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Ebselen Attenuates Lung Injury in Experimental Model of Carrageenan-Induced Pleurisy in Rats

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Abstract—The study evaluates the role of Ebselen (Eb), an organoselenium compound in animal model of acute lung injury induced by carrageenan (CG). *Wistar* rats received saline or 2 % λ-carrageenan in the pleural cavity, and treatment with Eb (50 mg/kg intragastrically) or dexamethasone (Dx) (0.5 mg/kg intraperitoneal) after CG administration. After 4 h, rats were euthanized and the pleural exudate removed for analysis of the total cell count, total protein, lactate dehydrogenase, and nitrite/nitrate. Moreover, lung tissue were removed to verify the myeloperoxidase activity and oxidative damage. Eb showed anti-inflammatory activity by inhibiting leukocyte influx, myeloperoxidase activity, and nitrite/nitrate concentration. Eb presented with an anti-inflammatory activity similar to Dx and an antioxidant activity better than Dx. This study suggests that Eb plays an important role against the oxidative damage associated with anti-inflammatory activity in animal model of acute lung injury, proving to be similar or potentially more effective than Dx.

KEY WORDS: acute lung injury; pleurisy; ebselen; inflammation; oxidative damage.

INTRODUCTION

Pleurisy induced by carrageenan (CG) is a well-characterized model of experimental inflammation, which allows the quantification of mediators involved in cell changes during the inflammatory process [1]. This model is characterized by infiltration of neutrophils, followed by lung injury by reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical (OH^-), and peroxynitrite ($ONOO^-$), as well as the liberation of other chemical mediators derived from active neutrophils [2].

During the inflammatory response, the ROS and RNS modulate phagocytosis, gene expression, and apoptosis. In pathological circumstances, such as acute lung injury and sepsis, the excess production of ROS and RNS can change epithelial and endothelial cells, contributing to the amplification of damage to the inflamed tissue [3]. The oxidative stress results in activation of redox transcription factors, such as nuclear factor-kB (NF-kB) and AP-1, which play a crucial role in the induction of inflammatory cytokines and intercellular adhesion molecule-1 (ICAM-1) [4]. However, in order to avoid the oxidative damage induced by ROS, the body is equipped with several lines of antioxidant defense, in which the enzymatic line consists of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) [5].

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Selenium, an element of the human diet, has been widely studied to prevent several diseases such as inflammatory disorders, associated with the action of ROS [6]. Trace elements of selenium plays an important role in the catalytic center of GPx, an enzyme which catalyzes the reduction of hydroperoxides to free radicals, protecting the cells from oxidative stress [7]. It has been observed that Ebselen (Eb) [2-phenyl-1,2-benzisoselenazol-3[2H]-1], a lipid-soluble organoselenium can potentially inhibit the lipid peroxidation through the action of GPx mimetic [8, 9].

Within this context, the present study was designed to investigate the effects of Eb on lung injury associated with carrageenan-induced pleurisy compared with glucocorticoid dexamethasone (Dx), known to have potent immunosuppressive effects as inhibitor of cytokine production and widely used in the management of inflammatory diseases [10]. For this purpose, we evaluated the following parameters: cell migration, lactate dehydrogenase (LDH) activity, total protein content, nitrite/nitrate concentration in pleural exudates; myeloperoxidase (MPO) activity and lipid and protein oxidative damage markers in lung tissue.

MATERIALS AND METHODS

Chemicals

Eb, thiobarbituric acid (TBA), dinitrophenylhydrazine (DNPH), 5,50-dithiobis (2-nitrobenzoic acid), hexadecyltrimethylammonium bromide, tetramethylbenzidine, 5,5-dithiobis (2-nitrobenzoic acid), and λ-carrageenan were purchased from Sigma Chemical Co. (St. Louis, US).

Animals

Adult male Wistar rats [weighing 250–350 g] obtained from Central Animal House of Universidade do Sul de Santa Catarina were used for induction of pleurisy. They were caged in groups of five, with free access to food and water, and were maintained on a 12-h light–dark cycle (lights on 7:00 a.m.), at a temperature of 22±1 °C. All experimental procedures were approved for Animal Care and Experimentation Committee of UNISUL (protocol number 12.015.4.03.IV), Brazil.

Induction of Pleurisy

Pleurisy was induced by CG as previously described [11]. The rats were anesthetized with ketamine hydrochloride and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected and saline (0.2 ml) or saline containing 2 % λ-carrageenan (0.2 ml) was injected into the pleural cavity. The skin incision was closed with a suture, and the animals received Eb (50 mg/kg, intragastrically i.g.) *via* gavage diluted in olive oil [12]. Four hours after the induction of pleurisy, the animals were killed and approximately 1 ml of pleural exudate from each animal was obtained for total cell count, LDH activity, total protein, and nitrite/nitrate concentrations. The lung was separated to evaluate inflammatory and oxidative damage parameters.

Experimental Groups and Treatments

The animals were divided into four groups ($n=6$ animals per group): group control was treated only with saline, receiving 0.2 ml saline into the intrapleural cavity; group CG received 0.2 ml of CG 2 % into the intrapleural cavity; CG+Eb received 0.2 ml of 2 % CG into the intrapleural cavity plus 50 mg/kg Eb intragastrically immediately after surgery; and group CG+Dx received 0.2 ml of 2 % CG into the intrapleural cavity plus 0.5 mg/kg Dx intraperitoneally immediately after surgery.

Inflammatory Parameters in Pleural Exudate

Neubauer chamber was used to count cells in the pleural exudate as an indicator of inflammation. It was performed by addition of the exudate solution with Türk liquid, for erythrocyte hemolysis. LDH activity was determined using commercial kits (Analisa—LDH UV-PP), and total protein quantity was measured by the Method of Lowry using bovine serum albumin as standard [13].

Nitrite/Nitrate Concentration in Pleural Exudate

Total nitrite concentration in the samples was measured using the Griess reaction, by adding 100 μl of Griess reagent (0.1 % [*w*/*v*] naphthylethylendiamide dihydrochloride in H_2O and 1 % [*w*/*v*] sulfanilamide in 5 % [*v*/*v*] concentrated H_3PO_4), vol. (1:1) to the 100 μl sample. Nitrite/nitrate concentration was measured spectrophotometrically with wavelength of 550 nm. The results were expressed as nitrite/nitrate concentration (nmol/mg protein) [14].

Myeloperoxidase Activity in Lung Tissue

Tissues were homogenized (50 mg/ml) in 0.5 % hexadecyltrimethylammonium bromide and centrifuged at 15,000×*g* for 40 min. The suspension was then sonicated

three times for 30 s. An aliquot of supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine and 1 mM H_2O_2. The absorbance was measured spectrophotometrically at 650 nm at 37 °C [15].

Lipid Peroxidation in the Lung Tissue

The formation of thiobarbituric acid reactive substances (TBARS) during an acid-heating reaction is widely adopted as a sensitive method for measurement of lipid peroxidation. Briefly, the samples of lung tissue were homogenized and mixed with 1 ml 10 % trichloroacetic acid and 1 ml 0.67 % TBA. Subsequently, they were heated in water bath at 100 °C during 30 min. The equivalent of malondialdehyde (MDA) was determined by absorbance of 532 nm using 1,1,3,3-tetramethoxypropane as an external standard. The results were expressed as equivalent of MDA [nmol/mg protein] [16].

Protein Carbonyl Content in Lung Tissue

The oxidative damage to proteins was assessed by the determination of carbonyl groups content, based on the reaction with DNPH. Briefly, the proteins were precipitated by the addition of 20 % trichloroacetic acid and redissolved in DNPH, and the absorbance was monitored at 370 nm. Carbonyl proteins were expressed as nanomoles per milligram protein [17].

Sulfhydryl Groups in Lung Tissue

Total thiol content in the lungs was determined using the 5,5-dithiobis (2-nitrobenzoic acid) (2-nitrobenzoic acid) method (DTNB). DTNB measurements were as described with some modifications [18]. Briefly, 30 μl of a sample was mixed with 1 ml of PBS/1 mM EDTA (pH 7.5). The reaction was started by the addition of 30 μl of 10 mM DTNB stock solution in PBS. Control samples, which did not include DTNB or protein, were run simultaneously. After 30 min of incubation at room temperature, the absorbance at 412 nm was measured and amounts of DTNB formed (equivalent to the amount of sulfhydryl (SH) groups) were calculated.

Quantification of Proteins

The quantity of proteins in determination of nitrite/nitrate concentration, MPO activity, lipoperoxidation, protein carbonyl, and sulphydryl groups was measured using the technique of Lowry [13]. When the Folin phenol reagent (phosphomolybdic-phosphotungstic reagent) was added, the Folin phenol reagent bound to the protein. The bound reagent was slowly reduced and changed in color from yellow to blue. Bovine serum albumin was used as standard and the absorbance was read at 700 nm.

Statistical Analysis

The results were expressed as mean ± SD, and $p < 0.05$ was considered significant. The differences among the groups were determined by one-way analysis of variance, followed by post hoc Tukey test. All the analyses were performed in SPSS 20.0 for Windows [SPSS, Chicago, IL].

RESULTS

The recruitment of inflammatory cells was higher in the pleural cavity in the group that received CG in relation to the control group (Fig. 1). However, these levels returned to the baseline following the administration of Eb, which was equivalent to Dx treatment results. Additionally, pleural exudate analysis showed that Eb reduced the LDH activity that was increased following CG administration, while Dx did not produce similar results (Fig. 2a). Eb and Dx presented a decrease in total protein levels when compared to the CG group (Fig. 2b). Furthermore, a significant decrease of nitrite/nitrate concentration in the pleural exudate was observed after the administration of Eb and Dx in relation to the animals that only received CG (Fig. 3).

The effects of Eb was verified by assessing MPO activity in neutrophilic infiltrate in the lung tissue. MPO activity was significantly increased 4 h after the administration of CG (Fig. 4). However, when Eb was administered, MPO activity was significantly reduced; similar to the results seen when treating with Dx. Lung injury induced by CG was also characterized by the increase of oxidative damage in lipids, carbonyl proteins and a decrease of integrity of sulfhydryl proteins (subpanels a, b, and c of Fig. 5, respectively). These levels were reversed with the administration of Eb, proving to be more effective than Dx.

DISCUSSION

In this study, we report the effects of the organoselenium compound Eb in a well established murine model for acute inflammation induced by CG. Acute administration of Eb exhibited pronounced anti-inflammatory actions, characterized by inhibiting influx of leukocytes into

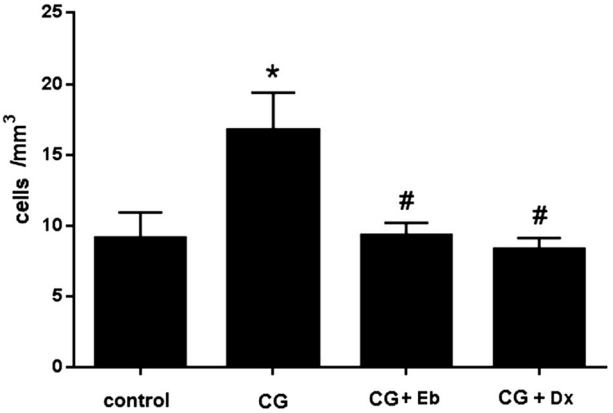

Fig. 1. Total leucocytes counts in the pleural exudate of the animals induced to pleurisy and treated with Eb. The results were expressed as mean ± SD, $n = 6$ by group and were considered significant for $*p < 0.05$ compared to the control group and $\#p < 0.05$ compared to the CG group. *CG* (carrageenan), *Eb* (ebselen), and *Dx* (dexamethasone).

the pleural cavity and decreasing nitrite/nitrate content. Moreover, Eb reduces oxidative damage in lipids, protein carbonyls, and free SH groups and increases cell viability. These findings suggest that the administration of Eb could effectively interfere with the inflammatory process in pleurisy induced by CG similarly, or often better than Dx .

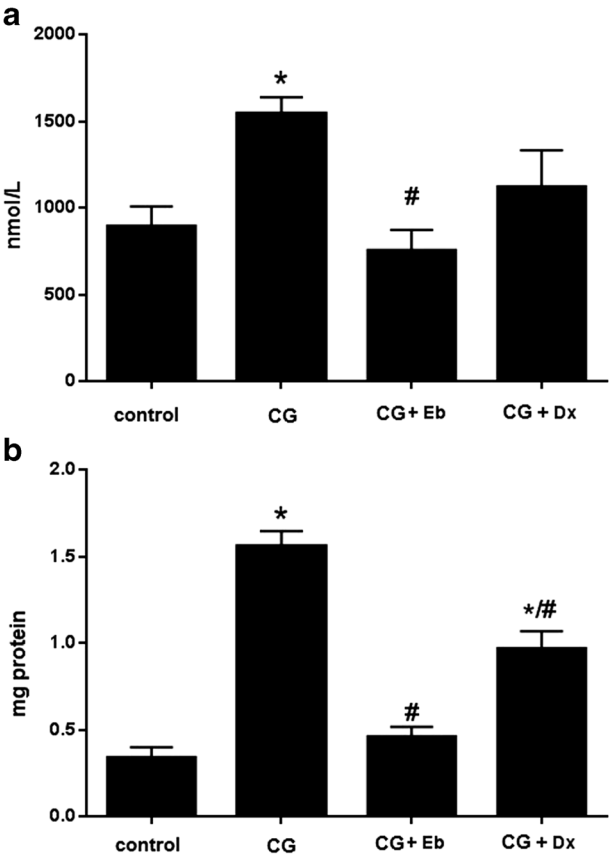

Fig. 2. Quantification of LDH (**a**) and total proteins (**b**) in the pleural exudate of the animals induced to pleurisy and treated with Eb. The results were expressed as mean ± SD, $n = 6$ by group and considered significant for $*p < 0.05$ compared to the control group and $\#p < 0.05$ compared to the CG group. *CG* (carrageenan), *Eb* (ebselen), and *Dx* (dexamethasone).

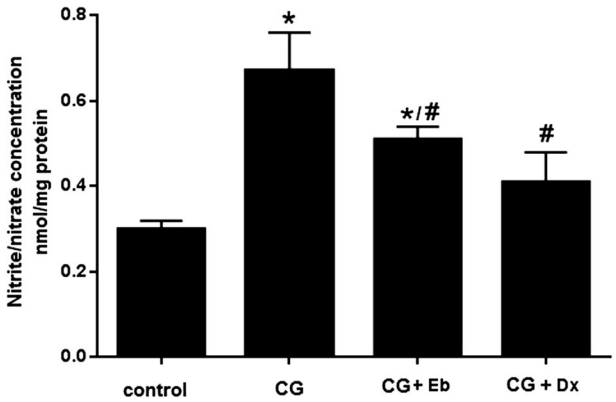

Fig. 3. Concentration of nitrite/nitrate in the pleural exudate of the animals induced to pleurisy and treated with Eb. The results were expressed as mean ± SD, $n=6$ by group and considered significant for $*p<0.05$ compared to the control group and $\#p<0.05$ compared to the CG group. *CG* (carrageenan), *Eb* (ebselen), and *Dx* (dexamethasone).

The model of pleurisy induced by CG has been widely used to investigate the mechanisms involved in the acute inflammation and also to test the effectiveness of anti-inflammatory drugs [19, 20]. The administration of CG results in an increased stimulus within the immune system, characterized by cell migration and, consequently, a continuous inflammatory process in the pleural serous layer, which leads to the pleura being highly permeable [21].

The predominance of neutrophils in pleural cavity is associated with the early inflammatory phases and can be found in important infectious and noninfectious conditions, such as parapneumonic effusion, viral infection, gastrointestinal disease, and acute tuberculous pleurisy [22]. We verified that the administration of Eb presented an anti-inflammatory response by inhibition of cell migration influx into the pleural cavity, with a similar effect to the commercial anti-inflammatory Dx. Furthermore, it is well recognized that levels of LDH and protein are markers of processes associated with the progression of pleural disease [23] and Eb in our study decreased these levels.

Evidences indicates that the increase in the formation of nitric oxide (NO) by nitric oxide synthase (NOS) may contribute negatively to the inflammatory process [24]. It has been proven that inhibitors of NOS activity reduces the development of inflammation induced by CG [24, 25]. Reduced quantities of NO decreases the permeability of endothelial membrane; which restricts the transmigration of polymorphonuclear (PMN) cells from the vascular compartment into the lung [26]. Even as the NO, the MPO activity is an indirect marker of active neutrophils and results in the exudation and migration of PMN cells [27]. After administration of CG, it was observed that the nitrite/nitrate concentration and the MPO activity were reduced in the pleural exudate and in the lung in animals treated with Eb in comparison to the nontreated group.

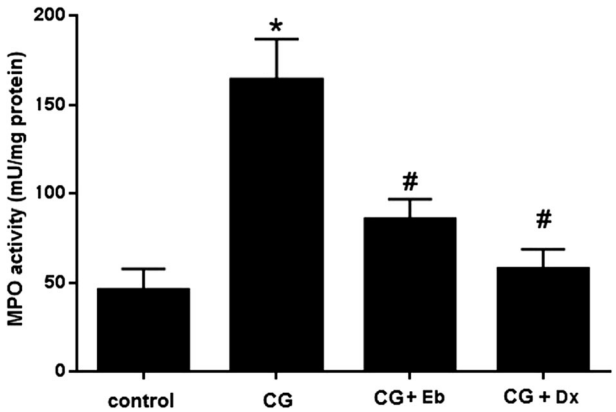

Fig. 4. Evaluation of MPO activity in the lung tissue of the animals induced to pleurisy and treated with Eb. The results were expressed as mean ± SD, $n=6$ by group and considered significant for $*p<0.05$ compared to the control group and $\#p<0.05$ compared to the CG group. *CG* (carrageenan), *Eb* (ebselen), and *Dx* (dexamethasone).

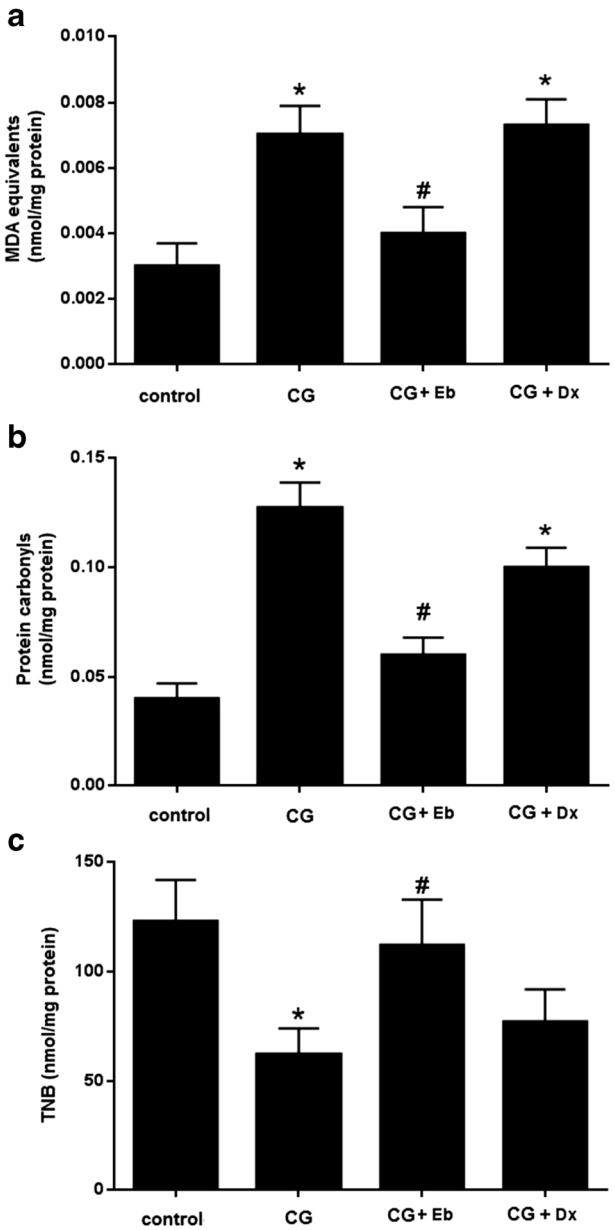

Fig. 5. Evaluation of lipid damage levels (**a**), carbonyl protein (**b**), and integrity of thiol groups (**c**) in the lung tissue of the animals induced to pleurisy and treated with Eb. The results were expressed as mean ± SD, $n = 6$ by group and considered significant for $*p < 0.05$ compared to the control group and $\#p < 0.05$ compared to the CG group. *CG* (carrageenan), *Eb* (ebselen), and *Dx* (dexamethasone).

Studies have suggested that oxidative stress is a result of inflammatory components of lung inflammation [27, 28]. Lung inflammation is generally characterized by extensive infiltration of PMN cells, which are important sources of ROS [28]. According to Guo and Ward [27], the lipid peroxidation plays an important role in lung inflammatory diseases. In the CG group, concentrations of malondialdehyde equivalent, a byproduct of lipid peroxidation, were increased in the lung. On the other hand, when Eb was administered, levels of malondialdehyde equivalent were substantially reduced with more evident results than Dx. Other oxidative indicators such as carbonyl proteins and the sediment of amino acids containing groups SH are sensitive targets for a variety of pro-oxidants [28]. Thus, in our model, it was verified that there is an increase in carbonyl proteins and a decrease of integrity of SH groups, and that Eb was effective in reversing these levels.

In conclusion, the current results supports the hypothesis that in CG-induced pleurisy, Eb has anti-inflammatory properties and antioxidant activity which can be related with previous studies of the mimetic role of glutathione peroxidase.

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