis model

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Abstract

Oxidant-mediated injury plays an important role in the pathophysiology of inflammatory bowel disease (IBD). Recently, antioxidants were shown to modulate colitis in mice. In this study, the protective effects of L-cysteine and glutathione (GSH) prodrugs are further evaluated against progression of colitis in a murine model. ICR mice were fed compounds incorporated into chow as follows: Group (A) received chow supplemented with vehicle. Group (B) was provided 2-(RS)-n-propylthiazolidine-4(R)-carboxylic-acid (PTCA), a cysteine prodrug. Group (C) received D-ribose-L-cysteine (RibCys), another cysteine prodrug that releases L-cysteine. Group (D) was fed L-cysteine-glutathione mixed sulfide (CySSG), a ubiquitous GSH derivative present in mammalian cells. After 3 days, the animals were further provided with normal drinking water or water supplemented with dextran sodium sulfate (DSS). Mice administered DSS developed severe colitis and suffered weight loss. Colonic lesions significantly improved in animals treated with PTCA and RibCys and, to a lesser extent, with CySSG therapy. Hepatic GSH levels were depleted in colitis animals (control vs DSS, $P < 0.001$), and normalized with prodrug therapies (control vs treatments, $P > 0.05$). Protein expressions of serum amyloid A and inflammatory cytokines [interleukin (IL)-6, IL-12, tumor necrosis factor- α (TNF- α), osteopontin (OPN)] were significantly increased in colitis animals and improved with therapies. Immunohistochemistry and Western blot analyses showed significant upregulation of the macrophage-specific markers, COX-2 and CD68, which suggests macrophage activation and infiltration in the colonic lamina propria in colitis animals. These abnormalities were attenuated in prodrug-treated mice. In conclusion, these data strongly support the novel action of the PTCA against colitis, which further supports a possible therapeutic application for IBD patients.

Inflamed gut from ulcerative colitis and Crohn's disease patients is rich in activated macrophages and neutrophils. These inflammatory cells generate excess amounts of reactive oxygen species (ROS) with subsequent increased oxidative stress.¹ The increased generation of highly toxic ROS exceeds the limited intestinal antioxidant defense system, thereby contributing to intestinal oxidative injury in ulcerative colitis patients.² Excessive production of ROS has been demonstrated in circulating phagocytic and polymorphonuclear leukocytes cells in inflammatory bowel disease (IBD) patients, using a chemiluminescence assay³ and after stimulation with the bacterial chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine.⁴ Thus, oxidant-mediated injury plays an important role in the pathophysiology of IBD.

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The endogenous tripeptide, glutathione (GSH), is the most important intracellular defense agent in mammalian organs, including the gut. GSH is involved in protein folding and protein synthesis as well as in intracellular signaling.⁵ GSH may be depleted during inflammatory insults, and GSH-deficient mice show severe degradation of the jejunum and colonic mucosa.⁶ One strategy for preventing this injury is to boost the levels of GSH within the cells. However, GSH is not taken up directly by most cell phenotypes and must first be broken down into its component amino acids (cysteine, glutamate, and glycine) by enzymes including δ -glutamyltranspeptidase.⁷ Administration of N-acetyl-cysteine in the trinitrobenzene sulfonic acid rat model attenuated this chemically induced colitis,⁸ which suggests that GSH precursors may be beneficial in the acute relapse of IBD. Dextran sodium sulfate (DSS) treatment of mice mimics IBD in that it provokes inflammation and macrophage activation, with subsequent loss of epithelial integrity, and increases luminal Gram-negative flora. Recently, antioxidants, namely 2(RS)-n-propylthiazolidine-4(R)-carboxylic acid (PTCA), a cysteine prodrug that stimulates GSH biosynthesis,⁹ were shown to modulate DSS-induced colitis in BALB/c mice.¹⁰ In this study, the protective effects of PTCA were further evaluated and compared with another L-cysteine prodrug and a GSH delivery agent against progression of colitis induced by DSS in an Institute for Cancer Research (ICR) mouse model.

MATERIALS AND METHODS

The antioxidants were synthesized in the HTN laboratory. PTCA is an L-cysteine prodrug with a masked sulfhydryl group in the form of a thiazolidine-4-carboxylic acid to stabilize it against air oxidation. L-cysteine-glutathione mixed sulfide (CySSG) is a ubiquitous GSH derivative present in mammalian cells and suggested to be a depot form of GSH.¹¹ CySSG, a glutathione delivery agent,¹² can liberate both GSH and L-cysteine by a glutathione reductase-catalyzed reaction with GSH.¹³ D-Ribose-L-cysteine (RibCys) is another cysteine prodrug that, like PTCA, can release L-cysteine *in vivo* by nonenzymatic, hydrolytic dissociation.¹⁴

Animals

Male IRC mice of about 6 weeks of age, purchased from Sprague-Dawley (Indianapolis, Ind), were housed in microfilter-top cages at the VA Medical Center Animal Research Facility at Lexington, Kentucky. They were placed in a room maintained at 22°C with a 12:12-h light:dark cycle and fed rodent chow and water *ad libitum*. This experimental study was approved and performed in accordance with the guidelines for Institutional Animal Care and Use Committee, the Veterans Administration Medical Center, and the University of Kentucky Research Resource Facility, Lexington, Kentucky, which were both certified by the American Association of Accreditation of Laboratory Animal Care. This study conforms to the relevant ethical guidelines for human and animal research.

Experimental procedures

After an acclimatization period, the mice were divided into 4 groups, and each group was provided with a different L-cysteine/GSH compound or vehicle (sucrose) incorporated (1% w/w) into rodent chow as follows:

Group (A) control was given chow supplemented with the vehicle (Sucrose). Group (B) was provided PTCA, a cysteine prodrug that stimulates GSH biosynthesis. Group (C) was provided RibCys, which is another cysteine prodrug that releases L-cysteine *in vivo*. Group (D) received CySSG, which is a GSH derivative present in mammalian cells. All animals remained on the assigned diets until they were euthanized on day 10.

Colitis induction

Three days after initiation of the experiment, the animals were further divided into 2 subgroups and given drinking water (normal controls) or water containing 3.5% DSS (ICN Biochemical, Aurora, Oh). The animals given DSS will develop a severe colitis after a period of 1 week. DSS-induced colitis is a well-accepted intestinal inflammation model and provokes colonic inflammation and macrophage activation with subsequent loss of epithelial integrity.^{10,15,16} Mice were randomized into subgroups (9/group) as follows:

Normal, water treated:

Control ICR mice with sucrose.

Control ICR mice treated with PTCA.

Control ICR mice treated with RibCys.

Control ICR mice treated with CySSG.

DSS treated:

DSS-treated ICR mice.

DSS-treated ICR mice with PTCA.

DSS-treated ICR mice with RibCys.

DSS-treated ICR mice with CySSG.

Evaluation of colitis

The progression of colitis was evaluated over the period of 1 week on the DSS treatment. Parameters monitored daily were body weight, physical appearance, food consumption, consistency of feces, diarrhea, and presence of gross blood in stool.

Tissue collection and histology

On day 10 after initiation of the experiment, the animals were euthanized by Halothane inhalation. A portion of the liver, spleen, heart, and small and large intestine were excised and processed for histopathologic analysis. The intestines were removed and perfused with phosphate-buffered saline (PBS), pH 7.4. A small cuff of the proximal and distal colon (within 1 cm from the rectum) was cut and fixed in 10% buffered formalin in PBS (Sigma Chemical Co., St. Louis, Mo). Tissues from the liver and pancreas were also subjected to the same procedure. The rest of the large intestine and a portion of the small intestine (ileum) were dissected, perfused with PBS, then flash-frozen in liquid nitrogen and stored at -80°C for further analysis of tissue antioxidants.

The fixed sections in formalin were stained with hematoxylin and eosin and evaluated by light microscopy for the presence of lesions. The severity of colitis was assessed by histologic grading score of the colon. The scores were based on histologic features with a numeric value (0 to 4) assigned to the specimen based on the following criteria:

Grade 0: No detectable lesions, no inflammatory cells, mucosa appears normal.

Grade 1: Few focal inflammatory infiltrate in the mucosa.

Grade 2: Mild multifocal inflammation with moderate expansion of the mucosa; crypt epithelium appears normal.

Grade 3: Moderate multifocal inflammation with moderate expansion of the mucosa, mild crypt epithelium disruption.

Grade 4: Severe diffuse inflammation with crypt epithelium disruption and ulceration.

Immunohistochemistry assay

To detect cyclooxygenase (COX)-1, COX-2, and macrosialin (CD68) expression in the colonic tissue, paraffin-embedded sections were cut and sequentially deparaffinized with hexane. The slides were rehydrated in alcohol baths, washed in PBS, microwaved in High pH Antigen Retrieval Solution (DAKOcytation, Carpinteria, Calif) for 5 min, washed with PBS, incubated in 0.3% H₂O₂-methanol for 10 min, and then rinsed with distilled water. The slides were then incubated at room temperature for 1.5 h in a blocking solution consisting of normal horse serum, rinsed with PBS, and incubated with primary antibody at 4°C overnight. Rabbit polyclonal IgG against CD68 was obtained from Zymed/Invitrogen (Catalog no. 1076154; San Francisco, Calif), and mouse monoclonal IgG1 against COX-2 was obtained from Cayman Chemical Company (Ann Arbor, Mich). The “Elite ABC” kit from Vector (Burlingame, Calif) was used with biotinylated secondary antibodies and biotin-conjugated horseradish peroxidase, and it was developed with a 3-3'-diaminobenzidine solution per the manufacturer's instructions. The slides were subsequently counter stained with Methyl Green (Dako).

COX-1, COX-2, and CD68 Western blot analysis

A portion of colon was homogenized, and the protein was solubilized in radioimmunoprecipitation buffer [1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 100-μg/mL phenylmethylsulfonyl fluoride, and 30-μL/mL aprotinin (Sigma Chemical Co.)]. Total soluble protein (35 μg) was resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to a 0.2-μmol/L pore-size polyvinylidene fluoride membrane (Schleicher and Schuell, Middlesex, UK), and detected with antiserum specific for COX-2 (Catalog No. 160109; Cayman Chemical Company) or COX-1 (Catalog No. 160106; Cayman Chemical Company) and CD68 with the use of an chemiluminescent detection system (ECL; Amersham Biosciences, Pittsburgh, Penn). Briefly, the membranes were blocked in Blotto (5% milk, 0.05% Tween 20, and PBS) overnight at 4°C. The membranes were incubated with the primary antibody for 1.5 h at room temperature and then washed in PBS with 0.05% tris-buffered saline tween-20 and incubated with the horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma Chemical Co.) for 1 h at room temperature. Visualization was effected using a chemiluminescence kit (ECL, Amersham Biosciences) and following the manufacturer's recommended procedure.

Whole blood and plasma isolation

Immediately after euthanasia, blood was collected from the right ventricle of the heart into a syringe containing a minute amount of heparin and placed on ice. Plasma was separated by centrifugation at 5000 × g for 5 min at 4°C. Samples were kept at -80°C until further analysis.

Immunoassays

Serum concentrations of SAA were determined using enzyme-linked immunosorbent assay (ELISA, Cytoscreen M SAA; Biosource, Camarillo, Calif), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-12 levels were measured with Quantikine M (R&D System, Minneapolis, Minn). Plasma levels of osteopontin (OPN) were detected by Assay Designs ELISA Kit (Ann Arbor, Mich).

Tissue and blood preparation for antioxidant determination

Blood samples were collected in heparinized tubes, and a 20% homogenate in 5% (w/v) metaphosphoric acid was prepared. After standing for 30 min on ice, the homogenate was centrifuged for 10 min (10,000 g) and the acid-soluble fraction was collected for measurement of sulfhydryl and disulfide compounds. Tissue homogenates (10%, w/v) were prepared in 5%

metaphosphoric acid, using all-glass Tenbroeck homogenizers, and kept on ice. After standing for 20 – 40 min, the homogenates were centrifuged for 1 min (10,000 g) and the acid-soluble fractions collected for measurement of free thiols/disulfides.

Analysis of GSH and other thiols (SH) and disulfides (SS) by high-performance liquid chromatography (HPLC)

GSH, oxidized glutathione (GSSG), cysteine, and cystine were simultaneously quantified by HPLC with dual electrochemical detection according to the method of Chen et al.¹⁷ In brief, 20- μ L samples were injected onto a 250 $\times$ 4.6 mm, 5 μ M, C-18 column (Val-U-Pak HP, fully end-capped ODS, 5 μ M, 250 $\times$ 4.6 mm; Chrom Tech, Inc., Apple Valley, Minn). The injected samples were eluted isocratically with a mobile phase consisting of 0.1 M monochloroacetic acid, 2 mM heptane sulfonic acid, and 2% acetonitrile at pH 2.8 and delivered at a flow rate of 1 mL/min. The compounds were detected in the eluant with a Bioanalytical Systems model LC4B dual electrochemical detector, using 2 Au-Hg electrodes in series with potentials of -1.2 V and 0.15 V for the upstream and downstream electrodes, respectively. Current (nA) was measured at the downstream electrode. Analytes were quantified from peak area measurements using authentic external standards.

Statistical analysis

All results are expressed as mean plus standard error of mean (SEM) unless otherwise stated. Data were evaluated using analysis of variance, followed by an appropriate *post hoc* test using GraphPad Instat version 3 for Windows (GraphPad Software, San Diego, Calif). Statistical significance was set at $P < 0.05$.

RESULTS

Male ICR mice administered 3.5% DSS developed severe colitis with bloody diarrhea and lost weight in a manner similar to BALB/C mice. Prodrug therapy significantly improved weight gains, with no significant differences noted between body weights from drug-treated animals and normal control mice (Table I).

Colon lengths of the colitis mice (DSS) were significantly decreased because of mucosal inflammation, edema, and thickening (Control 109 ± 2.8 mg vs DSS 66 ± 2.8 $P < 0.001$). In contrast, this decrease in colon length was significantly attenuated in prodrug-treated mice, which is consistent with reduced inflammation of their colonic tissue. Control mice showed normal mucosa with no detectable inflammatory activity. These data are presented in Table I.

Histologic evaluation revealed severe colonic lesions, with crypt epithelial disruption, ulceration, and diffuse infiltration of inflammatory cells in colitis mice (DSS 3.31 ± 0.18 , according to the score from 0 to 4). Colonic lesions significantly improved in treated animals with PTCA and RibCys ($P < 0.001$) and to a lesser extent with CySSG ($P < 0.05$) (Fig 1, A). The colonic brush border from treated animals exhibited more normal appearance, and the cytoskeletal showed less architectural distortion and fragmentation.

Immunohistochemistry revealed significant upregulation of macrophage-specific markers, namely CD68 (Fig 1, C) and COX-2 expression (data not shown) in colonic sections, which suggested macrophage activation and infiltration, particularly in the inflamed lamina propria, in colitis animals compared with normal controls (Fig 1, B). These abnormalities were attenuated by prodrug therapy, with the PTCA- and RibCys-treated mice exhibiting more improved appearance (Fig 1, D). Consistent with these findings, Western blot analysis demonstrated significant upregulation of COX-2 and CD68 protein expressions from activated macrophages in colitis mice (DSS), which was normalized in treated mice (Fig 2; Table I).

Antioxidant activity

GSH, the most important endogenous agent for cellular defense against oxidative stress, is vital for the integrity of the gut. Hepatic reduced GSH, which is the major source of gut antioxidant, was depleted (Fig 3) in colitis animals (DSS 4814 + 298 vs control 6530 + 318 nmol/g; $P < 0.001$) and was normalized with prodrug therapies (control vs treated, $P > 0.05$). The ratio of reduced-to-oxidized glutathione, GSH/GSSG, in the intestinal tissue was significantly reduced in colitis animals (DSS 5.6 vs control 9), which indicated accumulation of oxidative radicals and was normalized with PTCA therapy (Table I). Likewise, the cysteine accumulation in intestine was decreased in colitis mice (DSS vs control, $P < 0.05$) but significantly increased in prodrug-treated animals.

Plasma inflammatory markers

DSS-induced colitis was associated with a significant increase in acute phase protein expression of serum Amyloid A (SAA), which is an indicator for colitis and inflammatory response that was ameliorated in PTCA-treated mice (DSS vs PTCA < 0.001 , Fig 4). The levels of the inflammatory cytokine, IL-6, were significantly increased in colitis animals and normalized with PTCA therapy (Fig 5). In addition, DSS-induced colitis resulted in significant upregulations of IL-12 (Fig 6) and TNF- α protein secretion that were attenuated by prodrug therapy (Table I). Finally, plasma levels of OPN, an inflammatory mediator, were significantly increased in colitis animals and attenuated with therapies; however, they did not reach the significance.

Overall, these parameters of colitis were significantly improved with prodrug therapy, and PTCA demonstrated to be superior compared with the other cysteine/GSH prodrugs.

DISCUSSION

The purpose of this investigation was to determine the relative efficacy of 2 cysteine prodrugs and a mechanistically distinct GSH delivery agent against DSS-induced colitis in an ICR mouse model.

ROS have been implicated to contribute to tissue destruction in IBD. These ROS include hydroxyl radical, superoxide radical-anion, hydrogen peroxide, and nitric oxide. ROS are extremely unstable species because of their high reactivity and may result in lipid peroxidation and the oxidation of DNA and proteins.¹⁸ Antioxidant status has been described to be compromised in the intestinal mucosa from patients with ulcerative colitis.^{19,20} *In vitro* studies using enterocytes have also demonstrated the damaging effects of exposure to oxidants.²¹ Similar studies reveal that the colons of IBD patients produce more oxygen-free radicals compared with those of control subjects.^{3,4} In addition, using a chemiluminescence assay, significantly increased levels of reactive oxygen metabolites were found in the actively inflamed mucosa of IBD patients.^{3,22}

Glutathione is the most abundant cellular antioxidant synthesized by animal cells. GSH plays an essential role in cell biology and modulates cell responses to redox changes associated with the presence of ROS. GSH may be depleted during an inflammatory illness, and this GSH deficiency predisposes animals to organ failure and death after an otherwise nonlethal period of hypotension.²³ As GSH deficiency is associated with severe injury such as inflammation and sepsis, treatment strategies that maintain GSH levels may decrease the incidence of organ failure. Depletion of GSH induces enlarged lymphoid aggregates in intestine by recruitment of lymphocytes from the peripheral circulation.²² This depletion in tissue GSH has been implicated as a component of the inflammation that develops in IBD patients. On the other hand, supplementation with GSH monoethyl ester has been reported to prevent these lymphoid

aggregates.¹⁴ Accordingly, CySSG was used in this study to supply both GSH and cysteine to colitis animals.

Colitis animals showed an increase in plasma-oxidized proteins, which indicates that DSS induced oxidative stress to be at the systemic level that persisted until the end of the treatment.^{7,10,16,24} Oxidative stress is linked with the stimulation of the immune system.²⁴

Rebamipide, a drug that inhibits the production of free radicals, was shown to act as an anti-inflammatory agent in chronic DSS-induced colitis.²⁵ Previously, DSS-treated mice were shown to develop severe colitis, with decreased GSH concentrations in blood and colon and increased inflammatory markers such as SAA and TNF- α when compared with normal controls.^{10,16,26} In this investigation, DSS-treated animals developed severe colitis and lost a significant portion of their body weight. These animals also had decreased hematocrits, decreased GSH concentrations in the blood and liver, and increased inflammatory markers such as COX-2, CD68, SAA, cytokines IL-6, IL-12, TNF- α , and OPN when compared with normal controls. OPN is an important pro-inflammatory glycoprotein secreted by various cell types, including macrophages and intestinal epithelial cells.²⁷

In this study, no significant difference existed between intestinal GSH in colitis animals compared with normal controls; however, GSSG in the intestine was significantly increased in DSS-induced mice when compared with normal animals ($P < 0.01$). The control of oxidative stress involves multiple antioxidant mechanisms. Oral administration of the cysteine prodrug PTCA has been shown to protect against acetaminophen (APAP)-induced hepatic damage and necrosis as well as GSH/cysteine depletion.^{28,29} The results demonstrated a high degree of tissue selectivity in the APAP-induced depletion of GSH and cysteine concentrations and in the effectiveness of PTCA in maintaining and even increasing sulfhydryl levels in extrahepatic tissues of APAP-treated mice,³⁰ whereas the protective effect of PTCA was related to prevention of hepatic sulfhydryl depletion. Similarly, oral administration of PTCA provided protection against steatohepatitis by attenuating the expression of deleterious pro-inflammatory and fibrogenic genes in a dietary rat model.³¹

Other studies showed that PTCA significantly increased GSH biosynthesis in cultured rat lens compared with untreated controls³² and prevented naphthalene-induced experimental cataracts in mice.³³ In this study, PTCA-treated mice had significantly normalized levels of inflammatory cytokines (IL-6 and IL-12), acute protein SAA, colon lesions, with higher levels of blood and hepatic GSH, and lower GSSG concentrations compared with other treated and untreated DSS animals. In addition, PTCA, like its 2-methyl analog, MTCA,⁹ is expected to dissociate nonenzymatically to L-cysteine much more rapidly than RibCys,³⁴ hence, its efficacy in raising hepatic and plasma GSH levels. CySSG, on the other hand, requires a net enzymatic reduction of its disulfide bond to release cysteine or GSH.¹² Based on findings that antioxidants reduce disease activity in the murine model of DSS-induced colitis, these antioxidants are postulated to be useful dietary supplements in the treatment of IBD in humans.

CONCLUSIONS

PTCA, RibCys, and, to a lesser extent, CySSG provided protection against colitis in this murine model, which further supports a possible therapeutic application of GSH-repleting agents in IBD patients. These data strongly reinforce the previous report on the novel protective action of PTCA against colitis.

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Abbreviations

APAP	acetaminophen
CD68	macrosialin
COX	cyclooxygenase
CySSG	L-cysteine-glutathione mixed sulfide
DSS	dextran sodium sulfate
ELISA	

	enzyme-linked immunosorbent assay
GSH	glutathione
GSSG	oxidized glutathione
HPLC	high-performance liquid chromatography
IBD	inflammatory bowel disease
ICR	Institute for Cancer Research
IL	interleukin
OPN	osteopontin
PBS	phosphate-buffered saline
PTCA	2(RS)-n-propylthiazolidine-4(R)-carboxylic acid
RibCys	D-Ribose-L-cysteine
ROS	reactive oxygen species
SAA	serum Amyloid A
SDS	sodium dodecyl sulfate
SEM	standard error of mean
TNF-α	tumor necrosis factor-alpha

Fig 1.

(A) Colon sections were stained with hematoxylin and eosin, and lesions were graded from normal structure (0) to severe diffuse inflammation with crypt epithelium disruption and ulceration (4). Co represents control mice on normal drinking water. DSS represents colitis mice (those provided with DSS-supplemented water). PTCA, RibCys, and CYSSG represent those mice provided with DSS-containing water and treated with 1 cysteine/GSH prodrug. (B)–(D) Representative histologic colonic sections were incubated with anti-CD68 antibody and processed for immunohistochemical staining. (B) Colonic tissue from control (Co) mouse with normal structure compared with (C); a section from colitis mouse (DSS) shows distortion and thickening of mucosa with significant inflammatory deposits in inflamed lamina propria, and a (D) colonic section from PTCA-treated mouse shows significant improvement with less inflammation and reduced CD68 deposits (original magnification $\times 100$).

Fig 2.

Representative Western blot analysis and relative densities ($n = 3$), which indicates significant upregulation of COX-2 protein expression in colonic tissue from colitis mice (DSS) is normalized with prodrugs therapy, especially in PTCA animals.

Fig 3. Hepatic concentration of reduced GSH, the major source of gut antioxidant, was depleted in colitis animals (DSS) and normalized with prodrugs therapy (GSH was measured by HPLC).

Fig 4.

Concentration of SAA, an acute phase protein, was significantly increased in colitis animals (DSS) and considerably improved with therapies (ELISA, $\mu\text{g/mL}$).

Fig 5.
Serum concentrations of the inflammatory cytokine IL-6 (pg/mL) protein detected with ELISA.

Fig 6. Protein levels of IL-12 were upregulated in colitis animals and decreased with prodrugs therapy.

Table 1

Tissue and blood values from normal mice (control) were compared with colitis mice (DSS) and those treated with Cysteine/GSH produgs

Group	Control	DSS	PTCA	RibCys	CySSG	P value
Weight	29 ± 0.9 ^{##}	26 ± 1 [*]	29 ± 0.8 [#]	29 ± 0.9 [#]	28 ± 0.5	<0.05, >0.05 ^{##}
Hematocrit	49 ± 0.4	47 ± 0.5	50 ± 0.6	48 ± 0.6	50 ± 0.3	<0.01
Colon length (mm)	109 ± 2.8 ^T	66 ± 2.8 ^{*T}	80 ± 4 [*]	84 ± 4.2 [*]	87 ± 2.6 [*]	<0.001 <0.05 ^{T*}
Plasma GSH	1106 ± 37	881 ± 52	1148 ± 52 [*]	n/d	n/d	<0.05 [*]
Intestine GSH	1596 ± 35	1659 ± 214	1976 ± 157	1624 ± 207	1499 ± 276	>0.05 [#]
Intestine GSSG	178 ± 26 ^{##}	308 ± 26 [*]	200 ± 44 [#]	399 ± 56 [*]	444 ± 93 [*]	<0.05; >0.05 ^{##}
GSH/GSSG	9 [#]	5.4	9.9 [#]	4.1	3.8	>0.05 [#]
Intestine CYS	208 ± 27 [*]	167 ± 24 [*]	511 ± 9	755 ± 42	569 ± 66	<0.05
Intestine CSSC	555 ± 88	512 ± 49	510 ± 76	507 ± 72	317 ± 53	<0.01 [^]
TNF- α , Pg/mL	12	140 ± 40 [^]	68 ± 20 [^]	n/d	n/d	<0.05 [*]
OPN, ng/mL	52984 ± 6563 [*]	85338 ± 3160 [*]	74012 ± 5870	72511 ± 7249	65397 ± 6127	<0.05 [*]
CD68 density	152 ± 8.9	179 ± 7.6 [^]	133 ± 4.4 [^]	128 ± 4.5	142 ± 4.8	<0.01 [^]

[#] >0.05,

^{*} <0.05,

[^] <0.01,

^T <0.001.