The role of estradiol in the maintenance of brain-dead organ donors

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CHAPTER 4

17β-Estradiol Treatment Protects Lungs Against Brain Death Effects in Female Rat Donor

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ABSTRACT

Brain death (BD) affects the viability of lungs for transplantation. A correlation exists between high lung inflammation after BD and the decrease in female sex hormones, especially estradiol. Therefore, we investigated the effects of 17β-estradiol (E2) treatment on the lungs of female brain-dead rats.

Female Wistar rats were divided into 4 groups: BD (submitted to BD for 6 h), sham (falseoperated), E2-T0 (treated with E2 immediately after BD; 50 µg/ml, 2 ml/h), and E2-T3 (treated with E2 after 3 h of BD; 50 µg/ml, 2 ml/h). Lung edema, hemorrhage, and leukocyte infiltration were analyzed. Adhesion molecules were evaluated and analysis of NO synthase gene and protein

expression was performed using RT-PCR and immunohistochemistry, respectively. Release of chemokines and matrix degradation in the lungs were analyzed.

BD increased leukocyte infiltration, as shown by intravital microscopy (P=0.017), bronchoalveolar lavage cell count (P=0.016), the release of inflammatory mediators (P=0.02), and expression of adhesion molecules. BD also increased microvascular permeability and the expression and activity of MMP-9 in the lungs. E2 treatment reduced leukocyte infiltration, especially in the E2-T3 group, release of inflammatory mediators, adhesion molecules, and MMP activity in the lungs.

E2 treatment was successful in controlling the lung inflammatory response in females submitted to BD. Our results suggest that E2 directly decreases the release of chemokines, restraining cell traffic into the lungs. Thus, E2 has a therapeutic potential, and its role in improving donor lung quality should be explored further.

INTRODUCTION

Lung transplantation is often the only therapy option for many patients with end-stage failure. There is a low number of multiorgan brain death (BD) donors, among which only about 15-20% of potential lungs are considered suitable for transplantation.1 BD initiates a systemic inflammatory process with innate immune system activation,2 cytokine release and expression of adhesion molecules, which mostly affects the lungs.3 Important hormonal and metabolic changes occur after hypothalamic/pituitary failure in experimental models of BD associated with previous events such as trauma and hemorrhage, thereby adding to the final inflammatory result of the organ donor.4

While lungs from female donors are considered to have a greater risk for transplant in male recipients,5,6 lung transplantation between women shows potentially better results.5 An experimental study7 showed that female rats presented greater lung inflammation, associated with an acute reduction of female sex hormones. The reduction of female sex hormones is known to be detrimental to the cardiovascular system,8 and clinical and experimental studies reinforce the E2 protector effect on the immune system, pulmonary inflammation, and proinflammatory mediator release.9,10 In this context, this study evaluated E2 treatment as a therapeutic alternative to mitigate the deleterious effects of BD, by investigating the pulmonary inflammatory response to its application in female rats subjected to BD.

MATERIAL AND METHODS

Animals

Female Wistar rats (60 days, n=52) were kept in a 23 ± 2°C, 12 h light-dark cycle, with free access to food and water. All rats received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the ’Guide for the Care, and the “Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources,

published by the National Institute of Health (NIH Publication No. 86-23, revised 1996). The experiments were approved by the University of São Paulo Medical School Ethics Committee for Research Projects Analysis (SDC No. 4350/16/016). The animals were divided into 4 groups: *sham*—animals submitted to cranial perforation, BD—animals submitted to BD, E2-T0—animals treated with E2 immediately after BD induction, and E2-T3—animals treated with E2, 3 h after BD induction.

Anesthesia and BD Model

Female rats in estrus or proestrus phases of the estral cycle (identified by vaginal smears) were incubated under isoflurane anesthesia. A catheter was placed in the carotid artery for measurement of mean arterial pressure (MAP) and the jugular vein for treatment and/or fluid administration (saline solution, 2 mL/h with cyclodextrin (E2 vehicle – Sigma-Aldrich®, USA)).

A Fogarty-4F catheter (Baxter Healthcare Co., USA) was inserted intracranially, and BD was induced by rapid inflation of the balloon with 400 µL of saline solution. BD was confirmed by maximally dilated and fixed pupils, apnea, absence of reflexes, and a drop in MAP. After BD, anesthesia was interrupted, and fluid administration was started. All animals were maintained for 6 h. At the end of experimental period, arterial blood samples were obtained via catheter to analyze oxygen and carbon dioxide partial pressures using gas analyzer (ABL555, Radiometer Medical ApS, Denmark).

E2 Treatment and Serum Quantification of Hormones

The rats received 50 µg/mL (Sigma-Aldrich®) intravenous E2, i.v. in saline solution (2 mL/h). The E2-T0 group started fluid replacement with E2 right after BD confirmation. The E2-T3 group started fluid replacement immediately after BD confirmation but E2 treatment commenced after 3 h. The sham and BD groups received fluid replacement only. Serum E2 and corticosterone concentrations were quantified using an ELISA kit (Cayman Chemical Company, USA) following the manufacturer's protocols.

Cell Counts on Bronchoalveolar Lavage (BAL)

After euthanasia by exsanguination, 5 mL of culture medium (RPMI, Sigma-Aldrich®) was repeatedly injected in the airways. The BAL fluid recovered was then centrifuged (239 × g, 15 min), the pellet re-suspended in 1 mL of RPMI and the total cell number determined using a Neubauer chamber.

In vivo Analysis of Leukocyte Infiltrate on Lung Microcirculation

The intravital microcopy technique was based on Schmidt et al.[11]. Accordingly, an incision was made on the left lateral decubitus to expose the intercostal muscles, and a small incision was made between the ribs to expose the inferior right lung lobule. Leukocytes were stained based on Baatz et al.[12] with Rhodamine 6G (9 µmol/kg, i.v., Sigma-Aldrich®) injected 10 min before the end of BD. Leukocyte infiltrate was accessed in images extracted from 1 min videos, counted in 3 distinct frames of the video, obtained with an epifluorescence microscope coupled camera. The results are expressed as cell/mm^2 of lung tissue.

Histopathological Lung Evaluation

Lung sections were stained with hematoxylin/eosin then digitized. Tissue areas (n = 5 per group, two sections per animal, five areas per section) were randomly analyzed for leukocyte infiltrate, edema, and hemorrhage with NIS-Elements Software Basic Research (Nikon, Japan) by two examiners. The air space/lung tissue ratio was measured, the air space area was disconsidered and the leukocyte number reported in cells/μm^2 of lung tissue. A reticle with 130 intersecting lines (total area 27,648 μm^2) was placed covering the images and each intersection when over edema or hemorrhage was considered as a unit, the result was expressed as units/μm^2.

Ex vivo Lung Tissue Culture

Lungs were perfused via the pulmonary artery and tissue culture performed according to Breithaupt-Faloppa et al. [13]. Fragments of lung parenchyma (four pieces per well) were incubated in 1 mL of Dulbecco's Modified Eagle Medium (DMEM, Vitrocell, Brazil) for 24 h at 37°C in a humidified atmosphere with 5% CO_2. After incubation, the medium was stored at -80°C and lung pieces were dried and weighed.

Quantification of Inflammatory Mediators in Lung Cultures and Lung Homogenate Samples

Cytokine-induced neutrophil chemoattractant (CINC)-1, macrophage inflammatory protein (MIP)-1α, and -2 were quantified in lung culture medium aliquots using a Milliplex MAP kit (EMD Millipore Corporation, USA) and expressed as pg/mL. Measurements were performed using Luminex 200-Sofware xPonent/Analyst 4.2 version (EMD Millipore Corporation). Interleukin (IL)-1β and tumor necrosis factor (TNF)-α were quantified in lung homogenates by ELISA following the manufacturer's instructions (R&D Systems, Inc., USA).

Immunohistochemistry

The left lung was expanded with 1:3 optimal cutting temperature media diluted in PBS and snap frozen in nitrogen-hexane solution. Lung cryosections (10 µm) were fixed in acetone for 10 min. Endogenous peroxidase blocking (H_2O_2, 2%) was followed by unspecific site blockage with BSA (1%) in Tris-buffered saline-tween (TBS-T). The sections were incubated at 37°C for 2 h with primary antibodies in TBS-T/BSA: anti-intercellular adhesion molecule (ICAM)-1 (1:100; BOOSTER, USA), anti-vascular cell adhesion molecule (VCAM) (1:200, Abcam, UK), and anti-endothelial nitric oxide synthase (eNOS) (1:250; Novus Biologicals, USA). Next, a 1:200 conjugated horseradish peroxidase (HRP) secondary antibody was applied to the sections at 37°C for 1 h (EMD Millipore Corporation, USA). Finally, staining was performed using HRP as substrate (3-amino-9-ethylcarbazole; Vector

Laboratories, USA) for 5-10 min. Stained areas were identified in vessel walls (VCAM and eNOS) or whole lung tissues (ICAM-1) after determining a threshold and analyzing the staining area fractions with an image analyzer (NIS – Elements; Nikon, Japan). Background was determined by sections incubated in the absence of the primary antibody (negative control).

To investigate matrix metalloproteinase (MMP)-9 and inducible nitric oxide synthase (iNOS) expression in lung tissue, paraffin sections (4 µm) were rehydrated and treated for antigen retrieval (citrate buffer (pH 6,0), 20 min, 95°C). Endogenous peroxidase was blocked (H_2O_2, 2%), then goat serum (5%) was used for blocking non-specific sites. MMP-9 immunohistochemistry was performed with 1:100 primary antibody (anti-MMP-9, Abcam, UK) and secondary antibody linked to alkaline phosphatase (MULTIVIEW® IHC kit, Enzo Life Sciences, USA). iNOS immunohistochemistry was performed using a 1:100 primary antibody (anti-iNOS, Abcam) and secondary antibody conjugated to horseradish peroxidase (HRP; EMD Millipore Corporation). Negative controls were performed by incubating the sections in the absence of the primary antibody. Stained areas were quantified by analysis of tissue with NIS-Elements software (Nikon).

Gene Expression Analysis

Lung samples were stored in stabilization reagent at -80°C. Using commercial kit mirVana Kit (Ambion, USA) RNA was extracted. Then, cDNA was transcribed with reverse transcriptase (High-Capacity Reverse Transcriptase Kit, Applied Biosystems, USA). Real-time (RT)-PCR) was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan primers (Applied Biosystems) against glyceraldeyde-3-phosphate dehydrogenase (GAPDH), β-actin, ICAM-1, VCAM, and MMP-9 (Table 3). Targets were amplified over one cycle for 2 min at 50°C, one cycle for 10 min at 95°C, and 40 cycles for 15 s at 95°C and for 1 min at 60°C.

In parallel, β-actin, IL-1β, and TNF-α target genes (Table S1) were amplified with SYBR®Green primers (Applied Biosystems) and SYBR®Green PCR Master Mix (Applied Biosystems) over one cycle

for 2 min at 50°C, one cycle for 10 minutes at 95°C, and 40 cycles for 15 s at 95°C and for 1 min at 60°C.

MMP Activity Assay

The MMP activity assay was carried out with lung homogenate samples using a commercial fluorescent and specific substrate kit (Gelatinase Assay kit, BioVision, USA) according to the manufacturer's instructions. Analysis was performed using a fluorescence microplate reader (Multi-Mode Reader; Biotek, USA) in kinetic mode.

Lung Microvascular Leakage

Evans Blue (EB) dye extravasation was used to evaluate lung microvascular permeability[7]. The EB dye was injected (20 mg/kg, intravenously; Sigma-Aldrich®) 20 min before the end of the BD period. Dye was extracted from tissue then quantified by spectrophotometry (620 nm) with a standard curve (0.3-100 µg/mL). Data were expressed as µg/mg of dry weight.

Homogenization of the Lung Tissue

Lung fragments were weighed and dissociated in PBS containing 0.5% hexadecyltrimethylammonium bromide and 5-mM EDTA (pH 6.0, 3mL/kg; gentleMACS Dissociator (Miltenyi Biotec, Germany)). The supernatants were stored at − -80°C for analysis.

Determination of Total Nitric Oxide

Samples of lung homogenate were used to determine the total nitric oxide (NO_x^-), nitrite (NO_2^-), and nitrate (NO_3^-). We followed the method described by Breithaupt-Faloppa et al.[13]. Accordingly, nitrates were reduced to nitrites then measured with Griess reagent reaction (540 nm). The level of nitrites was determined with a standard curve of $NaNO_2$ (5-60 µM).

Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). The data were analyzed by ANOVA followed by Tukey, Dunnett's, or Sidak multiple comparison tests. Abnormally distributed data were analyzed using the Kruskal– Wallis test followed by a post hoc Dunn multiple comparison test. Statistical analysis was performed using GraphPad Prism Software v.8.3.1.

RESULTS

Arterial blood gases and serum concentrations of corticosterone and estradiol

The BD model applied here involves the sudden increase in intracranial pressure, leading to a transient hypertensive crisis followed by sustained hypotension, as previously shown[7]. This mean arterial pressure (MAP) pattern was observed in all studied groups (data not shown). Additionally, measurements of partial pressure of oxygen (O_2) and carbon dioxide (CO_2) were taken after the end of the 6h experiment in all groups (Table 1). Partial pressure of O_2 or CO_2 were not modified by the brain death, compared to Sham and remained similar in animals treated with E2.

Data on estradiol and corticosterone serum concentrations is shown in Table 1. Brain death rats presented reduction of both hormones in comparison to Sham. The E2 treated rats presented similarly elevated estradiol serum concentration compared to BD group.

Table 1 – Quantification of estradiol and corticosterone in serum samples (pg/ml), and oxygen and carbon dioxide partial pressure (mmHg). The female rats were subjected to BD (BD). In the treated groups, animals were submitted to BD and continuously treated with 17β-estradiol (E2) immediately after BD confirmation (E2-T0) or after 3h of BD (E2-T3). Controls were false operated animals (Sham).

Data expressed as mean ± SEM from 8 animals. $^{*,\gamma,\alpha}$ p ≤0.05 in relation to BD.

	Sham	BD	E2-T0	E2-T3	P ANOVA
Estradiol	482.2 ± 54.2*	68.2 ± 8.0	1713.5 ± 44.7$^{\alpha}$	1717.9 ± 29.2$^{\gamma}$	<0.0001
Corticosterone	3458.4 ± 929.9*	22.6 ± 10.4	16.8 ± 8.1	40.7 ± 31.1	0.0001
PaO_2	255.29 ± 34.48	195.15 ± 18.08	185.57 ± 22.16	186.68 ± 39.92	0.3176
$PaCO_2$	26.7 ± 3.75	33.56 ± 4.33	32.07 ± 3.84	38.84 ± 5.29	0.3630

The female rats were subjected to brain death (BD). In the treated groups, animals were submitted to BD and continuously treated with 17β-estradiol (E2) immediately after BD confirmation (E2-T0) or after 3 h of BD (E2-T3). Controls were false operated animals (Sham).
Data expressed as mean ± SEM from 8 animals.
aP ≤ 0.05 in relation to BD.

Estradiol effects on lung leukocyte infiltrate, edema and hemorrhage

Figure 1A illustrates the bronchoalveolar lavage (BAL) cell count. After BD, the BAL cell counts increased and the E2 treatments (E2-T0 and E2-T3) reduced the cell numbers in BAL. The lung in vivo quantification of leukocyte infiltration (intravital microscopy) after 6 h of BD showed increased leukocyte infiltration into the BD group and that the 3 h treatment (E2-T3) reduced leukocyte infiltration (Figure 1 B).

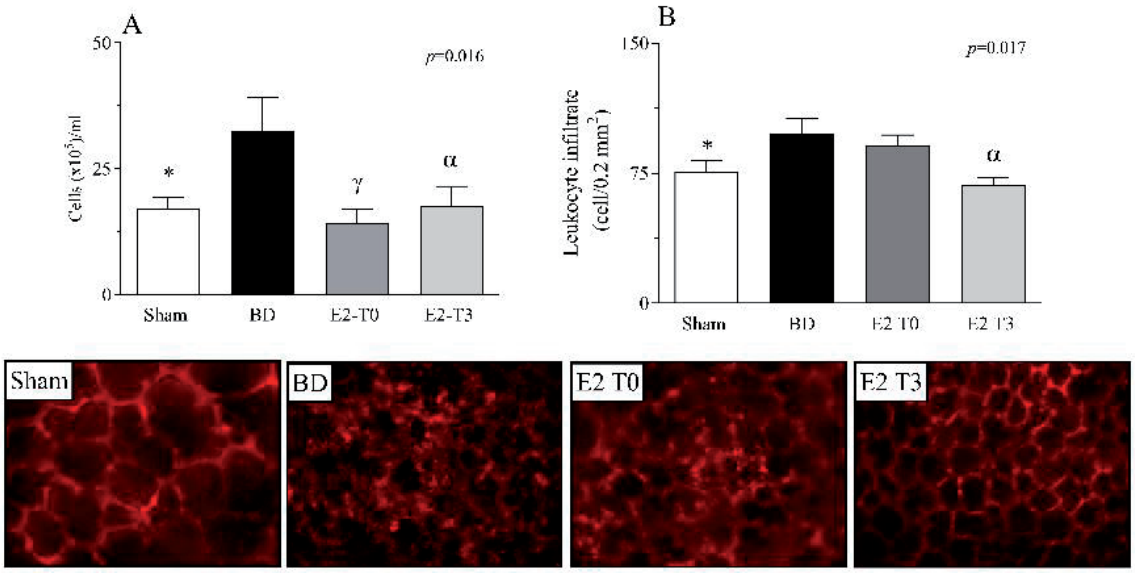
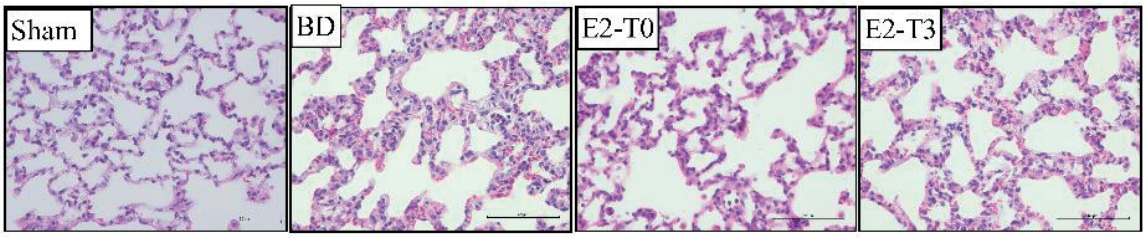


Figure 1 – Cell counts in the bronchoalveolar lavage (BAL) (A) and lung *in vivo* evaluation of leukocyte infiltrate using intravital macroscopy. The female rats were subjected to BD (BD). In the treated groups, animals were submitted to BD and continuously treated with 17β-estradiol (E2) immediately after BD confirmation (E2-T0) or after 3h of BD (E2-

T3). Controls were false operated animals (Sham). Data are expressed as mean ± SEM from 8 animals (A) and 5 animals (B). $^{*, \gamma, \alpha}p \leq 0.05$ in relation to BD.

Figure 2 presents the histopathologic analysis of leukocyte infiltrate, edema, and hemorrhage in lung sections. Both E2 treatments (E2-T0 and E2-T3) significantly decreased lung leukocyte infiltration. Furthermore, the lungs of E2-T3 animals presented significantly less edema and hemorrhage.



Lung histopathology

	Sham	BD	E2-T0	E2-T3	P ANOVA
Leukocytes infiltrate (leukocytes/mm^2)	2127 ± 149.5	2343 ± 96.27	1847 ± 102.4$^{\gamma}$	1851 ± 164.3$^{\alpha}$	0.0025
Edema (Units/mm^2)	3213 ± 277.3	3048 ± 140.8	2628 ± 160.4	2379 ± 97.8$^{\alpha}$	0.0087
Hemorrhage (Units/mm^2)	596.8 ± 50.4	681.3 ± 57.8	501.9 ± 58.4	488.7 ± 43.8$^{\alpha}$	0.0499

Figure 2 – Lung histopathology analysis of leukocyte infiltrate, edema, and hemorrhage. Photomicrographs of lung sections stained with standard hematoxylin and eosin staining. The female rats were subjected to BD (BD). In the treated groups, animals were submitted to BD and continuously treated with 17β-estradiol (E2) immediately after BD confirmation (E2-T0) or after 3h of BD (E2-T3). Controls were false operated animals (Sham). Data are expressed as mean ± SEM from 5 photos/sample, 3 samples/animal, and 8 animals/group animals on lung histopathology. Leukocyte infiltrate: $^{\gamma}p = 0.012$ in relation to BD, $^{\alpha}p = 0.005$ in relation to BD. Edema: $^{\alpha}p = 0.0022$ in relation to BD. Hemorrhage: $^{\alpha}p = 0.045$ in relation to BD.

Analysis of Long-lasting Chemokine Release

Table 2 shows the concentrations of MIP-1α, MIP-2, and CINC-1 in lung culture medium after 24 h. MIP-1α and CINC-1 concentrations were increased in media from BD lung culture, and MIP-2 concentration was higher in the BD group than in the sham group ($p = 0.063$). Conversely, both E2 treated groups (E2-T0 and E2-T3) significantly reduced the release of all studied chemokines.

Table 2 - Chemokines measured in culture (explant) medium of lung fragments for 24h at 37°. Quantified with multiplex assay. The female rats were subjected to BD (BD). In the treated groups, animals were submitted to BD and continuously treated with 17β-estradiol (E2) immediately after BD confirmation (E2-T0) or after 3h of BD (E2-T3). Controls were false operated animals (Sham).

(pg/mg)	Sham	BD	E2-T0	E2-T3	P ANOVA
MIP-1α	5198±1415*	21226±4626	5329±106.1γ	6484±1037α	0.0009
MIP-2	73756±39945	417038±206036	23967±8556γ	20731±7115α	0.0296
CINC-1	6754±264.5*	29657±9097	10062±1422γ	9988±2572α	0.0181

Macrophage inflammatory protein (MIP), cytokine-induced neutrophil chemoattractant 1 (CINC-1). Data expressed as mean ± SEM from 4-6 animals. *,γ, α p ≤0.05 in relation to BD.

Gene and Protein Expression of Adhesion Molecules

Figure 3 shows that gene expression of ICAM (A) and VCAM (B) was significantly increased by BD when compared to the sham group. E2 treatment decreased ICAM gene expression in both treated groups. Regarding protein expression, VCAM was increased in brain dead animals, but there was no difference in ICAM-1 levels. E2 treatment was able to reduce protein expression of both ICAM-1 and VCAM in group E2-T3 (Figures 3 C-D and Figure 4).

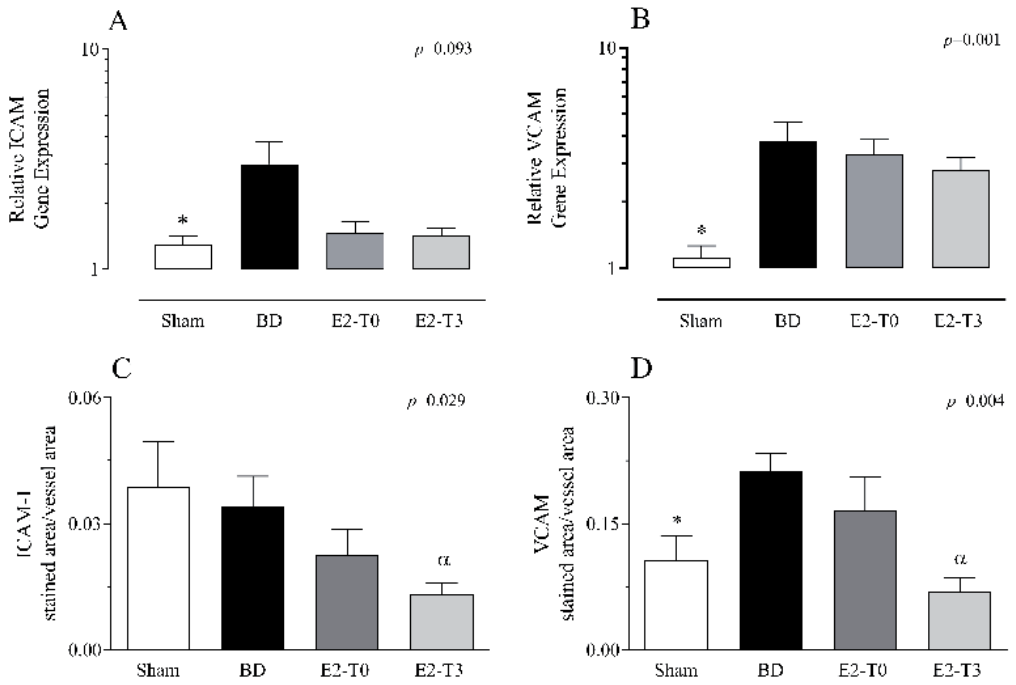


Figure 3 – Gene and protein expression of intercellular adhesion molecule (ICAM) -1 (A, C) and vascular cell adhesion molecule (VCAM) (B, D). The female rats were subjected to BD (BD). In the treated groups, animals were submitted to BD and continuously treated with 17β-estradiol (E2) immediately after BD confirmation (E2-T0) or after 3h of BD (E2-T3). Controls were false operated animals (Sham). Data are expressed as mean ± SEM from 8 (A, B) and 5 photos/sample, 2 samples/animal, and 5 animals/group in (C, D). *, αp ≤ 0.05 in relation to BD.

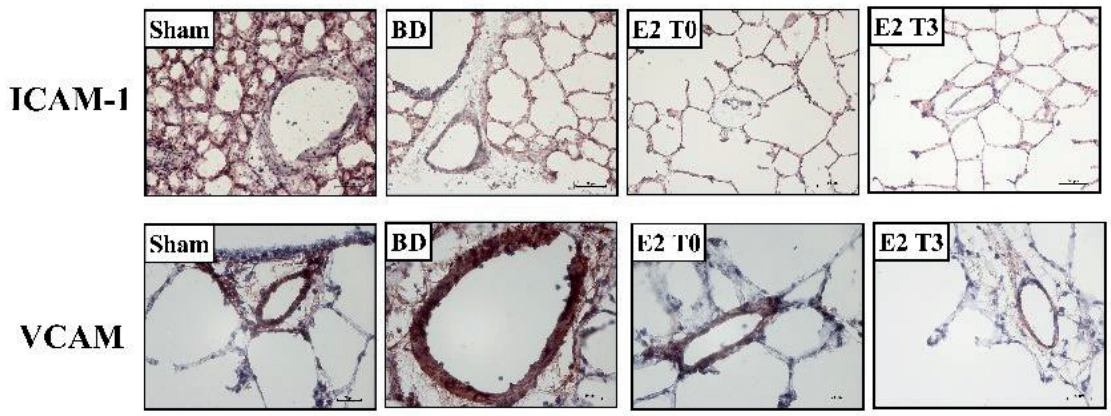


Figure 4 – Photomicrographs of immunohistochemical reaction showing ICAM-1 and VCAM on lung tissue (original magnification x40). The female rats were subjected to BD (BD). In the treated groups, animals were submitted to BD and continuously treated with 17β-estradiol (E2) immediately after BD confirmation (E2-T0) or after 3h of BD (E2-T3). Controls were false operated animals (Sham).

Analysis of Systemic Inflammatory Mediator Release

IL-1β and TNF-α were also analyzed for gene expression and protein concentration in lung tissue homogenates (Figure 5). In IL-1β, both gene expression and protein concentrations in lung tissue were increased after BD. E2 treatment reduced IL-1β gene expression in both groups (E2-T0 and E2-T3) and reduced IL-1β levels in the lung, showing a significant decrease in the E2-T0 group.

In relation to TNF-α, lung gene expression was not altered after 6 h of BD. Nevertheless, E2 treatment downregulated TNF-α gene expression in E2-T0 and E2-T3 animals, which was reduced in group E2-T3 compared to the BD group. The lung TNF-α protein concentration after 6 h was not increased by BD, but it was reduced in the E2-T3 group.

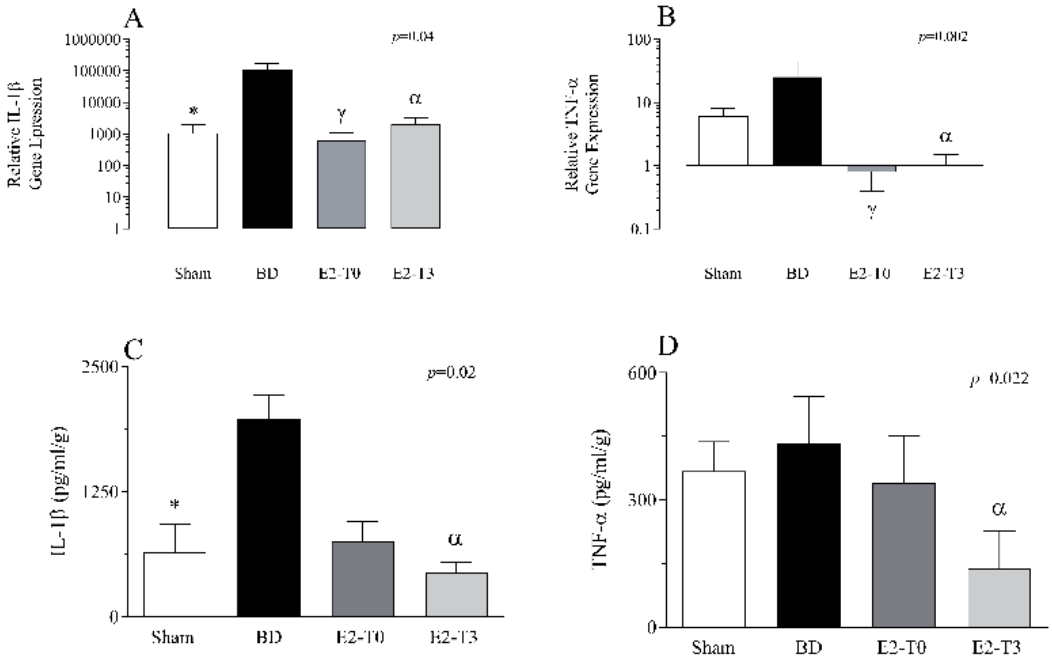


Figure 5 – Interleukin (IL)-1β and tumor necrosis factor (TNF)-α gene expression (A, B) and concentrations in lung homogenates (C, D). The female rats were subjected to BD (BD). In the treated groups, animals were submitted to BD and continuously treated with 17β-estradiol (E2) immediately after BD confirmation (E2-T0) or after 3h of BD (E2-T3). Controls were false operated animals (Sham). Data are expressed as mean ± SEM from 8 animals. $^{*, \gamma}p \leq 0.05$ in relative to BD.

MMP Expression and Activity

MMP-9 protein and gene expression levels were analyzed in the lungs (Figure 6A and B). After BD, the lungs showed increased expression of both the MMP-9 enzyme and gene. E2 treatment did not alter the enzyme expression, but the E2-T0 treatment reduced the gene expression of MMP-9. An activity assay for MMPs was performed (Figure 6C), and no differences were observed between sham and BD group animals. On the other hand, both E2 treatments reduced MMP (MMP-9 and 2) activities.

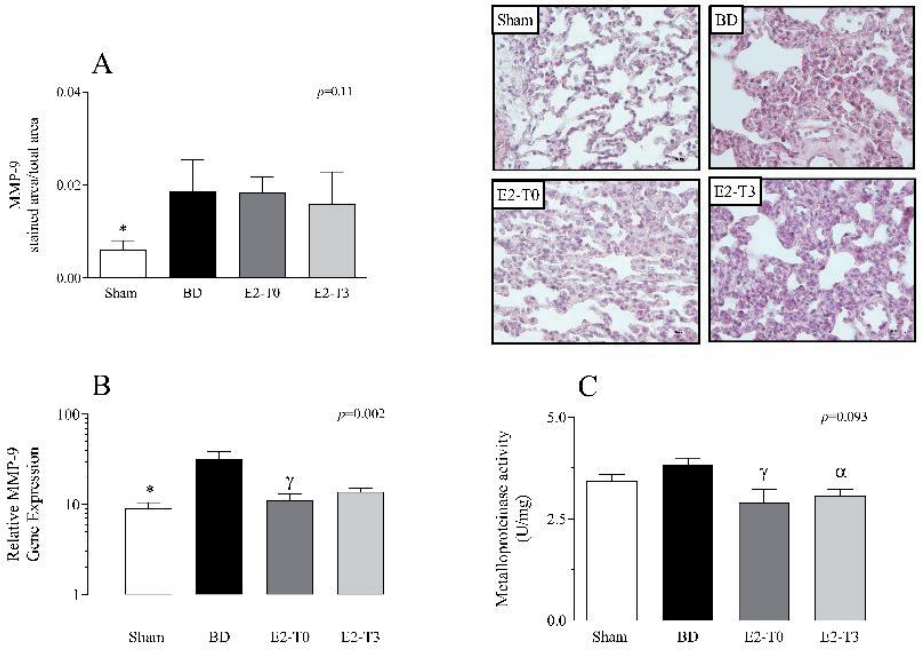


Figure 6 – Matrix metalloproteinase (MMP)-9 protein (A) and gene (B) expression, MMP activity (C), and lung photomicrography of MMP-9 protein expression. The female rats were subjected to BD (BD). In the treated groups, animals were submitted to BD and continuously treated with 17β-estradiol (E2) immediately after BD confirmation (E2-T0) or after 3h of BD (E2-T3). Controls were false operated animals (Sham). Data are expressed as mean ± SEM from 5 photos/sample, 2 samples/animals, and 5 animals/group (A), and 8 animals on (B, C). (B) $^{*, \gamma, \alpha}p \leq 0.05$ in relation to BD.

Analysis of Lung Microvascular Permeability and NO Production

BD increased the microvascular permeability in the lungs (Figure 7A), as observed by the increase in Evans blue dye extravasation. Concurrently, there was an increase in nitrate and nitrites in lung homogenates (Figure 7B). Nitric oxide synthase expression was analyzed, as well as eNOS protein expression in lung vessels (Figure 7C) and iNOS in lung tissue (Figure 7D). eNOS was found to be reduced after BD while iNOS was increased. E2 treatment had no effect on the permeability, NO synthases, or metabolites.

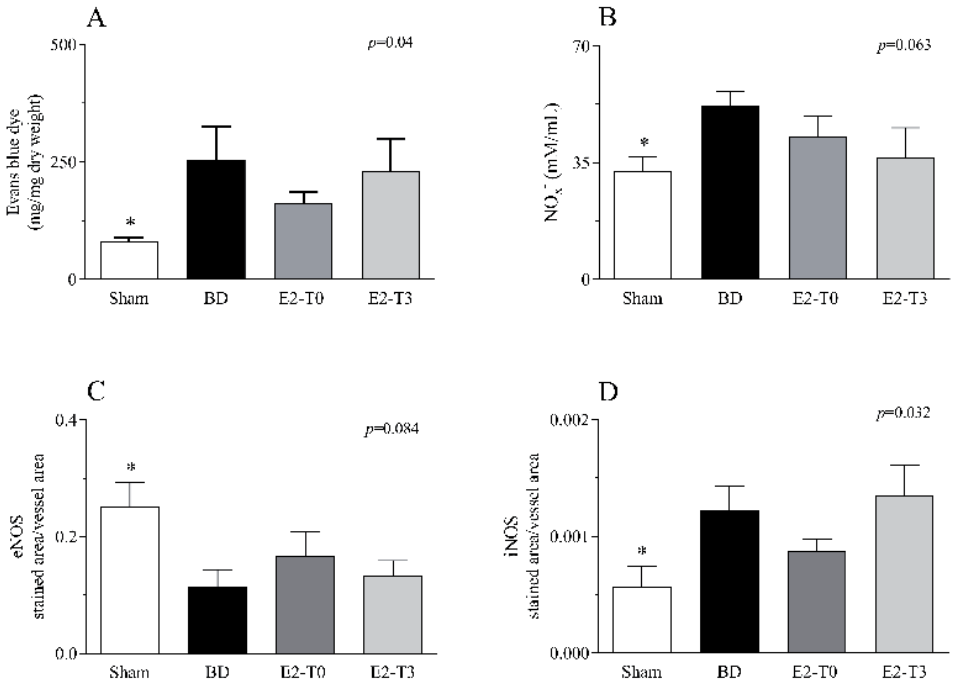


Figure 7 – Permeability assessment with Evans blue (EB) dye extravasation (A), NOx in lung homogenates (B), endothelial nitric oxide synthase (eNOS) (C), and inducible nitric oxide synthase (iNOS) (D) protein expression in the lungs. The female rats were subjected to BD (BD). In the treated groups, animals were submitted to BD and continuously treated with 17β-estradiol (E2) immediately after BD confirmation (E2-T0) or after 3h of BD (E2-T3). Controls were false operated animals (Sham). Data are expressed as mean ± SEM from 8 animals in (A, B), and 5 photos/samples, 2 samples/animal, and 5 animals/group in (C, D). $^{*}p \leq 0.05$ in relation to BD.

DISCUSSION

Brain death produces a systemic inflammatory response, of which the lung is one of the most vulnerable organs[3] through parenchyma degradation, activation of lung inflammatory cells, and recruitment of other leukocytes to the tissue[14]. The BD experimental model used by our study generated leukocyte infiltration as well as edema and hemorrhage in the lungs, resulting in lung injury. A study from our research showed that BD systemic injury affects men and women differently, whereby females have a greater inflammatory response characterized by a pronounced infiltrate in the lungs and increased permeability, associated with the acute reduction of E2, a known protector of lung inflammation[7]. The aim of this study was to understand the effect of E2 treatment on females submitted to BD, since it is known that E2 modulates lung inflammation by

regulating infiltration of inflammatory cells into the lung[15]. We used two different time points of E2 treatment to analyze its effects on BD-induced lung injury in females (E2-T0) and its possible therapeutic application (E2-T3). In order to maintain high E2 concentration during the studied period, a low therapeutic dose was used, as previously reported[16].

Our first analysis was BAL cellular content, which showed that BD is responsible for a higher cell count. It seems that not only were many leukocytes recruited to the lung, but they also passed into the alveolus. Leukocyte infiltration into the alveolar tree was confirmed by the increased number of cells observed in vivo on the lung parenchyma. It is important to point out that E2 treatment decreased leukocyte infiltration to the airways and lung parenchyma. Our study further investigated the production and release of cytokines, chemokines, and expression of adhesion molecules responsible for leukocyte mobilization during the inflammatory process. First, we analyzed the local release of MIP-1α, MIP-2, and CINC-1, which are relevant macrophage and neutrophil chemokines[17,18]. BD indeed caused a long-lasting production of MIP-1α and CINC-1, as observed in the lung in culture media. Similarly, a study from our laboratory observed an increased release of systemic CINC-1, influencing the blood white cell numbers (data not published). BD recruited leukocytes into the blood, predominantly neutrophils as a response. In turn, E2 treatment had a role in reducing recruited white blood cells, mainly by reducing the neutrophil population in the lung tissue in both treatments through reduction of chemokines in the lung.

In the event of lung inflammation, alveolar macrophages produce cytokines, such as IL-1 and TNF-α, which affect endothelial cells that express adhesion molecules and release chemokines[19]. Expression of adhesion molecules is a crucial step for leukocyte mobilization, as they are responsible for the firm adhesion and transmigration of leukocytes from the capillary endothelium into the lung parenchyma[19]. E2 regulates adhesion molecule expression, and there is a correlation between low levels of estrogen and higher expression of soluble ICAM-1 and VCAM, in females[20], and E2 is able to inhibit cytokine-stimulated VCAM-1 and ICAM-1 expression by endothelial cells[21]. In this regard, the acute reduction of E2 after the BD model would contribute to the increase in

adhesion molecule expression. BD increased both molecules on gene expression and VCAM on protein expression. Our data confirmed the effect of E2 on VCAM and ICAM-1 at the gene and protein levels. This indicates that in the BD model, E2 leads to a reduction in adhesion molecule expression in the lungs, similar to what is observed in females after ischemia and reperfusion or trauma –hemorrhage model[22,23].

Cytokine production plays a crucial role in mediating inflammatory injury. IL-1β and TNF-α cytokines are produced early in the inflammatory response by alveolar macrophages via activation of NF-κB and are released in the serum[24]. Our data show that BD elicits lung synthesis and release of IL-1β, which is involved in enhanced expression of both ICAM-1 and VCAM on endothelial cells[25,26]. More importantly, E2 treatment could have indirectly affected the expression of adhesion molecules in the lungs by reducing the local release of IL-1β and TNF-α, thereby contributing to lower leukocyte infiltration.

In addition to cytokines and chemokines during lung inflammation, MMPs are released to facilitate the clearance of foreign and harmful agents; however, when in excess, these enzymes may destroy the extracellular matrix, disrupt resident cells, and further stimulate the inflammatory process[27]. Here, MMP-9, which plays an important role in lung injury[27], was increased by means of genetic and protein expression as well as MMP activity after BD. MMP-9 is released from stimuli of TNF-α, IL-8, and IL-1β mainly on inflammatory cells, such as neutrophils, monocytes, macrophages, and even endothelial cells[28-30]. Lung MMP-9 was attenuated by E2 treatment, observed after both treatment time points on the MMP activity assay and gene expression. Other studies have confirmed the role of E2 in suppressing MMP-9 gene expression and attenuating hemorrhage, permeability, and leukocyte infiltration[31,32].

Additionally, the effect of E2 on lung edema formation after BD, parameters of vascular permeability, and nitric oxide synthases were assessed. In pathological situations, there is an overexpression of iNOS resulting in an excess of NO and imbalanced NO production[33]. Proinflammatory agents trigger iNOS expression in different cells, such as macrophages and smooth

muscle cells[34]. In our model, we observed that females registered increased total nitrites on lung homogenates accompanied by increased lung microvascular permeability after BD, which could be a result of increased iNOS expression. iNOS has proinflammatory properties, influencing the secretion of proinflammatory cytokines, endothelial expression of P-selectin and ICAM-1, increasing neutrophil infiltration and related oxidative and nitrosative stress[35].

It is known that apart from E2 levels decrease, corticosterone levels also decrease after BD[7,36], which could contribute to edema formation. Acutely, glucocorticoids inhibit vasodilation and increase vascular permeability that occurs following inflammatory insult[37]. E2 regulates the release of glucocorticoids in females during stress conditions, and females have higher basal levels of corticosterone and a faster secretion rate[38]. Our data show that E2 controlled the increase in parenchymal edema, but not alveolar edema. Thus, further studies should evaluate the association of E2 and corticosterone after BD, focusing on the control the edema formation and decrease the microvascular permeability.

Taken together the results point to E2 effects in reducing leukocyte infiltration, release of inflammatory mediators, adhesion molecules, and MMP activity in the lungs. E2 effects depend on several factors, including the estrogen receptors (ERs) involved in the response. Despite the fact that both classic ERs are required for pulmonary alveolar formation, lung cells were shown to express higher levels of ERβ than ERα mRNAs[39]. Conversely, ERα, and not ERβ, is expressed in alveolar macrophages and endothelial cells of lung vessels[40]. In parallel, E2 can also act through non-genomic mechanisms to rapidly activate signaling pathways and modulate protein expression, function and distribution. Here we could suggest that E2 treatment was able to elicit rapid effects by intracellular signaling pathways and to inhibit inflammatory gene expression by modulating transcription factors as NF-kB[41,42].

This study has some limitations, such as the possibility that the chosen time point for analysis at 6 h after BD limits the results that take effect before that period or after it. The study used E2, which is not a receptor-selective agonist. Lung function was not specifically addressed; nonetheless, our

study confirmed the important protective effects of E2 in the lungs after BD in females. The study adds to the important role of female sex hormones in the inflammatory response of females to BD, indicating that E2 may be a significant element in the improvement of donor treatment. Our results suggest that E2 exerts a direct control, decreasing the release of chemokines and restraining the cell traffic into the lungs. Considering these factors, there is a therapeutic potential of E2 that should be considered to improve donor lung quality and be further explored.

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