

# CO2 Pneumoperitoneum

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Laparoscopy (LS) has been shown to decrease the inflammatory sequelae of endotoxemia.  $\beta$ -arrestin 2 plays an important function in signal transduction pathway of TLR4. High mobility group box-1 (HMGB-1) is involved in the delayed systemic inflammatory response. We investigated the effects of CO<sub>2</sub> insufflation on liver, lung, and kidney expression of both  $\beta$ -arrestin 2 and HMGB-1 during sepsis. Cecal ligation and puncture (CLP) was performed in male rats and 6 h later the animals were randomly assigned to receive a CO<sub>2</sub> pneumoperitoneum or laparotomy. Animals were euthanized; liver, lung, and kidney were removed for the evaluation of  $\beta$ -arrestin 2 and HMGB-1 expression. Immunohistochemical detection of myeloperoxidase (MPO) was investigated in lung and liver and bacterial load was determined in the peritoneal fluid. CO<sub>2</sub> pneumoperitoneum reduced peritoneal bacterial load, increased the expression of  $\beta$ -arrestin 2, and blunted the expression of the potent proinflammatory HMGB-1 in liver, lung, and kidney compared with laparotomy. Liver and lung MPO was markedly reduced in rats subjected to LS compared with laparotomy. We believe that CO<sub>2</sub> exerts an early protective effect by reducing bacterial load and likely toll-like receptor activation which in turn leads to a preserved  $\beta$ -arrestin 2 expression and a reduced HMGB-1 expression.

Keywords: Hmgb ; Laparoscopy ; mobility ; inflammatory response ; arrestin ; liver ; lung ; pneumoperitoneum ; Myeloperoxidase ; X3b2

## 1. Introduction

The better preservation of immune function during laparoscopy compared with open surgery seems to be maintained under septic conditions but the underlying mechanisms that explain such results are still not completely understood. Several experimental evidences suggest that CO<sub>2</sub> pneumoperitoneum reduces the severity of sepsis and prolongs survival, compared with laparotomy in animal models of peritonitis <sup>[1][2][3]</sup>. However, little is known about the CO<sub>2</sub> pneumoperitoneum impact on early acute phase response in sepsis. Recently, we demonstrated that CO<sub>2</sub> pneumoperitoneum reduces inflammatory response by reducing early liver and lung interleukin (IL) 6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) expression in cecal ligation and puncture rat model <sup>[4]</sup>. Other studies have reported that CO<sub>2</sub> insufflation could also influence intra-abdominal bacterial contamination in murine models of abdominal sepsis <sup>[5][6]</sup>. In this regard,  $\beta$ -arrestins play a critical role as modulators of inflammatory response.  $\beta$ -arrestins are peculiar adaptor proteins involved in G-protein-coupled receptor (GPCR) desensitization and are also implicated in regulation of Toll-like receptor signalling and proinflammatory gene expression. Both  $\beta$ -arrestins 1 and 2 differentially seem to regulate TLR4 signalling pathways. In particular, it has been suggested that  $\beta$ -arrestin 2 is a negative regulator of inflammatory response in CLP-induced mortality <sup>[7][8][9]</sup>.

HMGB-1 is another potent proinflammatory cytokine involved in the delayed endotoxin lethality and systemic inflammatory response <sup>[10]</sup>. The majority of HMGB-1 secreted by activated monocyte/macrophages is from a preformed cellular pool within the first 16 h <sup>[11]</sup>; HMGB-1 mRNA levels are increased in various tissues such as muscle, liver, and lung following endotoxemia <sup>[12]</sup>. Subsequently, increased cellular synthesis of HMGB-1 further reinforces their release into extracellular milieu <sup>[11]</sup>. In addition, cells undergoing unprogrammed cell death can passively release HMGB-1, which worsens and prolongs inflammation <sup>[13]</sup>. It has been reported that HMGB-1 can induce cellular activation and generate inflammatory responses by interaction of both TLR2 and TLR4 <sup>[14]</sup>. Finally, HMGB-1 seems to function as an "alarmin" signal to recruit, alert, and activate innate immune cells, sustaining a potentially injurious inflammatory response <sup>[15]</sup>. In light of these considerations and supported by our previous data, we performed this study, in order to better understand for the first time whether the role of CO<sub>2</sub> pneumoperitoneum might be related to a reduced bacterial count and consequently to a decreased activation of the inflammatory cascade via TLR activation in an experimental model of CLP.  $\beta$ -arrestin 2 and HMGB-1 protein expressions are investigated in liver, lung, and kidney as target sites of septic injury.

## 2. Materials and Methods

### 2.1. Cecal Ligation and Puncture

For this study, a total of 21 animals purchased from Charles River (Calco, Lecco, Italy) were used. All procedures complied with the standards for the care and use of animal subjects, as stated in the Guide for the Care and Use of laboratory Animals, and were approved by the Committee on Animal Health and Care of the University of Messina. CLP was performed in 14 male Sprague-Dawley rats as previously described <sup>[8]</sup>. Specifically, rats were anaesthetized with ether, and a midline incision was made below the diaphragm to expose the cecum. The cecum was ligated at the colon juncture with a 2-0 silk ligature suture without interrupting intestinal continuity. The cecum was punctured twice with an 18-

gauge needle. The cecum was returned to the abdomen, and the incision was closed in layers with a 2-0 silk ligature suture. Six hours following CLP, animals were randomly selected to undergo CO<sub>2</sub> pneumoperitoneum (LS) at a pressure of 5–7 mmHg or laparotomy (LT) for 1 hour under general anaesthesia with sodium pentobarbital (50 mg/kg/i.p.). Pneumoperitoneum was achieved by introducing a Veress needle into the peritoneal cavity and by insufflating the abdomen with CO<sub>2</sub> using the Wisap CO<sub>2</sub> gas insufflator (Wisap, Sauerlach, Germany). The remaining seven animals did not undergo CLP, were left untreated, and were used as controls (naïve). At the end of the procedures, all animals were sacrificed: peritoneal fluid was collected and liver, lung, and kidney were excised and stored at –20°C or in 10% buffered formalin for further analysis.

## 2.2. Bacterial Load

To determine the bacterial load in the peritoneum, the peritoneal cavity was washed with 5 mL of sterile phosphate-buffered saline (PBS) and diluted with sterile PBS. One hundred microlitres of each dilution was then plated on chocolate agar plates (Fisher Scientific, Pittsburgh, PA) and incubated at 37°C for 24 hours under aerobic conditions. CFU were counted and results were expressed as CFU/mL of peritoneal fluid.

## 2.3. Isolation of Cytoplasmic Proteins and Western Blot Analysis for $\beta$ -Arrestin 2 and HMGB-1

Liver, lung, and kidney samples were homogenized in 1 mL lysis buffer (25 mM tris/HCl, pH 7.4, 1.0 mM ethylene glycol tetra-acetic acid, 1.0 mM ethylenediamine tetra-acetic acid, 0.5 mM phenylmethylsulfonyl fluoride, aprotinin, leupeptin, pepstatin A (10  $\mu$ g/mL each), and Na<sub>3</sub>VO<sub>4</sub> 100 mM). The homogenate was subjected to centrifugation at 15,000  $\times$ g for 15 min. The supernatant was collected and stored at –80°C. The concentration of total proteins was determined using a Bio-Rad protein assay kit (Milan, Italy).

$\beta$ -arrestin 2 and HMGB-1 were evaluated by western blot, according to the methods described previously [9]. Primary antibodies for  $\beta$ -arrestin 2 and HMGB-1 were purchased by Abcam (Cambridge, UK). Secondary antibodies peroxidase conjugated were obtained by Pierce (Rockford, Illinois, USA). The protein signals were quantified by scanning using a bioimage analysis system (Bio-Profil Celbio, Milan, Italy) and were expressed as integrated intensity in comparison with those of control animals measured with the same batch.  $\beta$ -actin (Cell Signaling) antibody was used on stripped blots to confirm equal protein loading.

## 2.4. Immunohistochemistry

An immunohistochemical study was performed to evaluate the presence (or absence) of an inflammatory reaction in liver and lung specimens. All specimens were tested with myeloperoxidase (Thermo, Fremont, CA) as an index of neutrophil accumulation. Slices of 5  $\mu$ m were rehydrated in graded alcohol and antigen retrieval was performed with pH 6.0 buffer citrate and endogenous peroxidase (i.e., from red blood cells) blocking with 1% H<sub>2</sub>O<sub>2</sub> in PBS. Primary antibody was incubated overnight at 4°C in a moisturized chamber and the day after secondary antibody was added and reaction visualized with DAB. Counterstaining was performed to identify nuclei in the specimens.

## 2.5. Statistical Analysis

All data are expressed as mean  $\pm$  standard deviation (SD). Significance of difference was assessed by two-way repeated-measures analysis of variance (ANOVA) followed by post hoc analyses where indicated. A value of  $P < 0.05$  was considered statistically significant. Graphs were produced using GraphPad Prism (version 4.0 for Windows).

# 3. Results

## 3.1. CO<sub>2</sub> Pneumoperitoneum Reduces Bacterial Load in Peritoneal Fluid

The bacterial load in peritoneal cavity was determined by CFU count. Cecal ligation and puncture following LT produced a marked bacterial load in the peritoneal fluid compared with naïve animals (Figure 1).

CO<sub>2</sub> pneumoperitoneum (LS) significantly reduced bacterial loads in peritoneal cavity compared with animals subjected to laparotomy (LT) (Figure 1;  $4 \times 10^6$  versus  $7 \times 10^6$  CFU/mL, resp.,  $P < 0.05$ ) suggesting that an antimicrobial effect may concur to the protective effect of CO<sub>2</sub>.

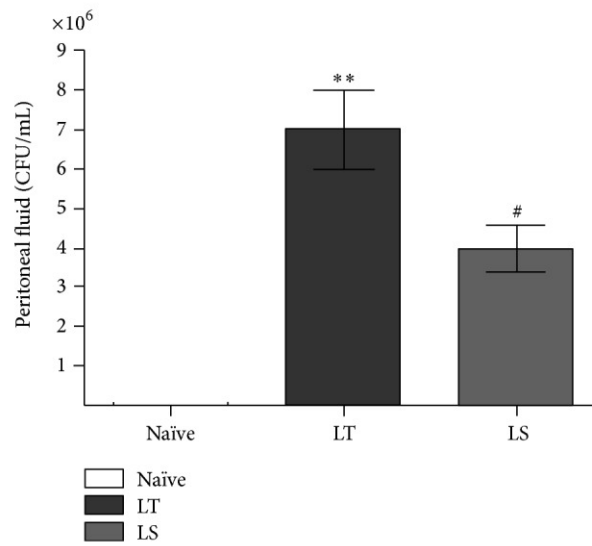


Figure 1. Bacterial count analysis in peritoneal fluid cultures (CFU/mL) from animals subjected to CLP followed by LT or LS procedures and naïve animals. Data represent the mean  $\pm$  SD of seven animals. \*\*  $P < 0.001$  compared with naïve; #  $P < 0.05$  compared with LT.

### 3.2. CO<sub>2</sub> Pneumoperitoneum Regulates Liver, Lung, and Kidney Expression of $\beta$ -Arrestin 2

To determine whether  $\beta$ -arrestin 2 plays a key role in modulating TLR signalling pathway in CLP-induced inflammation, we first evaluated CO<sub>2</sub> effects on  $\beta$ -arrestin 2 expression in liver, lung, and kidney. Animals that underwent laparotomy (LT) showed significantly reduced  $\beta$ -arrestin 2 protein expression in these organs compared with naïve group ( $P < 0.05$ ). CO<sub>2</sub> pneumoperitoneum preserved  $\beta$ -arrestin 2 expression in liver, lung, and kidney (LS), displaying higher levels of the protein compared with laparotomy (Figures 2(a), 2(b), and 2(c),  $P < 0.05$ ).

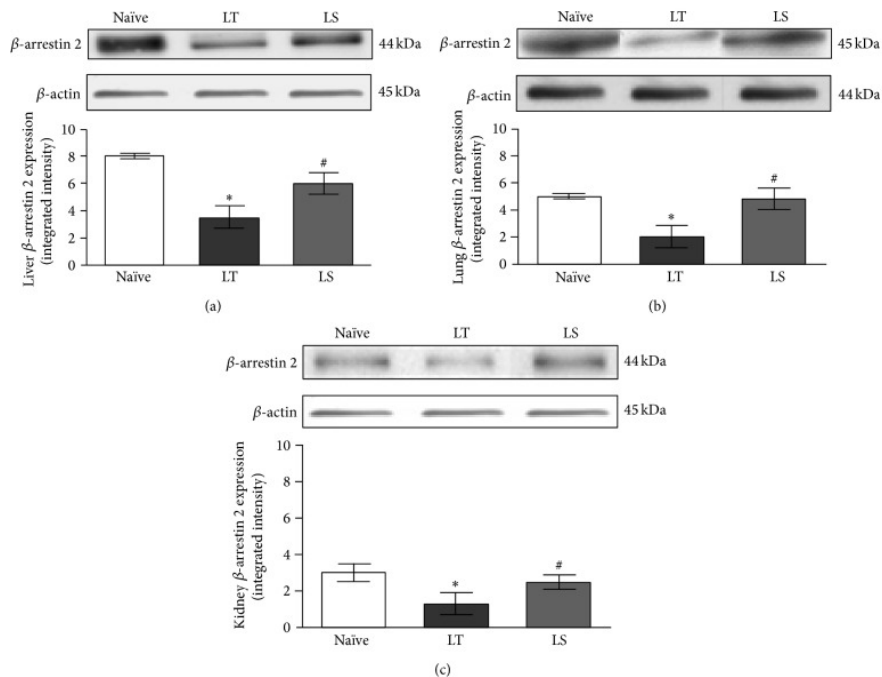


Figure 2. Representative western blot analysis of  $\beta$ -arrestin 2 protein in liver (a), lung (b), and kidney (c) from animals subjected to CLP followed by LT or LS procedures and naïve animals. Data represent the mean  $\pm$  SD of seven animals. \*  $P < 0.05$  compared with naïve; #  $P < 0.05$  compared with LT.

### 3.3. CO<sub>2</sub> Pneumoperitoneum Reduces Liver, Lung, and Kidney Expression of HMGB-1

HMGB-1 is known as late mediator in sepsis, but it can also be released early after a traumatic insult. Therefore, we studied the CO<sub>2</sub> pneumoperitoneum effects on CLP-induced HMGB-1 expression in liver, lung, and kidney of rats. Animals subjected to laparotomy showed a significant increase of HMGB-1 protein expression in liver, lung, and kidney ( $P < 0.05$ ) compared with naïve animals (Figures 3(a), 3(b), and 3(c)). CO<sub>2</sub> pneumoperitoneum markedly reduced the expression of this cytokine in liver, lung, and kidney tissues (LS) with respect to the LT ( $P < 0.05$ ), suggesting a CO<sub>2</sub> protective role during polymicrobial sepsis.

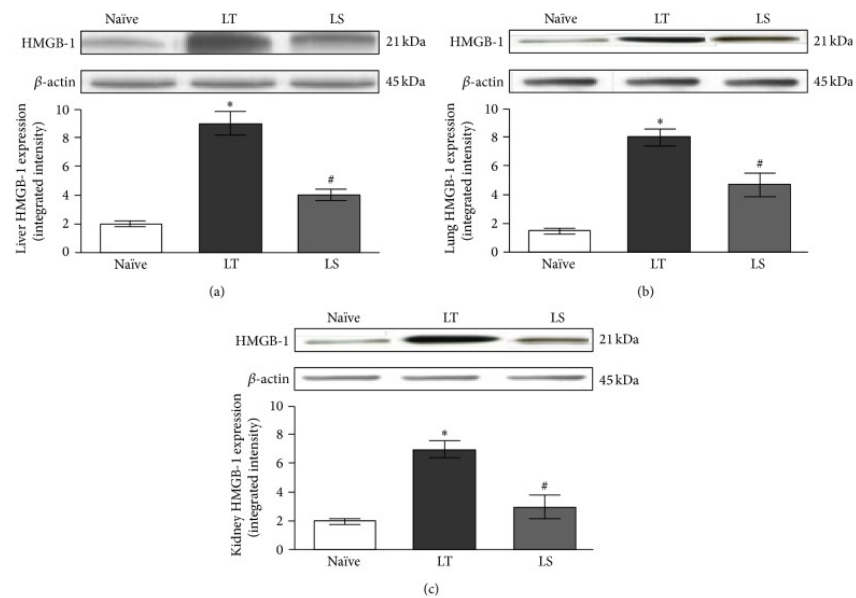


Figure 3. Representative western blot analysis of HMGB-1 protein in liver (a), lung (b), and kidney (c) from animals subjected to CLP followed by LT or LS procedures and naïve animals. Data represent the mean  $\pm$  SD of seven animals. \*  $P < 0.05$  compared with naïve; #  $P < 0.05$  compared with LT.

### 3.4. CO<sub>2</sub> Pneumoperitoneum Reduces Liver and Lung Neutrophil Accumulation

MPO staining for the evaluation of neutrophil infiltrate showed a strong inflammatory reaction in liver and lung of LT animals (Figures 4(b) and 5(b)) compared with naïve animals (Figures 4(a) and 5(a)). By contrary, rats subjected to CO<sub>2</sub> pneumoperitoneum (LS) revealed a reduced accumulation of inflammatory cells in liver and lung (Figures 4(c) and 5(c)), suggesting a protective role of this procedure regarding CLP.

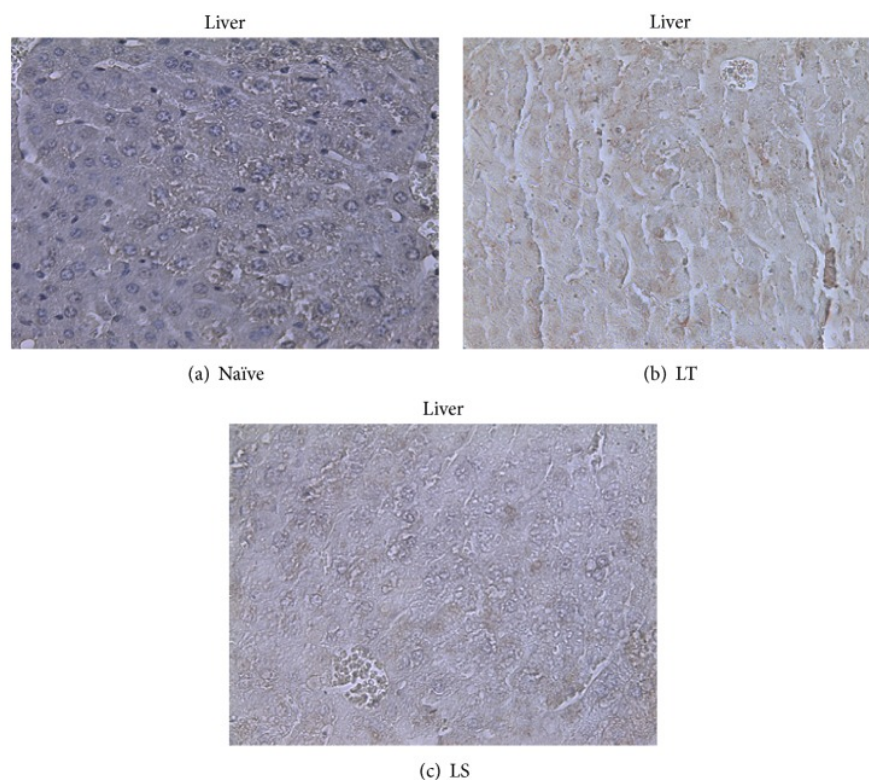


Figure 4. Immunohistochemical detection of myeloperoxidase in liver from naïve, LT, or LS animals. Samples collected from LT rats (b) show an increased inflammatory infiltrate and little, but still representative, edematous interstitial areas compared to naïve tissue samples (a). In contrast, samples from animals subjected to CO<sub>2</sub> pneumoperitoneum revealed a reduced accumulation of inflammatory cells (c). Original magnification  $\times 40$ .

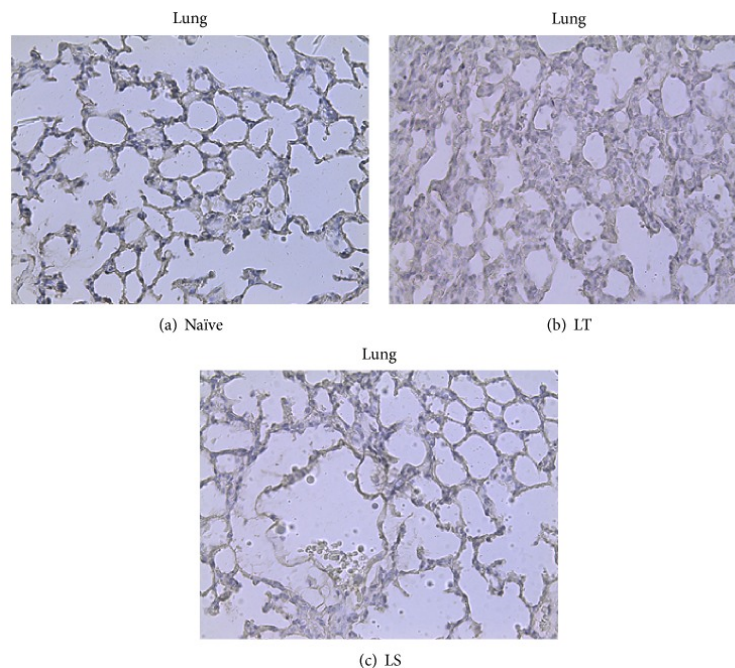


Figure 5. Immunohistochemical detection of myeloperoxidase in lung from naïve, LT, or LS animals. Samples collected from LT rats (b) show an increased inflammatory infiltrate and some grade of fibrosis of interalveolar septa compared to naïve tissue samples (a). In contrast, samples from animals subjected to CO<sub>2</sub> pneumoperitoneum revealed a reduced accumulation of inflammatory cells, as well as a substantial reduction of pulmonary fibrosis (c). Original magnification ×40.

## 4. Discussion

In today's surgical practice, laparoscopic treatment represents a regular part of therapeutic approach in septic surgical patients with localized and diffuse peritonitis [16]. Parallely, increasing studies delineate the physiopathological effects of pneumoperitoneum in septic conditions, where CO<sub>2</sub> seems to have a relevant biologic role. The immunological changes induced by pneumoperitoneum appear to contribute favourable outcomes to laparoscopy with increased survival in experimental model of sepsis [1,2,3]. However, this encouraging data is reflected on the final step of a common and complicated pathway. The upstream mechanisms by which CO<sub>2</sub> can affect the inflammatory process preserving immune function in septic state are not identified and not fully investigated. In sepsis, the massive deterioration of the immune response results in fact from an inappropriately amplified systemic inflammatory response to infection [17]. Exaggerated proinflammatory mediators production from macrophages, monocytes, or other host cells governs this crucial phase and can lead to the development of tissue damage until multiple organ failure and even death [18]. TLRs, expressed on the host cells, play a critical and primary role in the inflammatory responses [19]. Binding to TLR4 primes a series of signalling proteins that activate transcription factors and proinflammatory gene expression [20]. Recent studies have shown that  $\beta$ -arrestin 2, a member of the arrestin family, plays an important function in signal transduction pathway of TLR4 [7,21,22]. Similarly, HMGB-1, that has proinflammatory cytokine-like properties, can interact also with both TLR4 and TLR2 and prime inflammatory response that is similar to that initiated by LPS [14].

In a previous study, we have demonstrated an early and significant reduction of liver and lung TNF- $\alpha$  and IL-6 expression in CLP rat model undergoing CO<sub>2</sub> pneumoperitoneum. We also showed the correlation between early cytokine expression and histological alterations. In fact, in liver and lung specimens, these results were accompanied by a reduction in the inflammatory infiltrate, edema, venular ectasia, and cellular degeneration in animals subjected to CO<sub>2</sub> pneumoperitoneum compared with laparotomy. These data supported the possible protective role of CO<sub>2</sub> pneumoperitoneum in reducing the inflammatory cascade associated with sepsis via reduced expression of liver and lung proinflammatory cytokines [4]. Therefore, the main focus of the present study was to further investigate the possible upstream mechanisms of our preliminary findings consequent on CO<sub>2</sub> pneumoperitoneum in a murine model of polymicrobial sepsis that better reproduces human peritonitis. The current study is the first to explore the influence of CO<sub>2</sub> pneumoperitoneum on early  $\beta$ -arrestin 2 and HMGB-1 expressions in target organs of sepsis in correlation with peritoneal bacterial load and myeloperoxidase in a well-standardized rat model of abdominal sepsis. The data obtained by us demonstrate that CO<sub>2</sub> pneumoperitoneum exerts a dual beneficial effect both locally and on the organ level in CLP-induced inflammation.

Balagué et al. reported that the number of bacterial CFU obtained in peritoneal fluid was significantly lower in the laparoscopic groups than in the open group in a defined bacterial inoculum model [5]. In a similar experiment, Pitombo and coworkers demonstrate, 18 h after bacterial inoculum into the peritoneum, a significant increase of CFU/mL for the laparotomy group compared with CO<sub>2</sub> and control groups [6]. In our study, the microbiological evaluation of peritoneal fluid collected after CLP and pneumoperitoneum revealed that CO<sub>2</sub> significantly reduced the number of colony forming units in this group of animals compared to the LS group. These intriguing data induce thinking primarily that CO<sub>2</sub> can have a

specific and local action. Its prominent role in bacterial control in the storage and preservation of food products is known [23][24][25]. The potential antimicrobial effect of the gas if translated in the current study could explain the correlation between microbial inactivation (reduced bacterial count) and intra-abdominal exposure to CO<sub>2</sub>.

Looking at  $\beta$ -arrestin 2 results, we found a significant high expression in liver, lung, and kidney of animals subjected to laparoscopy following CLP compared with laparotomy animals and, in previous experiment, CLP-induced TNF-alpha and IL-6 expression turned out to be significantly higher in LT group with respect to the LS [4]. These data suggest that  $\beta$ -arrestin 2 is, in agreement with the literature, an essential negative regulator of inflammatory response in polymicrobial sepsis [7][8][9][26][27] via TLR signalling. In particular, our investigation reveals that CO<sub>2</sub> insufflation preserves  $\beta$ -arrestin 2 expression in the organs studied. This result if correlated with blunted CLP-induced increase in TNF-alpha and IL-6 in liver and lung tissues [4] supports the hypothesis that  $\beta$ -arrestin 2 improved expression is protective in a model of abdominal sepsis.

Regarding HMGB-1, we demonstrated, according to literature, an increase in liver, lung, and kidney expression of this proinflammatory protein in rats subjected to laparotomy following CLP. In contrast, after CO<sub>2</sub> insufflation, we had a significant reduction of this cytokine.

Our data also showed an increased neutrophil infiltration in liver and lung of LT animals. On the contrary, CO<sub>2</sub> pneumoperitoneum significantly reduced inflammatory cells, confirming its beneficial role in this animal model of peritonitis.

Altogether, our data might be taken into account in order to explain additional mechanism(s) of CO<sub>2</sub> beneficial effects in experimental sepsis. It is in fact likely that the reduced bacterial load induced by antimicrobial action of CO<sub>2</sub> leads to a reduced release of bacterial components as lipopolysaccharides. This could downregulate the Toll-like receptors activation that mediate antimicrobial responses. Consequently, the potential  $\beta$ -arrestin 2 and HMGB-1 interactions with TLRs could allow both a preservation of  $\beta$ -arrestin 2 and a reduction of HMGB-1 expression. We speculate that all these composite changes can correlate with a better anti-inflammatory effect exerted by  $\beta$ -arrestin 2 and in addition also justify the subsequent reduced proinflammatory cytokine expressions and tissue damage contributing to corroborating the previously reported results.

In conclusion, our study contributes to reinforcing the hypothesis that laparoscopic surgery better controls abdominal infection and favourably modifies the immune response reducing the inflammatory septic cascade. In particular, by our results, it can be evicted that CO<sub>2</sub> exerts a precocious and protective effect by reduced bacterial load with (and) likely Toll-like receptors activation which in turn lead to a preserved  $\beta$ -arrestin 2 expression, reduced HMGB-1 expression, and myeloperoxidase in this animal model of peritonitis.

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