

# POSITIVE CORRELATION BETWEEN DISEASE ACTIVITY INDEX AND MATRIX METALLOPROTEINASES ACTIVITY IN A RAT MODEL OF COLITIS

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**ABSTRACT - Context** - Inflammatory bowel disease, including ulcerative colitis and Crohn's disease, comprising a broad spectrum of diseases those have in common chronic inflammation of the gastrointestinal tract, histological alterations and an increased activity levels of certain enzymes, such as, metalloproteinases. **Objective** - Evaluate a possible correlation of disease activity index with the severity of colonic mucosal damage and increased activity of metalloproteinases in a model of ulcerative colitis induced by dextran sulfate sodium. **Methods** - Colitis was induced by oral administration of 5% dextran sulfate sodium for seven days in this group (n=10), whereas control group (n=16) received water. Effects were analyzed daily by disease activity index. In the seventh day, animals were euthanized and hematological measurements, histological changes (hematoxylin and eosin and Alcian Blue staining), myeloperoxidase and metalloproteinase activities (MMP-2 and MMP-9) were determined. **Results** - Dextran sulfate sodium group showed elevated disease activity index and reduced hematological parameters. Induction of colitis caused tissue injury with loss of mucin and increased myeloperoxidase ( $P<0.001$ ) and MMP-9 activities (45 fold) compared to the control group. **Conclusion** - In this study, we observed a disease activity index correlation with the degree of histopathological changes after induction of colitis, and this result may be related mainly to the increased activity of MMP-9 and mieloperoxidase.

**HEADINGS** - Colitis. Crohn disease. Metalloproteinases.

## INTRODUCTION

Inflammatory bowel diseases (IBD) have in common chronic inflammation of the digestive tract, which may or may not have a cause or specific pathogen<sup>(5,7)</sup>. Its etiology is multifactorial and complex, it is believed to have genetic involvement, environmental, immune and intestinal microbiota<sup>(26,30)</sup>.

For many years, studies on the understanding of the pathogenesis of IBD have been delayed by the lack of experimental models that corresponded to the disease<sup>(10)</sup>. But now more than 30 models have been developed, with the most varied clinical manifestations of IBD<sup>(4,18)</sup>. These models contribute to important advances in understanding the mechanisms of inflammation, the pathogenesis and the treatment of possible discoveries<sup>(11)</sup>. Among the models used, stands the induction of colitis by dextran sulfate sodium (DSS), due to its good reproducibility and also for presenting clinical symptoms, inflammatory

markers and histopathological features similar to IBD in humans<sup>(17,21)</sup>. The exact mechanism by which the DSS induced colitis is unknown but it is known that this compound is toxic to intestinal epithelial cells with a probable mechanism that has direct action on intestinal permeability allowing entry of luminal antigens resulting in an inflammatory response<sup>(22,29)</sup>.

Changes in the activity and expression of extracellular matrix metalloproteinases (MMPs) were described in IBD patients suggesting that these enzymes are involved in tissue degradation process<sup>(16,20)</sup>. MMPs are endopeptidases dependent of calcium and zinc and are considered the main enzymes involved in the control of the homeostasis of extracellular matrix (ECM) at various levels, including growth, division and cell function, regulation of immune response, controlled synthesis and matrix remodeling by cleavage of almost all of its components such as collagen, proteoglycans, fibronectin, elastin, and laminin<sup>(15,24)</sup>.

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Several MMPs (MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-12) have their activity increased in a variety of animal models colitis and in patients with inflammatory bowel disease. Thus, the present study investigated a possible correlation of DAI in the model of ulcerative colitis induced by DSS with the severity of colonic mucosal damage and increased activity of MMP-2 and MMP-9.

## METHODS

### Materials

Dextran sodium sulfate (DSS - MW: 36-50 kDa, MP Biomedicals, Solon, OH, USA), hexadecyltrimethylammonium bromide (HTAB, Sigma-Aldrich Co., St. Louis, MO, USA), *o*-dianisidine hydrochloride (Sigma-Aldrich Co., St. Louis, MO, USA); Acrylamide (Ludwig Biotechnology Ltd., Porto Alegre, RS, Brazil); *N,N'*-methylenebisacrylamide (Neon Comercial Ltda., São Paulo, SP, Brazil); Tris(hydroxymethyl)aminomethane (Biosolve Valkenswaard, Netherlands); Triton x-100 (Vetec Fine Chemicals Ltda., Duque de Caxias, RJ, Brazil); Gelatin (Sigma-Aldrich Co., St. Louis, MO, USA).

### Animals

Male Wistar rats (6-8 weeks old) were obtained from the Center of Biology for Reproduction (CBR) at Federal University of Juiz de Fora (UFJF, Juiz de Fora, MG, Brazil) for induction of colitis by DSS. The animals were kept in a bioterium of Laboratório de Análise de Glicoconjugados in the Biochemistry Department (UFJF), throughout the experiment in individual plastic boxes under, "Guide for the Care and Use of Laboratory Animals". The procedures were approved by the Ethics Committee on Animal Experimentation UFJF (002/2010-CEEA UFJF).

### DSS induced colitis

The induction of colitis DSS was done according Okayasu et al. with modifications. The animals were randomized into two groups: control group (n = 16), which was given water and the DSS group (n = 10) given only a solution of 5% DSS *ad libitum* for 7 days. The total volume of DSS consumed per rat was about 40 ml/day per group. At the end of the experiment (seventh day), rats were euthanized by deepening anesthesia with administration of sodium pentobarbital (100 mg/kg) and the colon was completely removed from the colon-cecal junction to the anal canal.

### Assessment of disease activity index

All the rats were observed once a day. The disease activity index (DAI) was determined by scoring body weight loss, trait of stool, and occult blood in stool or hematochezia from day 0 to day 7 in colitis induction according to the classic scoring system by Cooper in the process of modeling: body weight loss (0, none; 1, 1%-5%; 2, 5%-10%; 3, 10%-20%; 4, >20%), stool consistency (0, normal; 2, loose stool; 4, diarrhea), and stool blood (0, negative; 2, fecal occult blood test positive; 4, gross bleeding)<sup>(6)</sup>

### Hematological Evaluation

Blood samples were analyzed for the number of erythrocyte, hematocrit and hemoglobin for the evaluation of hematological parameters. The analysis was performed at the Center for Reproductive Biology (CBR/UFJF) using the veterinary hematology analyzer Poch 100i V-Diff (Sysmex).

### Histopathologic analysis

Initially, each colon segment was washed in 0.1 M phosphate buffer, pH 7.4, and its length and weight measured to evaluate the ratio length/weight. After the colon was opened longitudinally, using the technique of swiss roll, fixed with paraformaldehyde solution 10% in 0.05 M phosphate buffer, pH 7.4, for at least 24 hours, embedded in paraffin and processed so that cuts of 5 mm thick were performed. Besides the normal staining (hematoxylin and eosin, HE) it was also used a specific one mucopolysaccharides alcian blue at pH 2.5. The sections were graded histopathologically and evaluated as described by Cooper et al.<sup>(6)</sup>

### Myeloperoxidase activity (MPO)

The tissue MPO activity was measured according to the technique described by Bradley et al.<sup>(2)</sup>. The absorbance was determined at a wavelength of 450 nm. The MPO activity was calculated using the molar extinction coefficient ( $\epsilon = 11.48 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ )<sup>(8)</sup>. 1 UE (enzyme unit) of myeloperoxidase was considered as amount of enzyme that degrades 1  $\mu\text{mol}$ /min of hydrogen peroxide at 25 °C. The results were expressed in mUE/mg protein.

### Extraction of metalloproteinases

Fragments of the colon were macerated in liquid nitrogen and then added 1 ml of Tris-HCl 50 mM pH 7.4, containing 100 mM  $\text{CaCl}_2$  and 1 ml of Triton X-100 0.2% (v/v) for extraction the enzymes. The samples were centrifuged for 10 minutes at 12,000 xg, and the supernatant aliquoted. Protein was quantified by BCA kit (Thermo Scientific).

### Zymograms

Colon homogenates (10  $\mu\text{g}$  protein) were submitted to a electrophoresis on polyacrylamide gels (10% acrylamide-bisacrylamide solution T 30% C 2.7% gelatin containing 2 mg/ml) in Tris-glycine (25 mM / 192 mM ) pH 8.3 containing sodium dodecyl sulphate (0.1%). After the migration, gels were washed with Triton X-100 (2%) and incubated with 50 mM Tris-HCl, pH 8.2, containing 5 mM  $\text{CaCl}_2$ , 1  $\mu\text{M}$   $\text{ZnCl}_2$  and for 24h at 37°C. The gels were stained by Coomassie Brilliant Blue R-250 (0.5% dye, 30% methanol, 10% acetic acid) and destained (30% methanol, 10% acetic acid)<sup>(19, 27)</sup>. The activity of gelatinases was evidenced as bright regions (bleached) in the gel. To measure the intensities of the bands a program was used, TotalLab Quant<sup>®</sup>. The activities were corrected for protein content.

### Statistical analysis

Values are expressed as mean  $\pm$  standard error. Statistical analysis was performed using SPSS for Windows, version 19.

We evaluated the data normality by the Shapiro-Wilk test, the comparison of data distributed on the curve of normality was performed by t test student.  $P < 0.05$  was considered statistically significant.

## RESULTS

The clinical signs of DSS-induced colitis in rats were determined by assessing the disease activity index (DAI). This index is composed of three parameters (stool consistency, presence of rectal bleeding and weight variation of the animals) and provides the degree of severity of the inflammatory process that can be correlated with damage to the intestinal mucosa. We observed an increase in the DAI for the DSS group after the first day of induction with a significant increase from the fourth to the seventh day, in addition, significant weight loss during all days of the experiment (Figure 1. A, B), for the control group there was a gain of weight and no clinical changes. Weight loss was

the first symptoms observed from the third day on showed changes in stool consistency, while the presence of blood was viewed from the fifth day (Figure 1 C). Macroscopically, there was also a reduction in the length of the colon group DSS (Figure 1 D).

The DSS-induced colitis caused hematological decreasing of erythrocyte count ( $P < 0.01$ ), hematocrit ( $P < 0.05$ ) and hemoglobin ( $P < 0.01$ ), indicating severe loss of blood compared to the control group (Table 1).

TABLE 1. Hemathological parameters analysis with blood (EDTA) in the animals of both control and DSS group

Groups	Erythrocyte ( $\times 10^6/\text{mm}^3$ )	Hematocrit (%)	Hemoglobin (g/dL)
Control	$8.88 \pm 0.17$	$48.0 \pm 1.0$	$17.7 \pm 0.4$
DSS	<sup>a</sup> $6.75 \pm 0.58$	<sup>b</sup> $39.3 \pm 2.6$	<sup>a</sup> $13.1 \pm 1.0$

Data represents the values of the mean  $\pm$  standard error

Significant statistical differences are shown with <sup>a</sup> $P < 0,01$  and <sup>b</sup> $P < 0,05$  compared to the control

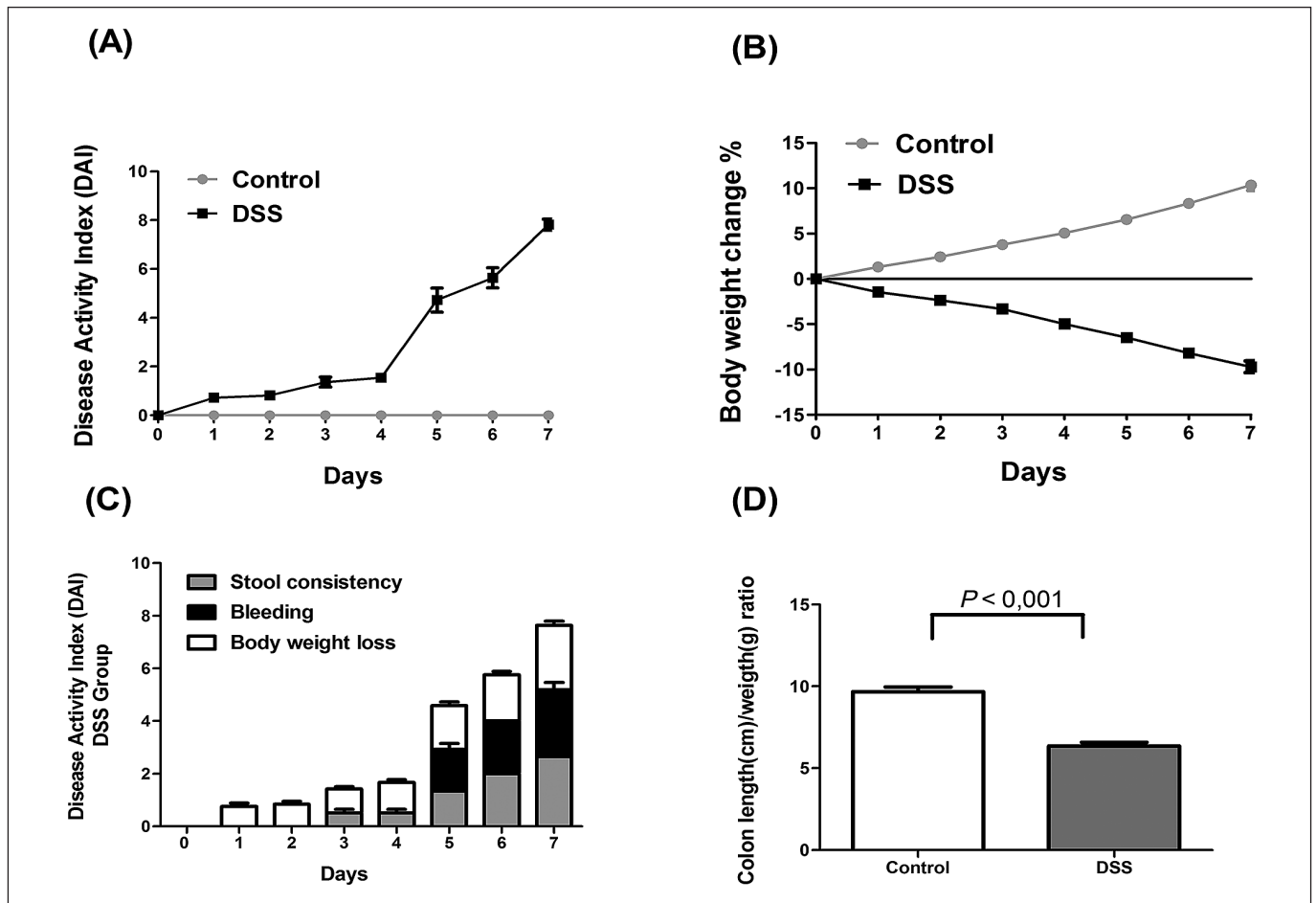


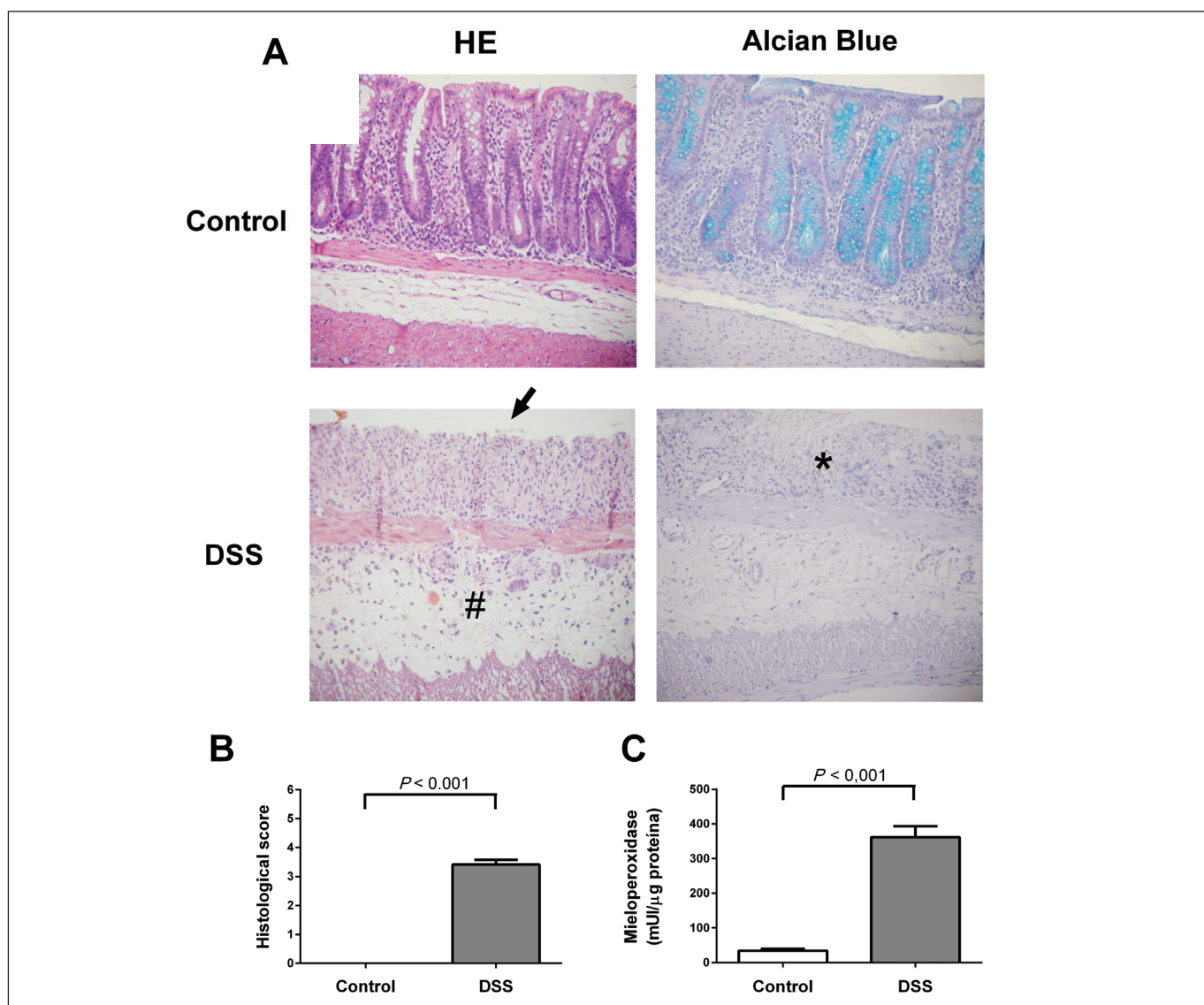
FIGURE 1. Assessment of disease activity index (DAI) in dextran sulfate sodium DSS-induced colitis model.

(A) The DAI of the DSS group presented a difference from the first day of the experiment and (B) a reduction in weight loss when compared to the control group. (C) DSS group began to demonstrate alterations in the consistence of their stool from the third day, and bleeding was noticed after the fifth day of the experiment while no alterations were observed on the control group. (D) The use of DSS caused shortening of the colon of the animals when compared to the control group. The data is demonstrated by the mean  $\pm$  standard error. DSS- dextran sulfate sodium

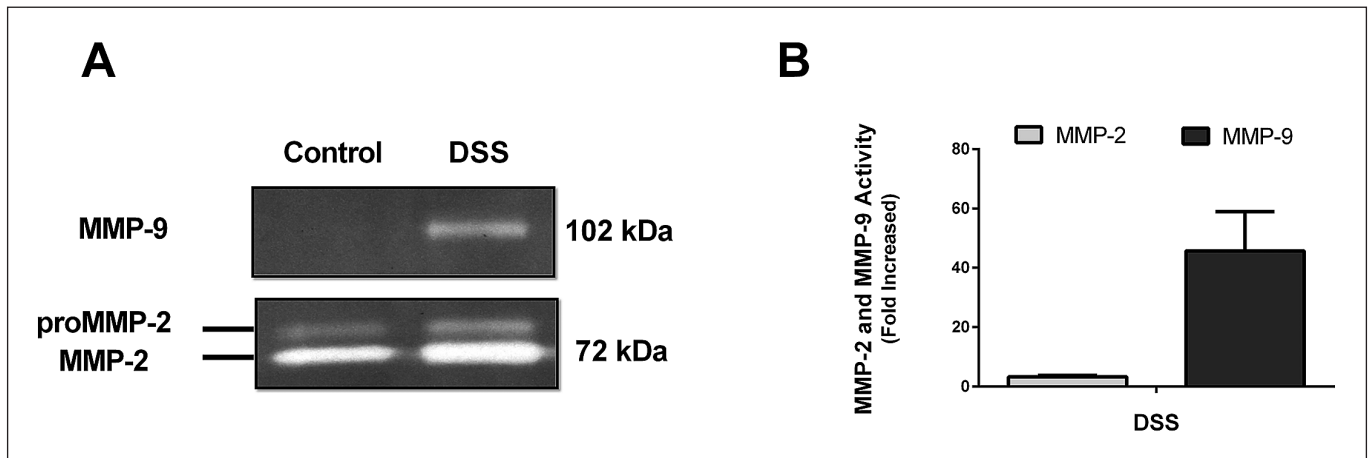
Histological analysis of colonic mucosa of animals in the DSS group showed multifocal areas of erosion and ulcers as well as regions of loss of more than 2/3 of the crypts. It was also observed vascular congestion, edema in the lamina propria and inflammatory infiltrate (Figure 2. A). Animals in the control group the colonic mucosa remained normal, with the epithelium and crypts intact (Figure 2. A). This result also observed in the analysis reflects the histological score (Figure 2. B,  $P < 0.001$ ). For alcian blue staining, we found out that the DSS group (Figure 2. A), had significant decreases in the number of goblet cells and mucin production while in the control group these numbers of crypts and goblet cells were maintained.

The activity of myeloperoxidase (MPO), a marker widely used to assess the tissue infiltration of neutrophils showed an increase in MPO activity compared to the control group (Figure 2. C). This result is consistent with the histopathological changes observed.

There is good evidence of the involvement of matrix metalloproteinases in inflammatory bowel disease, among them are two gelatinases (MMP-2 and 9). To evaluate changes in the activity of MMP-2 and MMP-9 in both controlled and induced group, densitometric analysis of zymograms was performed as described in materials and methods. It was observed in healthy animals only the activity of MMP-2 (Figure 3. A) while for the induced animals, besides the in-



**FIGURE 2.** Histopathological changes and enzymatic activity present in the colon of the dextran sulfate sodium DSS-induced colitis model. (A) Control group maintained normal colon, whereas DSS group shows the multifocal areas of the mucosal erosions with loss of epithelial cells (black arrow), goblet cell depletion (\*), crypt loss, inflammatory cell infiltration, edema and vascular congestion. (B) The histopathologic score demonstrated a significant increase in the DSS group compared to the control, (C) the same result was observed for the Myeloperoxidase activity (MPO) tissue activity. The colon was fixed and stained with HE and alcian blue pH 2,5, original magnification 10x).



**FIGURE 3.** A representative zymogram of matrix metalloproteinases in the colon of the DSS-induced colitis model. Aliquots (10  $\mu$ g of protein) were submitted to electrophoresis in polyacrilamida gel at 10%, containing gelatine 2 mg/mL, as substrate. The activities of the metalloproteinase activities MMP-2 and MMP-9 were quantified by densitometry of the bands in gel slabs (A) and compared to the control (B). The data represents the mean  $\pm$  standard error.

creased activity of MMP-2 compared to the control group (3 fold), we noticed the onset of the activity of MMP-9 (Figure 3. A, B).

## DISCUSSION

The experimental model using DSS has as main clinical manifestations: diarrhea, bloody stools, weight loss, bleeding, and anemia<sup>(29)</sup>. The histological changes in the colon most commonly found are the decrease mucin, epithelial degeneration, presence of erosions, infiltration of neutrophils and crypt abscesses<sup>(22)</sup>. These manifestations and histopathological changes were also observed in our study, in addition to shortening of the length of the colon evaluated macroscopically. The diarrhea observed in the animals of group DSS from the third day may be due to increased permeability of the intestinal cells or by hyperosmolarity in the lumen caused by DSS<sup>(31)</sup>. The weight loss observed and shortening of the colon shows high correlation with the histological and pathological changes of colitis and are good markers of the severity of bowel inflammation<sup>(29, 31)</sup>. Allied to these factors, the worsening of the disease occurs in the presence of acute bleeding evaluated by DAI and hematological measurements<sup>(12, 29)</sup>. We observed a significant reduction in erythrocyte count, hematocrit and hemoglobin in the group DSS, indicating that these animals suffer large losses of blood.

The histopathological features observed in this animal model are very similar to the disease in humans<sup>(22)</sup>. In DSS colitis it was observed the presence of edema and inflammatory infiltrate in the lamina propria, loss of crypts and goblet cells (loss of mucins observed by alcian blue) along with multifocal areas of erosions and ulcerations demonstrating great tissue damage in the colon. The inflammatory infiltrate was observed

and histologically confirmed by myeloperoxidase activity (MPO), a marker of inflammatory infiltrate often used in the model of colitis by DSS. MPO is a major enzyme found in the granules of neutrophils, it primarily catalyzes the oxidation of Cl<sup>-</sup> to hypochlorous acid and its increase is involved in tissue damage<sup>(23)</sup>. In our study, we observed a high activity of MPO in the DSS group compared to the control group ( $P < 0.001$ ). Recent studies also showed that reduction in the degradation of matrix present in the intestinal mucosa can be related to a reduction in MPO activity and improves the course of the disease in an experimental model of colitis<sup>(13, 14)</sup>.

Activity of extracellular matrix metalloproteinases is currently discussed in terms of its involvement with inflammatory bowel diseases. These enzymes and their inhibitors are produced by cells in the gastrointestinal tract, and a change in this balance can cause some inflammatory conditions in the intestine<sup>(15)</sup>. Within this group of enzymes stands, MMP-2 and MMP-9, known as gelatinases. MMP-2 is constitutively expressive while MMP-9 is absent in major human tissues<sup>(9)</sup>. Some lines of research suggests that these enzymes are involved in the process of destruction and tissue remodeling in inflammatory conditions<sup>(3, 28)</sup>.

Our study shows a high activity of MMP-9 (45 fold increase) in the group DSS, while there was a slight increase in MMP-2 (three fold increase). Studies in patients with ulcerative colitis showed greater activity of MMP-9 compared to the control patients<sup>(1)</sup>. Studies on experimental models have already shown that animals deficient on MMP-9 attenuated colitis demonstrating that gelatinase is involved in inflammatory processes and its inhibition can reduce inflammation<sup>(25)</sup>. This increase in MMP-9 may relate to the high neutrophil infiltration observed by tissue MPO activity as well as by high tissue damage visualized in histological analysis since these enzymes are involved in the degradation of ECM.

## CONCLUSIONS

Our results demonstrate that there is a correlation between the DAI and the degree of pathological alterations in the colon of the animals in the induced by DSS group,

and these changes can be caused principally by increased expression/activity mainly on the MMP-9 and myeloperoxidase produced by inflammatory cells. Being these enzymes a possible therapeutic target for the reduction of tissue injury in treating IBD.

Oliveira LG, Cunha AL, Duarte AC, Castañon MCMN, Chebli JMF, Aguiar JAK. Correlação positiva entre índice de atividade de doença e metaloproteínase de matriz em modelo de colite em ratos. *Arq Gastroenterol.* 2014;51(2):107-12.

**RESUMO - Contexto** - Doenças inflamatórias intestinais, entre elas colite ulcerativa e doença de Crohn, compreendem um amplo espectro de doenças que apresentam em comum inflamação crônica do trato gastrointestinal, alterações histológicas e um aumento de atividade de determinadas enzimas, tais como, metaloproteínases. **Objetivo** - Avaliar possível correlação do índice de atividade de doença em modelo de colite ulcerativa induzida por dextran sulfato de sódio com o grau de severidade de danos na mucosa colônica e aumento de atividade de metaloproteínases. **Métodos** - Colite foi induzida por administração oral de dextran sulfato de sódio 5% durante sete dias no grupo (n = 10), enquanto que o grupo controle (n = 16) recebeu água. Efeitos foram analisados diariamente pelo índice de atividade de doença. No sétimo dia, os animais foram sacrificados e as medições hematológicas, alterações histológicas (hematoxilina e eosina e coloração de azul Alcian), mieloperoxidase e atividades de metaloproteínases (MMP-2 e MMP-9) foram determinados. **Resultados** - Grupo dextran sulfato de sódio mostrou elevação no índice de atividade de doença e redução dos parâmetros hematológicos. A indução da colite causa lesão no tecido, com perda de mucina e aumento da mieloperoxidase ( $P < 0,001$ ) e as atividades MMP-9 (45 vezes) em comparação com o grupo de controle. **Conclusão** - Neste estudo, observamos uma correlação do índice de atividade de doença com o grau de alterações histopatológicas após indução da colite por dextran sulfato de sódio, podendo associar este resultado ao aumento principalmente da atividade de MMP-9 e de mieloperoxidase.

**DESCRITORES** - Colite. Doença de Crohn. Metaloproteínase.

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