# CHAPTER 2

# Differential Effects of Brain Death on Rat Microcirculation and Intestinal Inflammation: Female versus Male

**Adapted from Inflammation, Vol. 41, No. 4, August 2018**

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# ABSTRACT

Brain death (BD) affects organs by multiple mechanisms related to hemodynamic effects, hormonal changes, and the systemic inflammatory response, which reduce organ function and viability. BD reduces microcirculatory perfusion in rat mesentery; this disturbance is also observed in the pancreas and lungs. Sex hormones can affect microcirculatory function, altering tissue perfusion and influencing the inflammatory process. Here, we present differences between sexes in the microcirculatory alterations generated by BD and in inflammatory infiltrate. Male, female, and ovariectomized-female Wistar rats were submitted to BD by intracranial balloon catheter sudden inflation. BD was confirmed by maximally dilated and fixed pupils, apnea, absence of reflexes, and a drop in mean arterial pressure. Perfusion and flow of the mesenteric microcirculation were analyzed. Intestinal myeloperoxidase activity and leukocyte infiltration were quantified. ELISA quantified serum estradiol, corticosterone, and inflammatory mediators, whereas expression of eNOS, endothelin, and endothelial adhesion molecule was measured by immunohistochemistry. Male rats presented lower percentages of mesenteric perfused microvessels and reduced blood flow compared to females. The female group presented higher eNOS and endothelin expression. Leukocyte infiltration into intestinal walls was higher in females in comparison to that in males. Moreover, the female group showed higher mesenteric vessel ICAM-1 expression than males, whereas serum TNF-α, IL-1β, and IL-10 levels did not differ between sexes. The high estradiol concentration before BD and high eNOS expression apparently favored the maintenance of microvascular perfusion/flow; however, BD caused an acute reduction of female sex hormone concentration and higherICAM-1level; thus, the proinflammatory organ status after BD is favored.

# INTRODUCTION

Organ transplantation is the treatment of choice for end-stage organ failure. Most transplants use organs from heart-beating donors after brain death (BD), which is a state characterized by the absence of all central nervous system functions and the loss of pituitary function [1, 2]. Brain death affects organs by multiple mechanisms related to hemodynamic effects, hormonal changes, and systemic inflammatory response [3, 4]. The result is the deterioration of organ function and viability. Previous studies by our group evidenced that the impairment of microcirculation is characterized by a rapid, significant, and sustained decrease in the proportion of perfused small vessels in rat mesentery after BD [5], and suggested that this abnormality is independent of changes in mean arterial pressure (MAP) [6]. After brain death, microcirculatory disturbance is also observed in the pancreas of male animals [7].

In a parallel study, it was highlighted that organ inflammatory status can be influenced by sex hormones [8] and the female gender presents a higher lung inflammatory status associated with an acute reduction of female sex hormones. It is well established that estradiol exerts important vasoactive effects by activation of endothelial nitric oxide synthase (eNOS), as well as modulation of vasoactive substances released from endothelial cells or the direct vasodilation effect via relaxation of smooth muscle cells [1, 9]. Regarding injury, estrogen can inhibit vascular responses and thus prevent proatherosclerotic events [10].

The maintenance of organ homeostasis depends on a complex network of systems, including endocrine, immune, and neural systems, and reduction of blood flow may have profound consequences on organ status. Microcirculatory function following trauma can be affected by sex hormones [11], altering tissue perfusion and influencing inflammatory processes elicited by BD. BD is a clinical syndrome consisting of several clinical findings, some of which are similar to the characteristics assessed in rodent models. The evaluation of pathophysiological aspects of BD course using a rodent model can therefore precede clinical studies.

Thus, the aim of this study was to evaluate the differences between sexes in the microcirculatory alterations generated by brain death and in inflammatory parameters.

# MATERIALS AND METHODS

## Animals

Male and female Wistar rats (8 weeks old; 200–250 g) from our institutional animal facilities were used. They were allocated in groups of three rats per cage (12-h light-dark cycle, 21 ± 2°C) with access to water and food *ad libitum*. All rats received humane care, and the local Animal Care Committee approved the experiments performed (report no. 0344/12).

## Experimental Groups

A total number of 43 rats (15 male and 28 female) were assigned to three groups: Female, comprised of female rats in the proestrus or estrus phase of the estral cycle (n = 16); Female-OVx, comprised of ovariectomized (OVx) female rats (n = 12); and Male, comprised of male rats (n = 12). All animals were submitted to BD.

## Brain Death Model

Rats were anesthetized in a gas chamber with isoflurane (5%) and oxygen, and were intubated and ventilated with a rodent ventilator (Harvard Apparatus, Inc., Holliston,MA, USA) with a tidal volume of 10 mL/kg and a frequency of 70 breaths/min. All animals were maintained at FiO2 of 100% with continuous inhalation of 2% isoflurane and were monitored for 6 h through a catheter in the carotid artery. The jugular vein was catheterized for fluid administration (saline solution, 2 mL/h). A 1-mm hole was punctured through the cranium, and a Fogarty- 4F catheter (Baxter Healthcare Co., Irvine, CA, USA) was introduced intracranially. To elevate intracranial pressure and consequently induce BD, the balloon was abruptly inflated with 400–500 µL saline solution (0.9%). BD was confirmed by apnea, maximally dilated and fixed pupils, absence of reflexes, and a drop in MAP. After BD, the anesthesia was halted.

## Ovariectomy

Ten days before BD induction, female rats were anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg, i.p.). Then, after shaving and asepsis of the abdominal wall with iodized alcohol (3%), a median laparotomy was performed. An incision was made on the abdomen; the ovaries were identified, held tightly, and removed. Subsequently after surgery, tramadol was administered (5 mg/kg, i.p.) to analgesia and pentabiotics (540 mg/kg, i.m.). Analgesia was maintained for 3 consecutive days with paracetamol in drinking water (0.53 mg/ml).

## Quantification of Serum Levels of Estradiol and Corticosterone

Serum levels of estradiol and corticosterone were quantified using ELISA kits (Uscn Life Science Inc., Houston, TX, USA) in Male, Female, and Female-OVx rats in samples obtained 6 h after BD. Basal values were obtained in animals not submitted to BD.

## Intravital Microscopy of the Mesenteric Microcirculation

To analyze the mesenteric microcirculation, 3 h after induction of BD, rats were conserved in the lateral position on an acrylic plate heated to 37 °C. The mesenteric vascular bed was exteriorized and conserved by superfusion with warm Krebs-Henseleit solution (pH 7.2 to 7.4, 37 °C) saturated with a gas mixture (95% N2, 5% CO2). The equipment consisted of a trinocular microscope (Axioplan 2, Carl Zeiss AG, München, Germany) connected to a digital camera to capture moving images (Axiocam-HSc, Carl Zeiss AG, München, Germany) and a microcomputer. The collected data were analyzed using software (Axiovision 4.1, Carl Zeiss AG, München, Germany). The number of migrated leukocytes to the perivascular tissue was studied in chosen areas equivalent to 5000 µm2. The percentage of perfused small vessels (< 30 µm) was determined as previously described [6]. The blood flow in the mesenteric microcirculation in situ and in vivo was determined by use of a MNP110XP Fine Needle Probe (25 mm long, 0:48 mm diameter) connected to an IN191 Laser Doppler flowmeter (AD Instruments, Colorado Springs, USA). The obtainment signal is proportional to the flow of blood cells athwart the microvasculature. Thus, microvascular perfusion is the product of the average velocity of blood cells and the average number of blood cells read under laser, with the signals reported as blood perfusion (percentage of laser signal reflected).

## Immunohistochemistry for ICAM-1, PECAM-1, Endothelin-1 and eNOS

After 6 h of the induction of brain death, the rats were exsanguinated, and the mesentery was collected, submerged in hexane, and frozen in liquid nitrogen. Serial slices of the mesentery (8 µm) were placed on glass slides (Starfrost®, Knittelglass, Germany). The fragments were fixed in acetone, and non-specific sites blocked by incubation of the sections with Tris-buffered saline-Tween 20 (TBS-T) containing 2% bovine serum albumin for 1 h at room temperature. The segments were then incubated overnight in a humidified chamber (4 °C) with either of the following: monoclonal anti-rat ICAM-1 antibody (1:50; Cedarlane Laboratories, Ontario, Canada); monoclonal anti-rat PECAM-1 antibody (1:200; Novus Biologicals, Littleton, CO, EUA); monoclonal anti-endothelin-1 antibody (1:100; Abcam, Cambridge, UK); or polyclonal anti-eNOS

antibody (1:100; Abcam, Cambridge, UK). The endogenous peroxidase was blocked (2% hydrogen peroxide solution) for 15 min at room temperature. After washing the slides with TBST, the sections were incubated with secondary antibodies (anti-IgG HRP conjugate; Millipore, Billerica, MA, USA) for 1 h at room temperature. After further washes, the sections were developed with theHRPsubstrate3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Burlingame, CA, USA) for 10 min and counterstained with Mayer's hematoxylin. The background reaction was determined in sections incubated in the absence of primary antibody (negative control). The slides were mounted, and images were acquired with a DSRi1 digital camera (Nikon, Tokyo, Japan) connected to a microscope (Nikon, Tokyo, Japan) using NIS-Elements BR software (Nikon, Tokyo, Japan). The results are presented as the ratio of the stained area/vessel area.

## Gene Expression of eNOS and Endothelin-1 in Mesenteric Tissue

Fragments of mesenteric tissue were obtained 6 h after BD. Gene expression was quantified by real-time PCR using a 7500 Real-time PCR System® (Applied Biosystems, Foster City, CA, USA). RNA extraction of the tissue (mesentery) was performed using a commercial kit (Mirvana®, Ambion®, Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. The cDNA was transcribed using a High-Capacity Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA) and the PCR reaction was performed in real time. The primers used were all by TaqMan (Applied Biosystems, Foster City, CA, USA): GAPDH (Rn01775763_g1), β-actin (Rn00667869_m1 *), eNOS (Rn02132634_s1 *), endothelin 1 (Rn00561129_m1*). The cycling conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The relative gene expression presented was determined in relation to baseline values obtained from samples of non-manipulated male rats (n =3).

## Intestinal Myeloperoxidase Activity After 6 h of brain death, myeloperoxidase (MPO)

The activity was measured as an index of the presence of neutrophils in intestinal tissue. Intestine samples were prepared as previously described [12]. Tissue homogenates with phosphate buffered saline (PBS) containing 0.5% hexadecyltrimethylammonium bromide and 5 mM ethylenediamine tetraacetic acid (pH 6.0, 3 mL/kg) were incubated for 15 min with H2O2 and o-Dianisidine (Sigma, St. Louis, MO, USA), and the reaction was stopped by the addition of 1% NaN3. Absorbance was determined at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA) and expressed as activity per milligram of tissue.

### Histopathological Analysis

Intestinal tissue segments were spotted with hematoxylin and eosin and the presence of leukocytes was assessed by light microscopy. One section each from five rats per group was digitalized (Scanscope CS System, Aperio Technologies, Inc., Vista, CA, USA), and five fields from each section were extracted and exported in a format compatible with NIS Elements Software (NIS-elements, Nikon, Tokyo, Japan). The numbers of inflammatory cells observed per villus were counted and divided by villus height (µm).

### Statistical Analysis

Data are represented as means ± SEM. Comparisons between groups were made by ANOVA followed by Tukey's post test. The 6.0 version of GraphPad software (GraphPad Software Inc., La Jolla, CA, USA) was used for this purpose. Values of $P < 0.05$ were considered to be statistically significant.

# RESULTS

### Estradiol and Corticosterone Concentration

The concentration of estradiol 6 h after BD was significantly lower in the Female group than the respective basal values (Table 1). The ovariectomy was effective in reducing basal levels of estradiol. After 6 h of BD, there was no difference among the groups regarding serum estradiol concentrations.

In relation to corticosterone, the Female, Female- OVx, and Male groups presented significant reductions in corticosterone levels after BD, with no differences between these groups. However, Female-OVx presented lower basal levels of corticosterone than Female and Male groups.

**Table 1.** Serum Concentrations of Estradiol and Corticosterone

| | Female | | Female-Ovx | | Male | | P (ANOVA) |
|---|---|---|---|---|---|---|---|
| | Basal | BD | Basal | BD | Basal | BD | |
| Estradiol (pg/ml) | 47.9 ± 4.8 | 25.8 ± 2.3$^{\theta}$ | 22.9 ± 7.3$^{*}$ | 14.66 ± 5.7 | 23.7 ± 4.3 | 27.74 ± 6.4 | 0.0008 |
| Corticosterone (pg/ml) | 90.16 ± 17.3 | 9.2 ± 1.6$^{\theta}$ | 39.4 ± 11.6$^{*}$ | 18.3 ± 4.1 | 146.9 ± 25.8 | 11.9 ± 2.5 $^{\alpha}$ | < 0.0001 |

Values are means ± SEM
$^{\theta,*}$ P<.05 in comparison to Female basal values
$^{\alpha}$ P< 0.05 in comparison to Male basal values

## Differential Effects of Brain Death on Mesenteric Microcirculation

The percentage of mesenteric perfused microvessels was reduced after 3 h of BD in Male rats in comparison to Female (Fig. 1a). Relative to blood flow, the results also showed a reduction in the percentage of blood flow in the Male group when compared to that in the Female group (Fig. 1b).

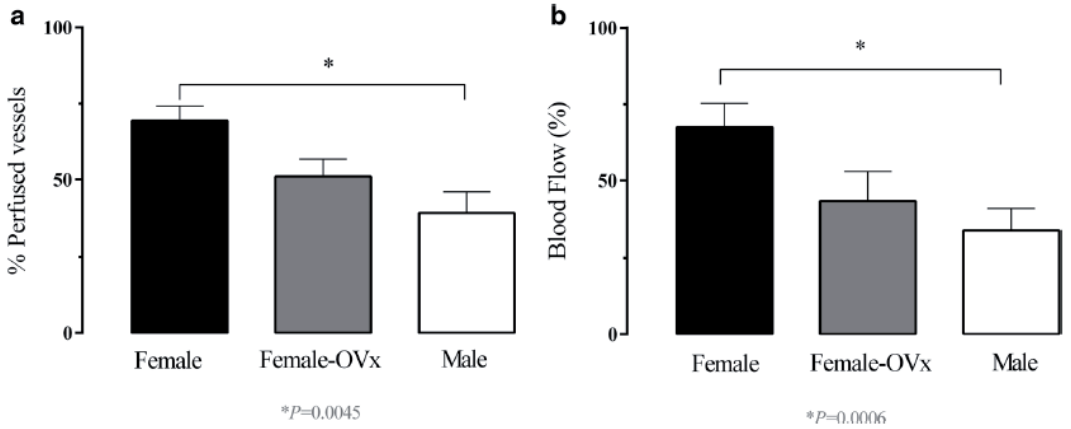


**Figure 1**. Mesenteric microcirculation perfusion and blood flow after brain death. **a** Percentage of perfused vessels. **b** Percentage of blood flow. Groups of females (n = 8), female-ovariectomized (OVx) (n = 5), and male rats (n = 5) were subjected to brain death and the mesenteric microcirculatory status was evaluated after 3 h. Data are expressed as mean ± S.E.M. ANOVA: **a** P = 0.0046 and **b** P< 0.0001.

In parallel, the presence of migrated leukocytes within the mesentery was analyzed and the results are shown in Table 2. As shown, despite the tendency of higher MPO activity presented by Female and Female-OVx groups in comparison to that by the Male group, no significant differences were found. However, when analyzing leukocyte infiltration into the intestinal wall, the Male group presented a lower number of inflammatory cells than the Female groups.

**Table 2.** Leukocyte Infiltration After Brain Death on Mesentery (3h) and on Intestine (6h) and Intestinal MPO activity (6h)

| | Female | Female-Ovx | Male | P (ANOVA) |
|---|---|---|---|---|
| Migrated leukocytes (5000µm²) | 2.75 ± 0.24 | 2.79 ± 0.32 | 2.09 ± 0.25 | 0.197 |
| Intestinal leukocyte infiltrate (cells/µm) | 0.23 ± 0.011$^{\theta}$ | 0.23 ± 0.018$^{\theta}$ | 0.14 ± 0.011 | < 0.0001 |
| Intestinal MPO activity (OD) | 0.84 ± 0.18 | 0.96 ± 0.16 | 0.53 ± 0.043 | 0.252 |

Values are mean ± SEM

$^{\theta}$ P< 0.001 in comparison to Male

## Sex Differences on eNOS and Endothelin-1 Protein and Gene Expression

To investigate microvascular perfusion-related elements, we analyzed the mesenteric expression of endothelial nitric oxide synthase (eNOS) and endothelin-1 (ET-1). Figures 2 and 3

show that the Female group presented higher eNOS and ET-1 protein expression relative to the Male group. In addition, the relative expression of the related genes was analyzed (Fig. 4). Regarding the eNOS gene, the Male and Female-OVx groups showed higher expression than the Female. Conversely, ET-1 gene expression was higher in the Female group than that in the Female-OVx.

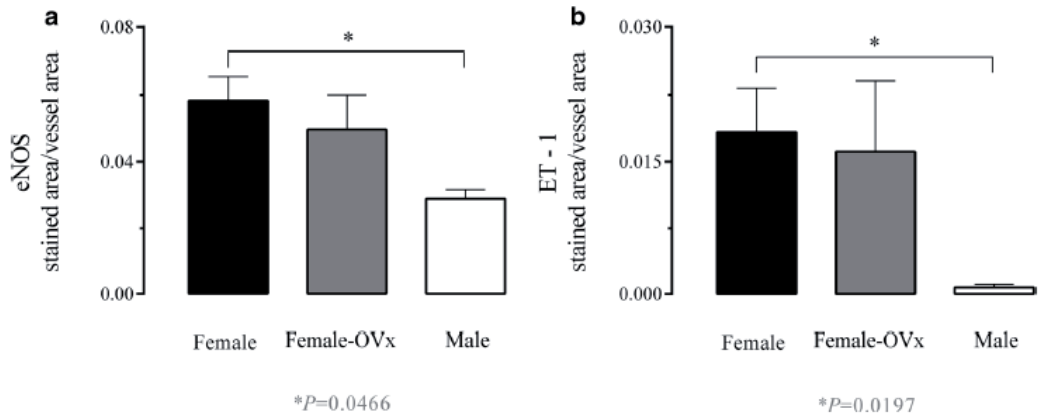


**Figure 2. a** Endothelial nitric oxide synthase (eNOS) and **b** endothelin-1 (ET) expression on the mesenteric tissue after brain death as determined by immunohistochemistry. Groups of female, female-ovariectomized (OVx), and male rats were subjected to brain death and the mesenteric tissue samples obtained after 6 h. Five stained areas of three sections from three slides per animal (n = 3) of each group were selected and the stained objects per area were quantified using an image analyzer. Data are expressed asmean ± S.E.M. ANOVA: **a** P = 0.0531 and **b** P = 0.0193.

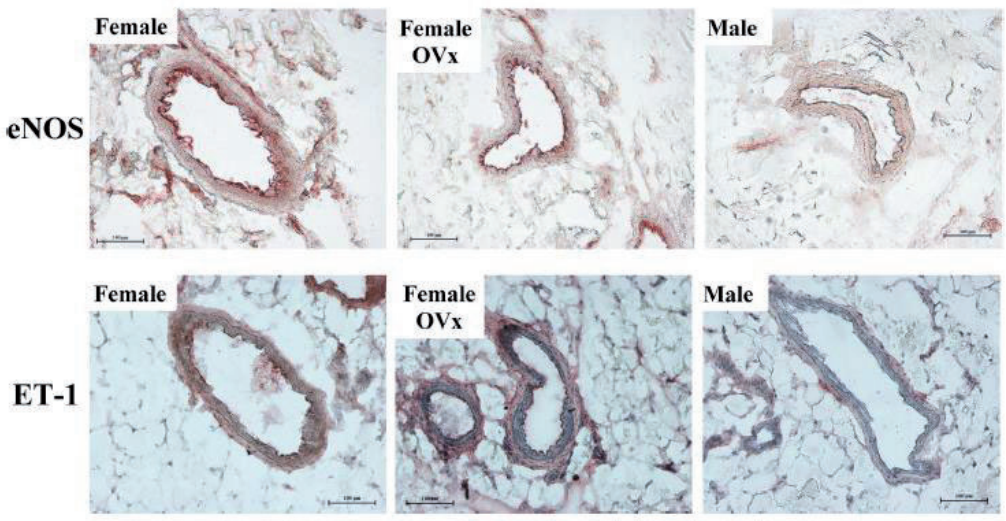


**Figure 3**. Photomicrographs of immunohistochemical reactions of endothelial nitric oxide synthase (eNOS) and endothelin-1 (ET-1) on mesenteric vessels after brain death. Groups consisted of female, female-ovariectomized (OVx), and male rats.

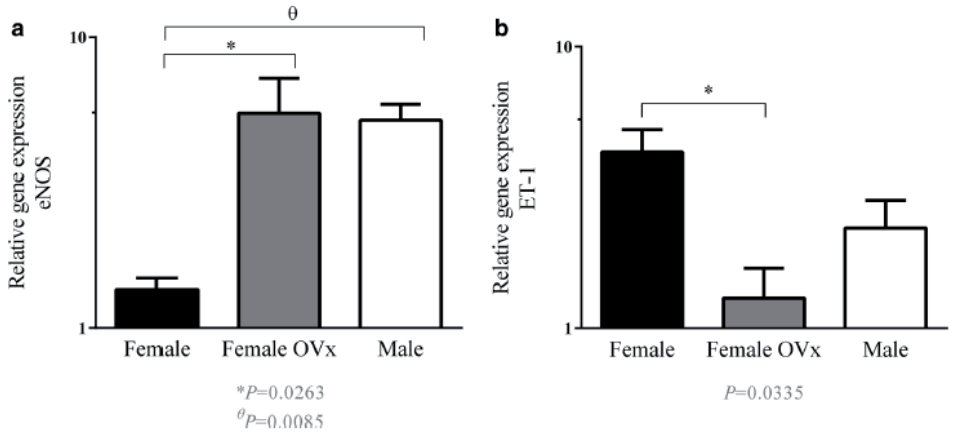


**Figure 4. a** Endothelial nitric oxide synthase (eNOS) and **b** endothelin-1 (ET) relative gene expression on the mesenteric tissue after brain death determined by RT-PCR. Groups of female (n = 8), female-ovariectomized (OVx) (n = 5), and male rats (n = 5) were subjected to brain death and the mesenteric tissue samples were obtained after 6 h. Data are expressed as mean ± S.E.M. eNOS. ANOVA: **a** P= 0.01 and **b** P = 0.0279.

## Mesenteric Protein Expression of Endothelial Adhesion Molecules and Serum Concentrations of Cytokines

The immunohistochemical analysis of ICAM-1 and PECAM-1 is shown in Figs. 5 and 6. With regard to ICAM-1 (Fig. 5a), the expression was higher in the Female group than in the other groups. With respect to PECAM-1 expression, there were no differences among groups (Fig. 5b). Furthermore, the serum concentrations of pro- and anti-inflammatory cytokines were quantified and the results did not indicate significant differences in TNF-α, IL-1β, and IL-10 levels among the groups (Fig. 7).

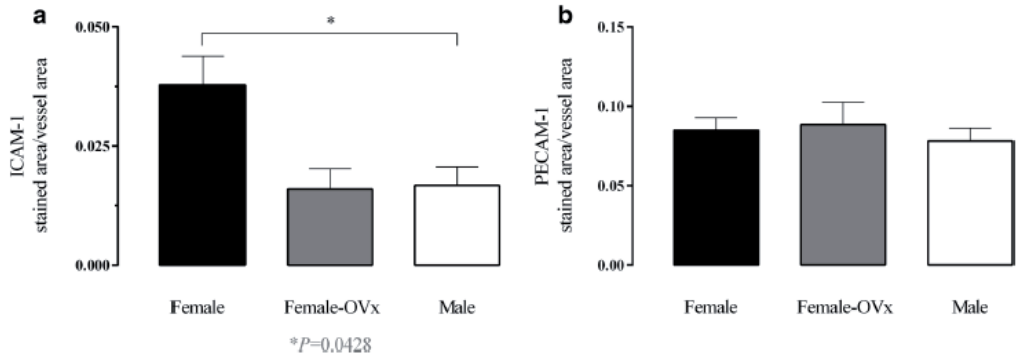


**Figure 5**. Endothelial adhesion molecules expression on mesenteric microvessels determined by immunohistochemistry: **a** ICAM-1 and **b** PECAM-1. Groups of female, female-ovariectomized (OVx), and male rats were subjected to brain death and the mesenteric tissue samples obtained after 6 h. Data are expressed as mean ± S.E.M. Ten vessels of three sections per animal of three animals of each group were selected, and the stained area per vessel was quantified using an image analyzer. ANOVA: **a** P = 0.013 and **b** P = 0.808.

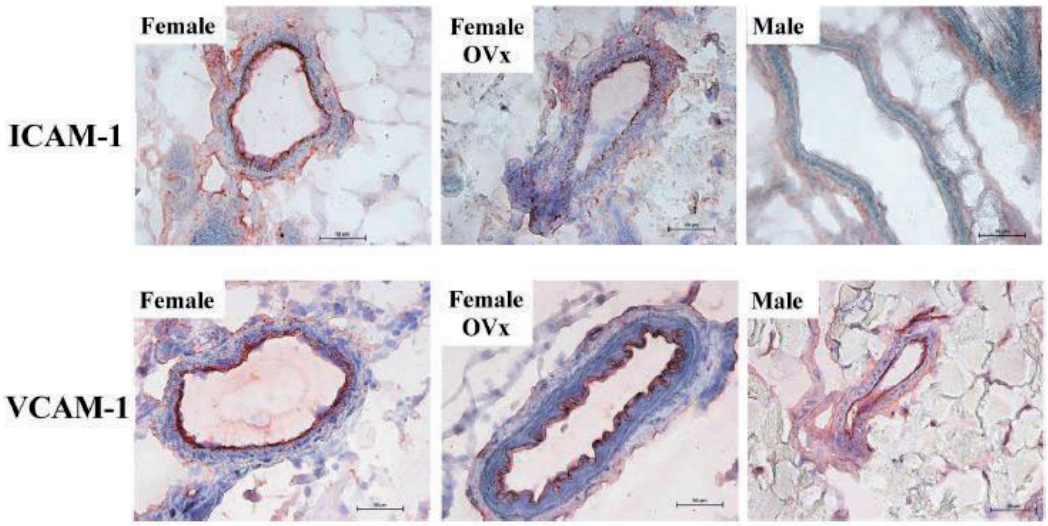


**Figure 6.** Photomicrographs of immunohistochemical reactions of endothelial adhesion molecules (ICAM-1 and PECAM-1) on mesenteric vessels after brain death. Groups consisted of female, female-ovariectomized (OVx), and male rats.

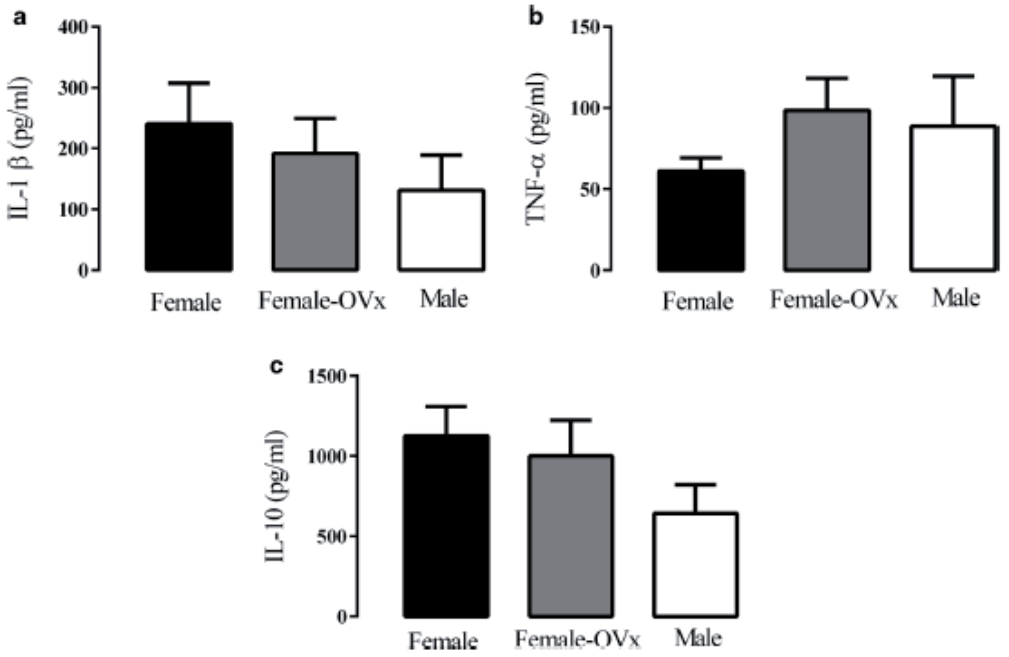


**Figure 7**. Serum concentration of cytokines after brain death: a TNF-α, b IL-1, and c IL-10. Groups of female (n = 8), female-ovariectomized (OVx) (n = 5), and male rats (n = 5) were subjected to brain death and the serum samples were obtained after 6 h. Data are expressed as mean ± S.E.M. ANOVA: a P = 0.462, b P = 0.382, and c P = 0.056.

# DISCUSSION

Recently, our group has shown that female rats submitted to BD present higher lung inflammation than males, associated with the acute reduction in female sex hormones following BD [8]. In the present study, we have expanded the analysis of sex influence on the effects of BD, focusing on the microcirculatory status. The data showed important sex differences in the

maintenance of flow and perfusion, observed in the mesenteric microcirculation, and in the intestinal inflammatory infiltrate. The female rats did not show the reduction of perfusion as found in male rats after BD. As shown previously, after BD, there is a prompt interruption in the perfusion of mesenteric microvessels measuring less than 30 μm in diameter [5]. The maintenance of perfusion by females, as observed here, is consistent with the well-established cardiovascular effects of estradiol [13] and its role in blood vessel dilatation and improvement of perfusion in trauma/sepsis models [9, 11]. As aforementioned, the Female group consisted of rats undergoing a high estradiol secretion period at the moment of BD induction. The better preservation of organ blood flow in female rats during a high estradiol concentration phase has also been observed in other models [14, 15]. In this sense, the sustained mesenteric blood flow presented by the Female group could be resultant of the hormonal environment, such as a high concentration of estradiol, at the moment of BD establishment. It was not possible to infer whether, once BD leads to a reduction in female sex hormones, after longer periods of observation, the perfusion and flow would be worsened.

The endothelium is an important regulator of blood vessel diameter by releasing endothelium-derived nitric oxide (NO), contracting prostanoids and peptides, and by initiating endothelium-dependent hyperpolarizations [16]. To understand the sex-dependent microcirculatory differences found after BD, we evaluated the vascular protein and gene expression of eNOS and ET-1 on the mesenteric vessels. The endothelial release of vessel-relaxing substances, such as NO and prostacyclin, is stimulated by estradiol [17], whereas ET-1-induced vasoconstriction is reduced [18].

eNOS exerts an important protective role by maintaining vascular tone and integrity [19]. Previous studies have shown sex differences in eNOS expression and activation in the endothelium, with higher protein expression in female animals [20]. There is also evidence that 17β estradiol increases eNOS protein expression and activity [21] and mRNA [22]. We have found higher protein expression of eNOS in the mesenteric vessels of the Female group in comparison to that in the Male, which could be a result of estradiol high concentration in Female group before BD induction. The maintained eNOS protein expression, despite the reduction of estradiol triggered by BD, might sustain NO synthesis, contributing to control of vascular tone. In this context, it is important to consider that proteins are subjected to extensive regulation, with half-lives that can vary from seconds to weeks. On the other hand, eNOS gene expression was lower in the Female group than that in the Male, a result which could indicate a future reduction of eNOS after longer periods of BD.

The vasoactive effects of ET-1 can be influenced by estradiol and are organ dependent [23], and estradiol and its metabolites inhibit ET-1 synthesis [24]. Ovariectomized female rats present higher ET-1, and estradiol replacement reduces ET-1 peptide expression [25]. In our study, after BD, females presented higher ET-1 protein expression than males. As females after BD presented reduced estradiol, and ET-1 synthesis and secretion are induced within minutes [26], we could suggest a link between the acute reduction of serum estradiol and the augmented ET-1 protein expression on mesenteric vessels. The present data also show that the ET-1 gene expression is upregulated in the Female group and is higher than the expression in Female-OVx. ET-1 contributes to the development of inflammation, increasing the expression of endothelial adhesion molecules and stimulating the aggregation of neutrophils [27].

To evaluate the inflammatory status of the intestine after BD, the presence of leukocytes in the intestinal tissue and the MPO activity were evaluated. The results showed higher inflammatory infiltrate on intestines of female rats, ovariectomized or not, in comparison to that in males. It is important to mention that the intravital microscopy was carried out 3 h after BD and the other parameters were analyzed 6 h after BD. The longer time period allowed us to better quantify the BD-induced inflammatory effects.

Under inflammatory conditions, specific glycoproteins that are expressed in endothelial cells and leukocytes mediate leukocyte rolling, adhesion, and migration. The capture and rolling of neutrophils along the microvasculature are mediated by members of the selectin family of adhesion molecules. Upon activation, endothelial cells upregulate the expression of ICAM-1, interacting with glycoproteins (β2 integrins) that are expressed in leukocytes to mediate adhesion and migration. Transmigration involves integrins and different junctional proteins, including platelet/endothelial cell adhesion molecule 1 (PECAM1) [28]. Considering that cell trafficking among compartments depends on endothelial adhesion molecules, the mesenteric vessel expression of ICAM-1 and PECAM-1 was evaluated. ICAM-1 plays an important role in leukocyte trafficking and our results imply an increased expression of ICAM-1 in the Female group. We previously observed that female rats submitted to BD also present increased serum CINC-1 levels [4], thus we could suggest that the leukocyte infiltration found in the intestine could result from the association of higher ICAM-1 expression and leukocyte activation through chemoattractant stimulus.

Additionally, the concentration of serum cytokines (TNF-α, IL-1, and IL-10) was evaluated. Although the Female group exhibited a higher intestinal inflammatory infiltrate, the systemic release of cytokines did not differ from other groups. Considering that all studied groups were

submitted to BD and that the BD-induced repercussions evolve with time, the systemic differences between genders might accentuate only after longer periods of BD.

The data presented herein allow us to reinforce the presence of important sex differences on the repercussions of BD and the higher inflammatory profile presented by the female sex. The initially high estradiol concentration before BD and high eNOS expression seemed to favor the maintenance of microvascular perfusion/flow; however, once BD is induced leading to the acute reduction of female sex hormone concentration and higher ICAM-1 expression, the proinflammatory organ status following BD is favored. Thus, we suggest that treatment with estradiol could be beneficial to female donors and improve organ quality.

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