Chapter II

Characterization of ulcerative colitis in Wistar rats
Introduction

Inflammatory bowel disease (IBD) is a complex multifactorial disease of unknown etiology which is not reproducible in cell culture systems. Substantial effort has been devoted to develop animal models of colitis that are being used in medical treatment of this debilitating inflammatory disorder. The ideal animal model should be very similar to human IBD and having the same contributing factors, pathophysiology, histopathology and clinical spectrum. Such models should be reproducible, not difficult to induce, have an anticipated time course of inflammation, resemble the clinical features, therapeutic response, and inflammatory cytokine profile of IBD (Strober 1985). Animal models of experimental colitis are classified into spontaneous and induced models that are used to study acute and chronic inflammation. In commonly used models, as discussed in the previous section, colitis is induced by administering toxic chemicals, such as acetic acid, trinitrobenzene sulfonic acid, indomethacin, formalin, or polysaccharides such as dextran sulfate sodium (DSS), carrageenan, or immune complexes (Elson 1995).

Effect of DSS load and duration of exposure on the severity of colitis

Colitis is usually induced by addition of DSS to drinking water of rats (ad libitum). The development of acute intestinal inflammation depends on the concentration, molecular weight, duration and frequency of administration of DSS (Solomon et al., 2010). Generally, acute colitis is induced by using high concentrations of DSS administered continuously in drinking water for short periods, typically 4–14 days. Each species and strain of experimental animals has a particular dose of DSS to induce colitis. For example, acute intestinal inflammation was induced in BALB/c mice using 3-10% DSS for up to 10 days (Okayasu et al., 1990), 3% DSS for 4 days in guinea pigs (Iwanaga et al., 1994), 5% DSS for 7 days in Wistar rats (Breider et al., 1997) and 4% DSS for 14 days in Sprague–Dawley rats (Mallon et al., 2006). A low dose of DSS for longer periods induces chronic colitis in the animals. For instance, continuous administration of 1% DSS for six months induces chronic colitis in Wistar rats (Chiba 1993).

Physical characteristics of Ulcerative colitis

Clinical manifestations of DSS colitis include severe watery diarrhea, occult blood in stools, decreased food intake, loss of weight restriction of movements, anemia and
eventually death. The onset and severity of these symptoms depend on the concentration of DSS, animal species, and the duration of administration. Fecal occult blood and diarrhea are the preliminary features of acute colitis and occur as early as day 2 (Kullmann et al., 2001). The inflammation may be peaked within 7–10 days (Mahler et al., 1998).

Clinical and histological features of Ulcerative colitis

The inflammation is specific to the colon, the macroscopic features include shortened colon, edema, hemorrhage, and ulceration. Histologically, diffused inflammation affects the mucosa and extends to the submucosa and the muscular layer, which is characterized by severe ulcers, mucosal edema, loss of goblet cells, crypt disruption and abscesses. Furthermore, there is massive infiltration of inflammatory cell into the mucosa and submucosa with neutrophils, macrophages and few lymphocytes. The earliest signs of histological damage were found to be loss of crypts followed by crypt shortening, preceding the development of inflammation (Cooper et al., 2000; Kitajima et al., 1999a). Chronic DSS colitis may be patchy, characterized by signs of epithelial erosions, mucosal fibrosis epithelial regeneration, and prominent lymphoid follicles (Cooper et al., 2000; Okayasu et al., 1990). The small intestine is largely unaffected by DSS and although there may be minor morphological changes in the ileum such as increased villous height, crypt cell proliferation and brush border activity (Geier et al., 2008).

Emerging evidence suggests that DSS but not dextran associated molecules establish linkages with medium chain fatty acids (MCFAs) that are present in the colonic lumen. The interaction of DSS with MCFAs forms 200 nanometer-sized vesicles that can fuse with colonocyte membranes. The arrival of DSS/MCFA vesicles in the cytoplasm may activate intestinal inflammatory signaling pathways. The deleterious effect of DSS is triggered by dextran moieties and is localized principally in the distal colon (Laroui et al., 2012).

Mechanism of DSS-induced Ulcerative colitis

The precise mechanism by which DSS causes colitis is unknown. Previous studies suggest that DSS induce colitis through direct alteration of gut mucosal barrier
permeability; since, tight junction proteins such as zona occludens (ZO)−1 are reduced by DSS as early as day 1, leads to increased permeability by day 3, changes that precede colonic inflammation (Kitajima et al., 1999a; Venkatraman et al., 2000). Uptake of small amounts of DSS by gut macrophages and mesenteric lymph nodes are noticed as early as day 1 in acute and chronic stages of colitis (Kitajima et al., 1999b). Collectively, these findings support an early role for a mucosal permeability defect in the pathogenesis of DSS colitis. Alternative mechanisms are also proposed in which DSS causes concentration-dependent direct cytotoxicity on the colonic mucosa, which leads to alteration of integrin-α4 and M290 subunit levels on epithelial cells disrupting their interaction with the γδ-intraepithelial T cells (Ni et al., 1996); γδ-intraepithelial T cells have an unknown function, but are thought to be involved in mucosal protection (and healing) against various stimuli including DSS (Chen et al., 2002).

The role of colonic flora in the pathogenesis of DSS colitis is unclear. It has shown that antibiotics, namely ciprofloxacin and metronidazole ameliorate DSS-induced acute colitis in mice (Hans et al., 2000). Conversely, mice grown under germ-free conditions develop severe colitis (Kitajima et al., 2001), whereas mice with normal intestinal flora antigens had reduced disease activity in colitis (Verdu et al., 2000). Hence, colonic microbiota may not play a crucial role in the initiation of DSS colitis. It has been evident that CD4+ and CD8+ T cells may not be important in the induction of acute DSS colitis. Acute colitic induced by DSS in mice has lower CD8+ cells while CD4+ cells remained unaltered (Stevceva et al., 2001). DSS-induced colitis is characterized by predominant Th1 type with increased levels IFN-γ and IL-4 in inflamed colonic tissues of mice (Dieleman et al., 1998). Notably, expression of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α and Interferon (IFN)-γ are upregulated, while synthesis of anti-inflammatory cytokines, such as IL-10, is downregulated (Rogler 1998, Kennedy 2000, Steidler 2000, Hofstetter 1998). Other parameters such as decrease in colon length and body weight, elevation in myeloperoxidase levels are the hall marks of severe intestinal inflammation.

**Advantages of DSS-induced Ulcerative colitis model**

The DSS model of colitis has many clinical and pathological similarities to human ulcerative colitis. It exhibits the inflammation–dysplasia–adenocarcinoma sequence and has also been shown to respond to standard human ulcerative colitis medical therapeutic
measures such as steroids, metronidazole (Hans et al., 2000), 5-aminosalicylates (Axelsson et al., 1998), cyclosporine (Murthy et al., 1993) and anti-TNF antibody (Murthy et al., 1999). There are also pathogenic similarities in the cytokine response of this model to human IBD, which is predominantly Th1 type. Like human IBD, there is also a variable genetic susceptibility of various mice strains to DSS-induced colitis and involvement of multiple genetic loci (Mahler et al., 1999). Both acute and chronic DSS colitis are highly reproducible (Elson et al., 1995). The model is also appealing due to its wide applicability to rats, mice, guinea pigs and hamsters. In addition, DSS is cheap and widely available. It is easy to store (at room temperature) and has a shelf life of up to 3 years. Preparation of DSS for induction of colitis is also quite simple: dissolves easily in tap water and can be administered ad libitum; hence unlike most chemical induction models, no general anesthesia is required. It is thus one of the most commonly used models of IBD and has been a better model to study various aspects of IBD such as therapy, pathogenesis (especially the role of permeability and epithelial destruction in the initiation of IBD), genetic predisposition of IBD loci.

The wide range of molecular weight of dextran corresponds to a wide variability in the induction of colitis by DSS (Table 1). This is a potential drawback of this model since the MW of DSS is an important factor that determines the site and severity of inflammation in this model (Axelsson et al., 1996). Kitajima and colleagues compared three commercial preparations of 5% DSS (MW 5,000, 40,000 and 500,000, respectively) administered for 7 days in BALB/c mice. They found that the three preparations led to variations in the disease site, that the severity of colitis observed was greatest with the 40,000 MW DSS and that no colitis developed with the 500,000 preparation (Kitajima et al., 2001). We compared the efficacy of 4% DSS (MW 9,000–20,000 versus MW 36,000–50,000) on the induction of acute colitis in male Sprague–Dawley rats and observed the development of a marked and mild colitis (median histological injury score 7 versus 13), respectively (unpublished). Hence, there is a variation in the severity of colonic inflammation with the MW of DSS.

We have selected 5% DSS (w/v, MW 36,000–50,000) dissolved in water for 7 days to induce ulcerative colitis in Wistar rats.
Materials and Methods

Animals

The protocol adopted for this study was approved by the Institute Animal Ethics Committee (IAEC), CFTRI, Mysuru, India. Female Wistar rats (n=24) weighing 180-190 g were procured from the Animal House Facility, CFTRI, Mysuru, India. The rats were kept in individual cages, acclimatized to constant conditions of humidity (70-80%), temperature (22-25°C), and light (12 h light and dark cycle), with food and water available ad libitum. The rats were randomly divided into 2 groups viz control (n=8), and DSS-treated colitic group (n=16).

Induction and assessment of colitis

Colitis was induced in the rats by administration of 5% DSS in drinking water for 7 days. Control rats received normal drinking water. Food and water intake of the rats were recorded during colitis. The disease was assessed based on the loss of body weight, gross rectal bleeding, and stool consistency during colitis. The severity of rectal bleeding and diarrhea were calculated using the scores described previously (Islam et al., 2008). Briefly, scoring for diarrhea or stool consistency (normal-0, soft-++, diarrhea- ++++) and rectal bleeding (0–4) was done based on the visual observation of fecal fluid and perianal area by two independent observers who were unaware of the experiment. The results are expressed as disease activity index (DAI), which is the cumulative mean of individual scores of stool consistency and rectal bleeding. Body weight loss was calculated based on the difference in the body weight of the rats at Day1 and the specific day of course of colitis. On Day 8, the rats were anesthetized under mild ether anesthesia and terminated by cardiac puncture. The colon tissues were excised extending from the cecum to the farthest accessible distal end in the pelvis. The length of the colon tissue was measured, and a sample of the tissue at the distal end was fixed in 10% phosphate buffered formalin for histopathological analysis. Later, the colon tissue was cut longitudinally, flushed with ice cold saline, frozen in liquid nitrogen, stored at -80°C, until further analysis.

Histopathology

Colon tissues were fixed in 10% phosphate buffered formalin, sectioned (10µm) using paraffin blocks and stained with hematoxylin and eosin (H&E). The stained sections were examined under Olympus BX51 microscope, and the images were captured using
the Progres Capture Pro 2.7 software (Jenoptik, Germany). The intensity of microscopic damage was graded in a blinded manner by two independent observers using a scale of 0-4 for each colon section for the extent of crypt damage, goblet cell erosion and presence of inflammatory infiltrates (Islam et al., 2008).

**Determination of MPO activity in the colon**

MPO activity was estimated according to the O-dianisidine method described previously (Ukil et al., 2003). The tissue was homogenized in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, centrifuged 5000 g for 20 min at 4°C. 100μl of the supernatant was added to the reaction mixture containing 2910μl of 50mM phosphate buffer (pH6.0), 30μl of O-dianisidine dihydrochloride (20mg/ml), and 30μl of 20 mM H₂O₂. After 10 min, the reaction was terminated by adding 30 μl of 2% sodium azide. The absorbance of the solution was read at 460 nm using a spectrophotometer. 1U of the enzyme was considered as the amount of enzyme that produces a change in absorbance of 1·0 /min/mg of protein.

**Catalase activity**

Catalase activity was measured in the colon homogenate based on the method of Clairborne 1985. The colon samples were homogenized in 0.01M phosphate buffer saline (PBS) pH 7.4 and centrifuged at 10000 g at 4°C for 20 min. 0.01 μl of supernatant was added to 1 ml cuvette containing 980 μl of 50mM potassium phosphate buffer and 100 μl of 20 mM H₂O₂. The reduction of absorbance was measured at 240 nm for 3 min at 30 sec interval. Catalase activity was expressed as μm of H₂O₂ degraded/ min/ mg of protein. The extinction coefficient of H₂O₂ was 0.0436 μm/ml/cm.

**Superoxide dismutase (SOD) activity**

SOD activity was measured according to the method of Sun et al., 1988. SOD estimation was based on inhibition of NBT reduction by the superoxide radicals produced by xanthine and xanthine oxidase system. The reaction mixture (50 mM sodium phosphate, 0.1 mM EDTA, 0.05 mM NBT, and 0.05 mM xanthine, pH 7.8, 25°C) was mixed with xanthine oxidase (0.05 units), and the change in absorbance at 550 nm was followed. 0.01ml of the sample was then added, and the concentration of sample required to decrease the rate of reaction by 50% was calculated. SOD activity was expressed as
U/mg of protein; where, IU of the enzyme is the amount of protein required to inhibit 50% of the reaction.

**Tissue glutathione assay**

Glutathione levels were estimated based on the method described previously (Sedlak and Raymond 1968). An aliquot of 0.1ml of the sample was mixed with 1 ml of distilled water and 1 ml of 50% TCA. The tubes were vortexed and centrifuged for 15 min at 3000 g. An aliquot of the (0.2 ml) supernatant was mixed with 0.7 ml of 0.4 M Tris buffer (pH 8.9), 0.1 ml of 0.01M DTNB. The absorbance of the sample was read at 412 nm against a reagent blank. The amount of glutathione was calculated using a calibration curve.

**Determination of lipid peroxides in the colon**

Thiobarbituric acid reactive substances (TBARS) in serum were estimated as an indicator of lipid peroxides based on the method described previously (Ukil et al., 2003). Briefly, 0.05 ml of the tissue homogenate was added to a reaction mixture containing 0.2 ml of 8.1 % SDS, 1.5 ml of 0.8 % thiobarbituric acid and 0.7 ml of distilled water. Samples were boiled for 1 h at 95° C and centrifuged at 3000 × g for 10 min. The absorbance of the solution was measured spectrophotometrically at 532 nm. The lipid peroxides were quantified using tetramethoxy propane as a standard.

**Determination of glutathione S-transferase (GST) activity**

GST activity was measured by monitoring the enzyme catalyzed conjugation of glutathione with 1-chloro, 2, 4-dinitrobenzene (CDNB) according to the method of Habig et al., 1974. An aliquot (10µl) of the colon homogenate was added to the reaction mixture containing 900 µl of phosphate buffer (0.1M, pH-6.5, 0.5mM EDTA), 50µl of CDNB (1.5mM) and 50µl of glutathione (1mM). The increase in absorbance was monitored for 3 min at 340 nm. GST activity was expressed as µmol of GSH-CDNB conjugate formed/min/mg protein (extinction coefficient 9.6 mM/cm).

**Statistical analysis**

Statistical analysis was performed using Graph Pad statistical software (Graph Pad Instat). The results were expressed as a mean ± standard deviation. Unpaired t-test was
done to compare the means between the different groups by Welch’s corrected test. One-tailed p < 0.01 was considered significant in the present study.

**Results**

**Macroscopic features of DSS-induced colitis in rats**

Administration of DSS resulted in severe diarrhea, rectal bleeding and body weight loss in the rats. The disease activity index (DAI), calculated based on diarrhea and rectal bleeding, was significantly higher at day 3, peaked at day 5 and persisted till day 7 of the course of colitis. There was drastic decrease in body weight of DSS-treated rats, calculated as 26.2% overall from day 1 to day 7. Furthermore, DSS treatment resulted in 40% mortality in rats. The colon length was significantly lower in DSS group (9.5±2.5 cm) compared to control group (16.1±1.3 cm). The spleen weight was significantly higher in DSS group (0.61±0.08 g) compared to control group (0.43±0.02 g) (Figure 2.1).

![Graphs showing disease activity index, body weight loss, mortality, colon length, and spleen weight](image)

**Figure 2.1:** Disease activity, body weight loss, mortality, colon length and spleen weight of rats challenged with 5% DSS for 7 days. Values are mean ± SD of six rats. Significantly different at * p<0.05.

**Histopathological features of colitis**

Histological examination of colon tissue sections of DSS-treated rats revealed that there was a denuded surface with severe ulceration, extensive loss of goblet cells, crypt
distortion, edema and massive infiltration of inflammatory cells including neutrophils and lymphocytes (Figure 2.2). The histopathology score was remarkably higher in the DSS group (3.9± 0.4 versus 0.1 in the control group).

Figure 2.2: Histopathological images (4x) of colon tissue sections of rats stained with hematoxylin and eosin.

**Myeloperoxidase (MPO) activity**

Myeloperoxidase is abundant in azurophilic granules of the neutrophils and is a marker of neutrophil infiltration. MPO activity was significantly higher in the colon tissues of the DSS-treated group (3.7±0.8 U/mg protein) compared to control group (0.6±0.2 U/mg protein) (Figure 2.3).

Figure 2.3: Myeloperoxidase activity in the colon tissues of rats. Values are mean ± SD of six rats. Significantly different at * p<0.05
Lipid peroxides in the colon tissue

Tissue levels of malondialdehyde (MDA), a byproduct of lipid peroxidation, were estimated in the colon, as an indicator of oxidative damage. MDA levels were remarkably higher in the colon tissues of DSS-treated rats (1075±198 nmol/mg protein) compared to control rats (135±33 nmol/mg protein) as shown in Figure 2.4.

Antioxidant enzyme status in the colon tissue

The antioxidant enzymes such as catalase, SOD, GST, and GSH were estimated in the colon to assess the antioxidant status of the colon tissue. Catalase activity was four-fold lower in the colon tissues of rats of DSS group (13.6±9.5 μmol of H$_2$O$_2$ degraded/min/mg protein) compared to control group (55.2 ± 5.1 μmol of H$_2$O$_2$ degraded/min/mg protein). SOD activity was near three-fold lower in DSS group (1.2 ± 0.4 U/mg protein) compared to control group (3.5 ± 0.7 U/mg protein). GST activity was 2.34 fold lower in DSS group (6.1 ± 4.1 nmol GSH-CDNB conjugate formed/min/mg protein) compared to control group (14.3 ± 3.2 nmol GSH-CDNB conjugate formed/min/mg protein). GSH levels were remarkably lower in DSS group (8.7 ± 5.1 nmol/mg protein) compared to control group (27.8 ± 3.1 nmol/mg protein) (Figure 2.5).

Figure 2.4: Malondialdehyde (MDA) levels in the colon tissues of rats. Values are mean ± SD of six rats. Significantly different at * p<0.05
Figure 2.5: Catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST) and glutathione levels in the colon tissues of rats. Values are mean ± of six rats.

Discussion

Administration of 5% DSS for 7 days in rats exhibited adverse symptoms such as severe diarrhea, rectal bleeding and body weight loss, which are similar to human ulcerative colitis. Diarrhea is reported to be due to the increased permeability of intestinal cells or hyper osmolarity due to administration of DSS (Schwartz et al., 2008). Weight loss and the shortening of the colon, which are indicators for the severity of intestinal inflammation correlate with the pathological and histological changes, occur during colitis (Okayasu et al., 1990). In agreement with these findings, there was a remarkable decrease in the survival rate at the end of course of colitis. There was a significant shortening of colon length and splenomegaly in rats of DSS group. Histologically, the colon sections of DSS-treated rats exhibit mucosal repletion and exudative edema. The infiltration of neutrophils was observed in the mucosa and the submucosal layers. This was correlated with an increase in MPO activity in the colon tissue. MPO activity specifies the intestinal inflammation to assess the tissue damage and extent of infiltration by inflammatory cells (Yamamoto et al., 2008). Furthermore, the antioxidant status was
significantly affected by the DSS treatment, as evidenced by lower catalase, SOD, GST activities, and GSH levels in the colon tissue.

It is considered that DSS-induced colitis may be partially mediated by cytotoxic reactive oxygen metabolites (ROM). Increased mucosal ROM generated from stimulated macrophages and neutrophils as well as lipid peroxidation byproducts have been reported in colon biopsy specimens of patients with ulcerative colitis (Cross et al., 1987; Braganza JM et al., 1989; Simmonds et al., 1992; Keshavarzian et al., 1992). MPO is an enzyme found abundant in neutrophils and is a marker of inflammation and tissue injury (Nieto et al., 1998). In the present study, MPO activity was found six-fold higher after DSS treatment. The results are in concurrent with the previous studies where DSS treatment elevates MPO activity and contribute to inflammation and tissue injury in the colon. It has been reported that rats fed a diet enriched with tocopherol has lower MPO activity, indicates that restoring antioxidant status as a treatment for ulcerative colitis (Kruidenier et al., 1998). The elevation of MPO activity in the present study is positively correlated with an increase in neutrophil infiltration in the colon.

Human colon has low levels of catalase, SOD and glutathione peroxidase (GPX), and any imbalance in the ROM generation and antioxidant enzymes leads to tissue injury (Verspaget et al., 1991). It has been found that GSH levels and SOD activity were lower in patients with UC (Inauen et al., 1988, Mulder et al., 1991). DSS-induced colitis has been characterized by a decrease in catalase, SOD, GST activity, and GSH levels in the colon (Hossam et al., 2009). MPO produces toxic hypochlorous acid (HOCl) from \( \text{H}_2\text{O}_2 \) and \( \text{Cl}^- \) anion (Eiserich et al., 1998). The degradation of \( \text{H}_2\text{O}_2 \) requires catalase enzyme. Also, inflammatory cells present in the inflamed mucosa are capable of producing superoxide anion and hydrogen peroxide. Therefore, in the present study, the reduction of catalase, SOD activity, and increase in MPO activity contributes to the intestinal injury caused by DSS.

DSS significantly altered the oxidant/anti-oxidant balance as evident by increased lipid peroxides and reduction in colon GST activity and GSH content (Hossam et al., 2009). These results are in agreement with the previous reports on DSS colitis (Barbosa et al., 2003; Mustafa et al., 2006; Osman et al., 2008). The decrease in colonic GSH content observed in the current study could be partially attributed to the increased MPO activity. Supporting this perspective, GSH is an important target of HOCl generated by the MPO
system. The action of HOCl on GSH gives the disulfide GSSG as well as products identified as internal sulfonamides and thiosulfonates. These products can deplete the cellular GSH level and further compromise the colonic antioxidant defense system (Winterbourn and Brennan, 1997). Furthermore, depletion of GST has been linked to the pathogenesis of UC (Korkina et al., 2003). Lower GST detoxification capacity has been reported in the mucosa of UC patients (Berkhout et al., 2006). The data on GSH and GST activity in the current study are consistent with the above reports.

In addition, an enhanced production of inflammatory mediators was reported in DSS treated mouse colon during the colitic phase. Proinflammatory cytokines are local inflammatory mediators, produced by macrophages, lymphocytes as well as by epithelial and mesenchymal cells, involved in the development and pathogenesis of inflammation and immunity (Sartor et al., 1994). In general DSS induced colitis, produced differential expression of proinflammatory mediators at different regions of colon. All the cytokines were expressed at much lower levels in the proximal colon than that of distal colon thus suggesting that the distal colon is more affected by DSS (Azuma et al., 2008; Yan et al., 2009).

Based on these observations, it can be concluded that administration of 5% DSS through drinking water for 7 days induced colitis with characteristic clinical and histological alterations in Wistar rats. There was significant increase in MPO activity and lipid peroxidation in colon mucosa. Further, the antioxidant enzymes and GSH levels were severely impaired in colitic rats which were correlated with increased MPO activity and lipid peroxidation in the colon. Thus, it can be suggested that DSS induces inflammation in the colon mucosa through disruption of oxidant/antioxidant balance in Wistar rats.