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INTRAPERITONEAL INSUFFLATION OF CARBON-DIOXIDE RESCUES INTESTINAL DAMAGE IN AN EXPERIMENTAL MURIN MODEL OF COLITIS

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ABSTRACT

Objectives

Necrotizing enterocolitis (NEC) is a severe neonatal surgical condition, associated with a prolonged pro-inflammatory state, leading to high mortality and morbidity rates. Carbon-dioxide (CO₂) insufflation during laparoscopy may have an anti-inflammatory effect. We aimed to evaluate the effects of CO₂-insufflation on experimental colitis.

Methods

Acute colitis was induced in 6 weeks-old Balb/c mice by the administration of 2%-Dextran Sulfate-Sodium (DSS) during 7 days (n=45). On day 4, 2 groups received intraperitoneal insufflation (duration: 30mn, pressure: 5 mmHg) of CO₂ ("DSS+CO₂") or air ("DSS+air"). A group received no insufflation ("DSS"). Groups were compared for clinical severity using the disease activity index (DAI - body weight loss, stool consistency, bleeding), histological severity (histopathological activity index, colon length, ulcerations), colonic mucosecretion, and inflammation.

Results

DAI was significantly decreased in DSS+CO₂ group, compared to DSS (p<0.0001) or DSS+air (p<0.0001) groups. Colon length was increased in DSS+CO₂ treated mice compared to DSS (p=0.0002). The histopathological activity index was lower in DSS+CO₂ (vs DSS, p=0.0059 /vs DSS+air, p=0.0389), with decreased ulcerations (3.77 vs 10.7, p=0.0306), and persistent mucosecretion with increased mucin-secreting cells.

Conclusions

CO₂-insufflation attenuates DSS-induced colitis and improves both clinical and histological scores. Laparoscopy with CO₂ insufflation represents a therapeutic anti-inflammatory strategy for NEC.

Key Words: CO₂-insufflation; Necrotizing enterocolitis; laparoscopy; Dextran Sulfate Sodium; acute colitis;

What is known:

- Necrotizing enterocolitis (NEC) is a severe neonatal surgical condition, associated with a prolonged pro-inflammatory state, leading to high mortality and morbidity rates.
- CO₂-insufflation during laparoscopy may have an anti-inflammatory effect.

What is new :

- This study is the first to demonstrate a beneficial effect of CO₂-insufflation on an experimental model of acute colitis, both on clinical and histological levels. CO₂-insufflation attenuates DSS-induced colitis and improves both clinical and histological scores.
- Laparoscopy with CO₂ insufflation represents a therapeutic anti-inflammatory strategy for NEC, which could have beneficial effects on short-term and long-term outcome of these fragile babies

I. INTRODUCTION

Necrotizing enterocolitis (NEC) is a severe gastrointestinal surgical emergency, that occurs in one out of 1000 neonates¹. NEC is associated with a high mortality rate, reaching 50% in neonates requiring surgery¹⁻³.

Despite advances in neonatal care and surgery, NEC remains a devastating disease, as both diagnosis and treatment are challenging in this fragile cohort of preterm neonates^{1,4,5}. Conservative treatment without surgery used to be the gold-standard, but this paradigm has shifted in the last decades, due to recent research highlighting the effects of prolonged systemic inflammation on the child's neurodevelopmental outcome⁶.

The anti-inflammatory effect of laparoscopy compared to laparotomy has already been reported in several clinical studies, notably in patients with complicated appendicitis⁷⁻⁹. This effect could be explained by the insufflation of carbon dioxide (CO₂) during laparoscopy, as experimental studies have demonstrated that CO₂ may modify the inflammatory response to injury¹⁰⁻¹³. This effect has also been reported on an experimental model of peritonitis exploring the direct anti-inflammatory effect on the peritoneum itself¹⁴. To the best of our knowledge, none of these experimental studies reported the effect of CO₂ on bowel epithelium and the underlying mechanism is only partially understood. This potential anti-inflammatory effect mediated by CO₂-insufflation during laparoscopy may represent a real therapeutic strategy in neonates with NEC.

The aim of this study was to investigate the effect of intra-peritoneal CO₂ insufflation on an experimental model of acute colitis.

II. METHODS:

- **Experimental outline**

All procedures were reviewed and approved by the ethical committee Paris-Nord n°121 (APAFIS No #17452). Five weeks-old male Balb/c mice wild type (Janvier Laboratories, France), weighing between 18 and 24 g, were housed in cages where standard chow and water were available *ad libitum*. The animal housing environment was maintained at a temperature of 21°C with a 12-hour light/dark cycle. Mice were allowed to acclimate to these conditions for at least 7 days before inclusion in experiments.

- **Experimental model of colitis in mice (Figure 1)**

Acute colitis was induced by dextran sodium sulfate (DSS) administration¹⁵. Mice were treated with 2%-DSS in drinking water (molecular weight 40 kDa; Sigma-Aldrich Saint Louis, Missouri, USA) which was given *ad libitum* for 7 days. The volume drunk by each mouse was monitored every day.

- **Intraperitoneal insufflation**

All procedures were performed under aseptic conditions. On day 4 of DSS administration (time period that corresponds to the inflexion point of the weight curve (onset of inflammation), anesthesia was induced in an isoflurane chamber and maintained using vaporized isoflurane delivered through a nosecone. Two randomized mouse groups received a treatment with intraperitoneal insufflation of either CO₂ (DSS+CO₂, n=15) or air (DSS+air, n=15) (Figure 1). CO₂ pneumoperitoneum was achieved by delivering CO₂ through a 22-gauge angiocatheter (BD Insite Autoguard BC) placed percutaneously through the abdominal wall. Insufflation pressure was maintained at 5 mmHg during 30 minutes using a laparoscopic insufflator (Laparoflator 26012, Storz, Germany). Air pneumoperitoneum was achieved using a sphygmomanometer, with an insufflation pressure maintained at 5 mmHg during 30 minutes. For the control group (DSS alone, n=15), an angiocatheter was introduced in the peritoneum through the abdominal cavity and remained clogged for 30 minutes, to prevent any exchange

of gas with the environment. All animals were euthanized by cervical dislocation after 7 days of DSS administration for colonic harvesting.

- **Clinical assessment of colitis**

After DSS administration, clinical severity was assessed daily by a single observer, who carried out this observation blindly, without knowing the affiliation of the mice. This was performed using the disease activity index (DAI), a composite score described by Cooper et al., including body weight loss, stool consistency, and gross bleeding¹⁶. Each parameter was scored as shown in Table 1. The total score ranged from 0 (unaffected) to 12 (severe colitis).

- **Histological assessment of colitis**

Histological severity was assessed using colon length, histopathological activity index (HAI), and percentage of ulcerated area.

After harvesting on day 7, colon length was measured from the ileocecal junction to the end of the distal rectum after applying a gentle stretch on the colon. Subsequently, specimens were rapidly collected and washed with PBS to remove stools and then fixed in 4% paraformaldehyde (Sigma) for 24 h. Segments from proximal and distal colon were removed and washed with cold PBS. The collected fragments were fixed with 10% neutral buffered Formalin. Colon samples were then embedded in paraffin blocks using the Swiss Roll technique, and cut into 4 μ m sections¹⁷. Paraffin-embedded sections were stained with haematoxylin-eosin and for mucin with Alcian blue/periodic acid-Schiff (AB/PAS).

Histological severity was characterized by the presence of ulcerations, inflammatory cell infiltration, signs of edema, crypt loss, epithelial cell hyperplasia, and decrease in goblet cells. The histopathological activity index (HAI) was calculated as described by Erben et al.¹⁸ and Laroui et al.¹⁹ using the presence of inflammatory cell infiltrate, crypt damage, and ulceration. The total score ranged from 0 (unaffected) to 11 (severe colitis) (Additional Table 2). Histological grading was performed by an experienced pathologist in a blinded manner.

Ulceration was defined as the complete loss of a crypt associated with total loss of surface epithelium. All slides were scanned and analyzed using the NDPViewer software to assess the proportion of ulcerated mucosa surface (in mm²) related to the total surface. The proportion of ulcerated area was expressed as a percentage of the total area of the Swiss Roll for each mouse¹⁷.

- **Assessment of secretion of mucus and pro-inflammatory cytokines**

Alcian Blue/Periodic Acid Schiff (AB/PAS) was used to detect mucopolysaccharides such as mucins. AB/PAS positive cells were counted using the NDPViewer software, in order to evaluate the possible loss of mucosecretion, in 3 areas of 0.1mm². Each 0.1 mm² rectangle indicates territories where the basal loss of the crypts was at least a third, thus making it possible to study the number of AB/PAS positive cells reflecting functional mucosecretion.

- **Immunohistochemistry**

To determine the effect of intraperitoneal CO₂ insufflation on inflammation in DSS-induced colitis, expressions of inflammatory markers, including Interleukin 6 (IL-6), Mucin-2 (MUC-2) and Myeloperoxidase (MPO), were studied.

IL-6 is a pleiotropic cytokine whose actions include modulation of proliferation, differentiation and maturation of hematopoietic progenitors, and control of cellular metabolic activities. It is considered as a pro-inflammatory cytokine, and its concentration is elevated in DSS colitis models.

MUC-2 is specifically expressed in caliciform cells of the small intestine and colon. Early loss of mucosecretion, particularly MUC-2, is observed in inflammatory bowel disease and other ulcerative colitis.

MPO, a lysosomal enzyme, is a heme-containing peroxidase expressed mainly in neutrophils and, to a lesser extent, in monocytes. MPO has been shown to be a local mediator of tissue damage and resulting inflammation in various inflammatory diseases.

The expression of these proteins were analyzed by immunohistochemistry, according to the protocol described below:

Paraffin-embedded slides were deparaffinized and incubated in 3% hydrogen peroxide.

Antigen retrieval was obtained by boiling sections for 2 minutes in citrate buffer (pH 6.0). Slides were then incubated with the following primary antibodies: anti-IL-6 (1:50; ab208113, Abcam, United Kingdom), anti-MUC-2 (1:100; sc-15334, Santa Cruz Biotechnology, United States) or anti-MPO (1:100; ab9535, Abcam, United Kingdom). Appropriate secondary antibodies (Horse Anti-Rabbit, Vector Laboratories, United States) were then added and slides were incubated at room temperature for 30 min. Color was developed using the AEC substrate kit (GBI Labs, United States) followed by counterstaining with Mayer's hematoxylin (Sigma Aldrich). Staining intensity was assessed using ImageJ (software version 2.0.0), with ICTN plugin (Image-based Tool for Counting Nuclei).

- **Statistical analysis**

All data were expressed as mean \pm SEM. Statistical analysis was performed using GraphPad Prism software version 8 (GraphPad Software Inc., La Jolla, CA, USA). Data were compared using Mann-Whitney U tests, Kruskal-Wallis with Dunn's multiple comparison tests, or one way ANOVA with Tukey's multiple comparison tests when appropriate.

Statistical significance was defined as p-values <0.05 .

III. RESULTS:

- **CO₂ insufflation attenuates the clinical severity of DSS-induced colitis**

All the animals survived until the 7-day experimental period. Drinking volume was checked every day and was similar for the three groups. Acute colitis induced by DSS manifested as diarrhea and bleeding associated with a significant decrease in body weight. Body weight loss

significantly improved after CO₂ insufflation (1.02 ± 0.010), compared to DSS alone mice (0.975 ± 0.013 ; $p=0.027$) (Figure 2A).

At day 7 of DSS-administration, clinical severity of colitis assessed using DAI was decreased in mice treated with CO₂ insufflation (1.50 ± 0.429), compared to the DSS alone group (4.47 ± 0.456 , $p < 0.0001$) and to DSS-treated mice with air insufflation (4.33 ± 0.36 ; $p < 0.0001$) (Figure 2B). Colon length was significantly increased in the DSS+CO₂-treated group (8.54 ± 0.177) compared to the DSS alone (6.97 ± 0.262 , $p=0.0002$) and DSS+Air-treated group (7.53 ± 0.277 , $p=0.0166$) (Figure 2C) Macroscopically, more inflammation was observed in colon to DSS+Air and DSS alone groups, compared to the DSS+CO₂-treated group, with a redder and bloodier edematous appearance, in agreement with the clinical score found previously (Figure 2D).

- **CO₂ insufflation attenuates the histological damage in DSS-induced colitis**

Histological damage related to colitis was assessed using the histological activity index (HAI), and the percentage of ulcerated area.

Histological analysis of colons of mice treated with DSS alone showed severe inflammation with ulcerations and leukocyte infiltration spread throughout the mucosa and sub-mucosa layers (Figure 2E). The HAI significantly decreased after CO₂ insufflation (5.9 ± 0.82) compared to the DSS-alone (9.2 ± 0.53 , $p=0.0059$) and DSS+Air-treated groups (8.4 ± 0.79 ; $p = 0.0389$) (Figure 2F).

Furthermore, with CO₂ pneumoperitoneum, histological scores revealed a decrease in colonic architectural disruption at day 7 with partial preservation of the epithelial barrier and goblet cells associated with decreased destruction of the crypts and immune cell infiltration.

The percentage of ulcerated colonic surface was decreased after CO₂ insufflation, compared to DSS-treated mice (3.77 ± 1.41 vs 10.7 ± 2.58 , $p = 0.0306$). There was no significant difference between the DSS +air and DSS alone groups (9.38 ± 2.14 vs 10.7 ± 2.58 , $p > 0.05$) (Figure 2G).

- **CO₂ insufflation reduces goblet cell loss and decreases pro-inflammatory cytokine production in DSS-induced colitis**

Colons from CO₂-treated mice showed a persistence of mucus-secreting cells in ulcerated areas unlike the two other groups. As a result, CO₂ insufflation was associated with an increased number of AB/PAS positive cells compared with the DSS+Air (79.8±4.19 vs 36.8 ±4.21; p=0.0006) and DSS alone group (79.8±4.19 vs 36.1±4.34; p=0.0007) (Figure 3A). There was no difference between the DSS+Air and DSS alone groups (36.8 ±4.21 vs 36.1±4.34; p>0.05). Moreover, CO₂-insufflation was associated with an increase in MUC-2 positive cells, compared to DSS only mice (117±17 vs 55±9; p=0.0146) (Figure 3B). The number of MUC-2 positive cells was also higher in the DSS+CO₂ group compared to the DSS+Air group (117±17 vs 64±5; p=0.0892), even if this difference was not statistically significant. These results are in agreement with those found previously when counting cells marked with AB/PAS, and confirm the positive influence of CO₂ on mucosecretion.

Furthermore, a trend towards decreased local inflammation after CO₂ insufflation was observed with a reduced number of both IL-6 or MPO positive cells compared to DSS alone and DSS+Air groups (Figure 3C). The number of IL-6+ cells was lower in the colons of mice exposed to CO₂ than in in the DSS+Air group (683±133 vs 949 ±191; p=0.32) and DSS alone (683±133 vs 1308±285; p=0.077), although these differences were not statistically significant. Results for MPO-positive cells were not statistically significant between groups, probably due to weak immunostaining.

IV. **DISCUSSION**

In this study, we showed that intra-peritoneal CO₂ insufflation attenuates clinical and histological severity of colitis.

These findings were obtained using a well-described model of colitis in 5-weeks old mice. The choice of this model is related to the fact that mice are the smallest animals suitable for this work on inflammation and intraperitoneal insufflation. Intraperitoneal CO₂ insufflation in mice is easily performed, reproducible, and requires only two abdominal opening points for catheters. Previous studies on the effects of carbon dioxide pneumoperitoneum were performed in Balb/c mice²⁰. This murine model is perfectly suited for our study. Indeed, Balb/c mice, due to their sensitivity to DSS treatment, develop colitis with a constant severity²¹.

Previous studies have showed a beneficial effect of CO₂ insufflation in several clinically relevant models, such as pancreatitis or peritonitis. Machado *et al.* showed that abdominal insufflation with CO₂ before induction of acute pancreatitis caused a significant decrease in ascites volume, cells, and TNF alpha in the peritoneal cavity and in serum TNF alpha and IL-6²². In the pancreas, CO₂ insufflation reduced MPO activity, acinar, and fat necrosis suggesting that CO₂-pneumoperitoneum reduces pancreatic inflammation and attenuates systemic inflammatory response in acute pancreatitis. Others have shown that CO₂-pneumoperitoneum reduces levels of TNF-alpha mRNA, IL-6, IL-1 receptor antagonist, and soluble TNF receptor-1 in mice²³. In a murine model of polymicrobial peritonitis, CO₂ pneumoperitoneum improved survival compared to laparotomy, whereas helium pneumoperitoneum failed to show this effect¹⁴. Moreover, CO₂-pneumoperitoneum has been shown to increase survival in rodents challenged with endotoxin²⁴. In this latter study, abdominal insufflation of CO₂, but not helium or air, significantly reduced mortality among animals with LPS-induced sepsis.

However, to the best of our knowledge, our study is the first to address the effects of CO₂ pneumoperitoneum on a model of severe colitis. We found that insufflating CO₂ within the peritoneal cavity during 30 minutes significantly improved the clinical phenotype of colitis and rescued the histological damage induced by DSS.

The beneficial effects of CO₂ could be mediated by inflammation modulation. Indeed, CO₂ administration has been reported to decrease pro-inflammatory cytokines, such as IL-6 and TNF-alpha, and to increase levels of the anti-inflammatory cytokine IL-10^{12,22,24}. CO₂-pneumoperitoneum has been reported to modify the inflammatory response to injury. *In vitro* experiments have shown that CO₂ administration inhibits the production of pro-inflammatory cytokines by peritoneal macrophages in response to LPS stimulation¹¹. Similarly, this inhibition was specifically obtained on macrophages incubated with CO₂, whereas this effect was not observed with helium or air²⁵. In our study, CO₂ pneumoperitoneum reduced histological signs of inflammation, such as the inflammatory cell infiltrate, and the number of IL-6 and MPO positive cells.

Acute ulcerative colitis hampers the general homeostasis of gut, resulting in decreased mucus production and epithelial cell renewal. Our results indicated that CO₂ pneumoperitoneum promoted MUC-2 expression, which is a high-molecular weight glycoprotein, specifically expressed in intestinal goblet cells. Goblet cells are specialized secretory cells found throughout mucosal epithelia, and play an important role in maintaining tissue homeostasis by secreting various factors, including proteins and mucins, all of which contribute to the mucus layer that protects the mucosal epithelium²⁶. Our study demonstrated mucus modulation as another aspect of the protection rendered by CO₂ pneumoperitoneum against acute inflammation. A higher number of goblet cells quantified histologically as compared to the DSS indicated a mucus-mediated protective mechanism during acute inflammation. Immunohistochemistry of MUC2 supported these histopathological findings. The expression of MUC-2 was more important in the colon of CO₂-treated mice, with significantly more positive cells quantified compared to DSS only mice.

The beneficial effects of CO₂ insufflation on the outcome on colitis and local inflammation suggests that CO₂ insufflation during laparoscopy could represent an innovative therapy for

neonates with NEC. Indeed, inflammation yields a crucial role in the local and systemic damage associated with NEC. NEC is associated with an increased expression of inflammatory cytokines, such as TNF- α , IL-1 β , IL-6 and IL-8 in both affected tissues and plasma²⁷. IL-6 is predominantly generated by activated macrophages, T-cells and the endothelium, but is also expressed by enterocytes in response to infection. IL-6 stimulates the production of acute phase proteins in the liver, and plays a role in B cell proliferation and antibody production. Morecroft et al. studied IL-6 levels in neonates with NEC and reported that plasmatic IL-6 levels correlated with the clinical severity of NEC²⁸. In our study, CO₂ pneumoperitoneum showed a trend towards a decreased expression of IL-6 in colons of DSS-induced mice.

The mechanism underlying the beneficial effects of CO₂ insufflations remains only partially understood. Creating a more acidic environment and impairing the cytokine production by the macrophages could be an explanation. The upstream mechanisms of this anti-inflammatory profile are yet to be identified, but interestingly, studies described increased peripheral IL-10 production in animals submitted to acidification of the peritoneal cavity, whether induced by CO₂-pneumoperitoneum or by peritoneal acidic buffered lavage²⁹. Several pathways, that have a demonstrated role in the development of enterocolitis, such as oxidative stress, are yet to be explored, in the setting of CO₂ pneumoperitoneum³⁰.

We acknowledge that this study has some limitations. The experiments were designed to assess the clinical and histological changes that occur after CO₂ insufflation in the acute phase of a model of colitis, but not the long-term. Further studies should be performed to assess whether this effect is maintained in the long-term. In future studies, assessing the effects of CO₂ insufflation on the parameters of blood gases and respiration, as well as the long-term cerebral impact of colitis-related inflammation may be considered. Moreover, additional studies are being performed to identify the pathways involved in the effects of CO₂ insufflation.

In conclusion, this study is the first to demonstrate a beneficial effect of CO₂-insufflation on an experimental model of acute colitis, both on clinical and histological levels. CO₂-insufflation was associated with improved clinical and histological scores, colon length, and mucosecretion. CO₂-insufflation rescues DSS-induced colonic damage, by modulating mucosecretion by goblet cells and reducing the secretion of pro-inflammatory cytokines.

Introducing laparoscopy in the management of preterm infants with necrotizing enterocolitis may reduce both local and systemic inflammation, which could have beneficial effects on short-term and long-term outcome of these fragile babies. Further investigations are needed to better understand the underlying mechanisms associated with CO₂ insufflation, in order to optimize the management of children with NEC.

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Figure legends

Figure 1. Experimental outline and intra-peritoneal insufflation under general anesthesia, using a 22-Gauge catheter. A: before insufflation. B: during insufflation.

Figure 2. Clinical assessment and histological assessment of colitis severity.

A. Percentage of body weight loss. Results are expressed as mean \pm SEM for each group.

*P < 0.05, versus DSS only-treated mice.

B. Disease Activity Index. Results are expressed as mean \pm SEM for each group.

**** P < 0.0001, versus DSS-treated mice.

C. Colon length.

D. Macroscopic appearance of colons.

E. Severe inflammation with ulcerations and leukocyte infiltration spread throughout the mucosa and sub-mucosa layers in DSS only-treated mice. Images were taken with a 3 \times objective.

F. Histological activity index. Results are expressed as means \pm SEM for each group.

*P < 0.05, **P < 0.01, ***P < 0.001.

G. Percentage of ulcerated colonic surface.

Figure 3. Mucosecretion and inflammation

A. AB/PAS positive cells. On the left, persistence of cells stained with AB/PAS in an ulcerated area of the colon of CO₂ mice at D7. On the right, absence of cells stained with AB/PAS, in an ulcerated area of control mice (DSS alone) at D7.

B. MUC-2 IHC staining. Images were taken with a 6 \times objective.

C. IL-6 . IHC staining. Images were taken with a 16 \times objective.

Table legends

Table 1. Disease activity index.

Animals were assessed daily for weight loss, stool consistency, and bleeding. The total score ranged from 0 (unaffected) and 12 (severe colitis).

Additional Table 2. Histological scoring.

Colon samples were assessed for inflammatory cell infiltrate, crypt damage, and ulceration. The total score ranged from 0 (unaffected) to 11 (severe colitis).



