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Tratamento hormonal no transplante: estratégias de manejo do doador e do enxerto

Hormonal treatment in transplantation: donor and graft management strategies

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ABSTRACT

Vidal dos Santos M. Hormonal treatment in transplantation: donor and graft management strategies [thesis]. São Paulo: "Faculdade de Medicina, Universidade de São Paulo"; Groningen: Rijksuniversiteit Groningen; 2025.

The shortage of suitable organs for transplantation and the exponential increase in patients on the waiting list demands improvements in current guidelines for the management of brain-dead donors, as well as new treatment strategies with the purpose of ameliorating the impacts of brain death (BD) in the graft and improving its quality. Understanding the type of donor, especially the sex and etiology of BD, may provide new information on how each donor, or even an organ, responds differently to the systemic imbalance triggered by BD. Overall, this thesis evaluated the impact of a new treatment option by evaluating the combined effects of 17β-estradiol (E2) and methylprednisolone (MP) on graft quality for transplantation. Throughout the chapters, we evaluated the effects of the proposed treatment in the lungs (**chapter 2**) and kidneys (**chapter 3**) when administered to female animals after the induction of BD. In both chapters, we observed a positive effect of the treatment, specially by reducing leukocyte infiltration to the airways and the renal parenchyma. Later, we evaluated how the slow induction of BD affects males and females (**chapter 4**), and observed that lung and kidney injury vary between the sexes, with female lungs presenting a more exacerbated inflammation, while the males presented worst renal function an increased apoptosis. Finally, we treated lungs (**chapter 5**) and kidneys (**chapter 6**) from rats of both sexes during *ex vivo* machine perfusion with E2 and MP. In male lungs, treatment was able to improve lung function, especially by improving compliance, while in females, treatment decreased pulmonary inflammation. Regarding the kidneys, treatment was detrimental to females by reducing perfusion flow leading to worst renal function. No difference was observed in male.

Keywords: Brain death. Sex differences. Estradiol. Glucocorticoids. Machine perfusion.

RESUMO

Vidal dos Santos M. Tratamento hormonal no transplante: estratégias de manejo do doador e do enxerto [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; Groningen: Rijksuniversiteit Groningen; 2025.

A escassez de órgãos adequados para transplante e o aumento exponencial de pacientes na lista de espera exigem melhorias nas diretrizes atuais para o manejo de doadores com morte encefálica, bem como novas estratégias de tratamento com o objetivo de amenizar os impactos da morte encefálica (ME) no enxerto e melhorar sua qualidade. Compreender o tipo de doador, especialmente o sexo e a etiologia da ME, pode fornecer novas informações sobre como cada doador, ou até mesmo um órgão, responde de maneira diferente ao desequilíbrio sistêmico desencadeado pela ME. No geral, esta tese avaliou o impacto de uma nova opção de tratamento, avaliando os efeitos combinados de 17β-estradiol (E2) e metilprednisolona (MP) na qualidade do enxerto para transplante. Ao longo dos capítulos, avaliamos os efeitos do tratamento proposto nos pulmões (**capítulo 2**) e rins (**capítulo 3**) quando administrados a animais fêmeas após a indução da ME. Em ambos os capítulos, observamos um efeito positivo do tratamento, especialmente pela redução do infiltrado leucocitário nas vias aéreas e no parênquima renal. Posteriormente, avaliamos como a indução lenta da ME afeta machos e fêmeas (**capítulo 4**), e observamos que a lesão nos pulmões e rins varia entre os sexos, com os pulmões femininos apresentando uma inflamação mais exacerbada, enquanto os machos apresentaram pior função renal e aumento da apoptose. Finalmente, tratamos os pulmões (**capítulo 5**) e rins (**capítulo 6**) de ratos de ambos os sexos durante a perfusão *ex vivo* com E2 e MP. Nos pulmões masculinos, o tratamento foi capaz de melhorar a função pulmonar, especialmente ao melhorar a complacência, enquanto nas fêmeas, o tratamento diminuiu a inflamação pulmonar. Quanto aos rins, o tratamento foi prejudicial para as fêmeas, reduzindo o fluxo de perfusão e levando a uma pior função renal. Nenhuma diferença foi observada nos machos.

Palavras chaves: morte encefálica; diferença entre os sexos; estradiol; glicocorticoides, perfusão.

I dedicate this thesis to my parents

Eu dedico esta tese aos meus pais

Table of contents

Chapter 1

General introduction and scope of the thesis

General introduction

Organ transplantation and brain death

For many terminally ill patients, organ transplantation remains the main treatment option. However, the gap between organ necessity and the number of transplants performed is a matter of great concern, and several countries struggle to reduce the waiting list and improve graft quality. Data from Eurotransplant annual report (2023) show that 6,815 organs were successfully allocated in 2023, while 13,498 patients were still active on the waiting list. In the United States, Health Resources and Service Administration (2023) shows that, even though more than 40,000 transplants have been performed, approximately 100,000 patients are still waiting for an organ. In Brazil, data from the Brazilian transplant registration (2023) reports that, from 14,073 potential donors in 2023, only 4,035 became effective donations. Among those potential donors, more than 16% were not considered due to medical contraindications.

Brain-dead donors are among the main sources of organs worldwide. Brain death (BD) is associated with metabolic, hemodynamic, and hormonal changes that result in a systemic inflammatory process that may reduce graft quality [4,5]. BD is characterized by an increase in intracranial pressure (ICP), which can occur as a result of a stroke, head trauma, or polytrauma. The increase in ICP is followed by herniation of the brainstem and an increase in systemic blood pressure through the release of catecholamines, as means to ensuring adequate perfusion of

the brain. As a consequence, a phase of compensatory hypotension occurs, resulting from catecholamine depletion, peripheral vasodilation, loss of vasomotor nuclei in the brainstem, and activation of baroreceptors located in the carotid arteries [6 - 8]. Hemodynamic instability impairs the perfusion of various organs, resulting in a shift from aerobic to anaerobic metabolism and activation of the systemic inflammatory response, where there is the release of inflammatory mediators, chemokines, and cytokines and an increase in the expression of endothelial adhesion molecules. This process results in elevated levels of inflammatory mediators in the donor, which are associated with worse outcomes after transplantation [9 - 12].

Several studies have utilized experimental models of BD [13 - 16]. The literature describes two distinct BD models, defined as fast- and slow-induction. Fast-induction is used to simulate acute brain trauma, with rapid balloon expansion, whereas slow-induction mimics hemorrhagic stroke through the gradual balloon insufflation [17].

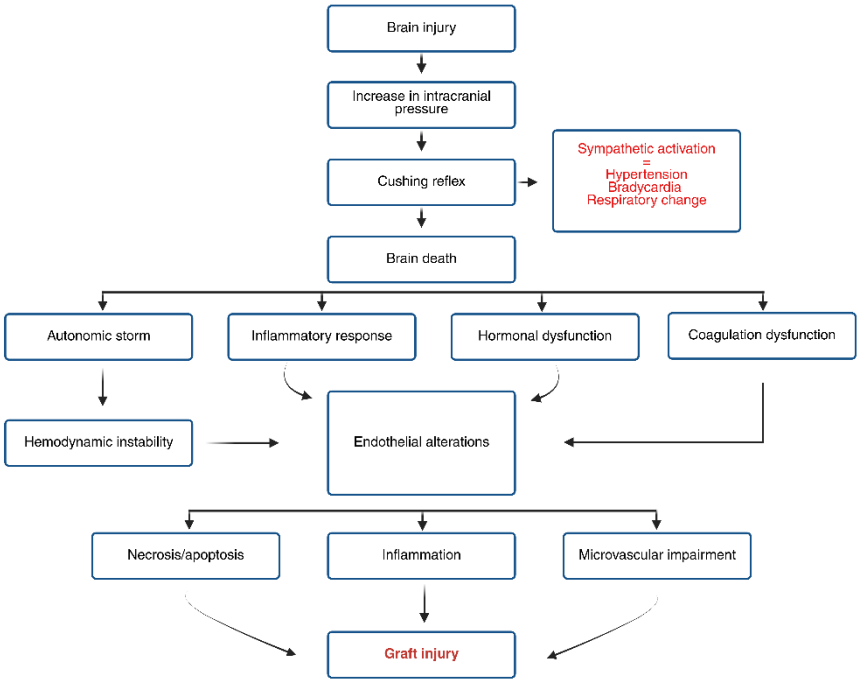

Figure 1 - Pathophysiology of brain death. Created by BioRender.com.

The lungs are among the most vulnerable organs to the deleterious effects of BD and may develop neurogenic pulmonary edema (NPE). The autonomic storm resulting from BD causes hemodynamic changes and altered microvascular permeability [18–20]. The increase in peripheral vascular resistance and mean arterial pressure caused by BD leads to the redistribution of blood volume to the lungs, causing loss of capillary integrity and resulting in pulmonary edema [6]. In parallel, the lungs develop an inflammatory response, which may progress to acute lung injury. Alveolar macrophages release tumoral necrosis factor-α (TNF-α) and interleukin (IL)-1, and there is an increase in interleukin

concentrations such as IL-2 and IL-6 [9, 21]. Furthermore, endothelial cells are activated and there is an increase the expression of adhesion molecules. Studies by Weaver et al. (2017) revealed that increased levels of cytokines such as IL-1 and TNF-α in the lung tissue immediately after BD lead to increased expression of adhesion molecules such as ICAM-1, VCAM, E-selectin, and P-selectin on endothelial cells.

The kidneys are also widely affected by BD, which can lead to acute kidney injury (AKI) or acute renal failure (ARF) [23]. They play a very important role in the homeostasis regulation of ions and water contents in the blood, serving as the primary means for waste products excretion [24]. Specifically, owing to excessive secretion of catecholamines and volume depletion during brain death, the kidneys are exposed to hypoperfusion and ischemic damage, affecting the quality of the organ to be transplanted [25].

Hormonal dysfunction

Importantly, BD leads to the reduction of several hormones, including cortisol [26]. Hormonal changes resulting from interruption of the pituitary gland function impair the donor's response to BD. Pituitary failure leads to significant reduction in hormones such as vasopressin, thyroid hormones, and cortisol, which intimately affect metabolism related to the inflammatory process [27 – 29]. In parallel, studies point to reduced concentrations of female sex hormones after BD [30 - 32]. This hormonal imbalance could interfere with the inflammatory process initiated by BD and be responsible, as evidence shows, for the worse

outcomes of organ transplants from female donors to male recipients when compared to transplants between donor and recipient of the same sex [33 - 36].

Sex differences

Few studies have assessed the impact of sex on transplantation with clinical or experimental data. The existent clinical studies indicate that lungs from female donors are considered to be at increased risk [37, 38]. In kidney transplants, Miller et al. (2017) also reported worse outcomes on grafts from female donors. Sex-mismatched kidney transplants have inferior outcomes, which has been associated with nephron underdosing due to female smaller kidneys [40]. However, high rates of acute rejection suggest that this may be related to immunological features [40].

In an experimental model of fast BD induction, Breithaupt-Faloppa et al. (2016) also observed the role of the immune system in female grafts, with greater leukocyte infiltration and increased vascular permeability in the lungs of females than in those of males. Moreover, Rebolledo et al. (2016) and van Zanden et al. (2020), after slow induction of BD, observed that thoracic and abdominal organs are differently affected. This study, however, was performed only in male animals, and further investigations on how slow induction can affect female organs are necessary.

Although studies on sex differences focused on the immunological response to transplantation are limited, potential mechanisms have been elucidated. Sex dimorphism begins at the genetic level under the

influence of sex chromosomes. Despite the Y chromosome presenting coding genes related to inflammation and immunity, the X chromosome is responsible for harboring multiple genes involved in regulating immune pathways [43]. In females, the process of X inactivation occurs with the aim of balancing the expression of X-linked genes to that of males and is random in each individual cell. This process, however, is not 100% effective, and approximately 23% of genes may escape silencing, leading to increased expression of genes related to immunological processes in females, i.e., genes related to TLR7 expression [44 – 46].

Additionally, the hormonal profile plays an important role in sex immune differences. In males, studies have shown that testosterone suppresses immunity, usually by decreasing Th2 and Th17 differentiation. In females, estradiol (E2) has shown immunoenhancing effects, marked by a Th2 response in high-E2 environments and an increased Th1 response with low E2 [47].

Hormonal therapies

Although there is no consensus on the efficacy of hormone replacement in patients with BD [48], studies indicate that donor treatment with hormones such as corticosteroids, T3 and T4 contributes to a decrease in the amount of vasopressor required to maintain hemodynamic stability, while increasing the number of organs considered ideal for transplant [49, 50].

The administration of T3 reestablished aerobic metabolism in BD patients [50], and Joseph et al. (2014) associated the early administration of levothyroxine with an increased number of organs procured per donor. Specifically, the beneficial effects of glucocorticoid (GC) administration on brain-dead donors comes from the attenuation of the release of proinflammatory cytokines [29]. In addition, GC administration contributes to improved oxygenation and pulmonary recovery and decreases the release of TNF-α and IL-1β [15, 51].

Moreover, studies have shown that female sex hormones have potential therapeutic effects on the pathophysiology of BD and ischemia-reperfusion injury. Vieira et al. (2018) reported that E2 treatment in brain-dead male rats improved lung quality by reducing edema, hemorrhage and iNOS activation, as well as promoting endothelial nitric oxide (eNOS) protein expression. Estrogens have direct effects on endothelial cells. E2 promotes vasodilation by increasing eNOS transcription through genomic activation and NO release via nongenomic activation [55]. Additionally, E2 counteracts the vasoconstriction induced by endothelin-1 [55] and vascular relaxation is associated with the activation of estrogen receptor (ER)-α [56, 57] whereas endothelin-1 attenuation is associated with ER-β [55]. E2 has also shown antioxidant properties by increasing NO availability and decreasing the generation of reactive oxygen species [58].

In a BD model, studies showed that E2 treatment reduces the expression and release of inflammatory markers, adhesion molecules and local leukocyte infiltration and improves vascular tone by

increasing protein expression of eNOS in the heart, lungs and kidneys from females [60 - 63].

Even progesterone treatment, although not used in a donation model, has shown positive results in reducing the levels of inflammatory markers, such as NK-kB, IL-1β, IL-6 and TNF-α and attenuating gut damage, in a traumatic brain injury model in males [64, 65].

Glucocorticoid and female sex hormone combination

In the context of BD in females, it is important to consider that the reduction in GC and female sex hormone concentrations in the donor significantly influences the systemic inflammatory process and the functional state of various organs.

The hypothalamic-pituitary-adrenal (HPA) axis plays a crucial role in regulating the inflammatory response through the release of GCs, which acts by inhibiting the synthesis and release of cytokines and inflammatory mediators through the activation of glucocorticoids receptors (GRs) [66]. The interaction of this hormone with its receptors modulates the inflammatory response by stimulating the transcription of anti-inflammatory factors such as annexin-1 and IL-10, inhibiting the production of prostaglandins and the expression of induced nitric oxide synthase (iNOS), stimulating the expression of eNOS, and blocking the transcription of NF-kB and AP-1, thus reducing the expression of key proinflammatory factors such as IL-1β, IL-2, IL-6, IL-8, TNF-α, and IFN-γ. At the cellular level, glucocorticoids reduce

the recruitment of inflammatory cells by inhibiting the expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin [66 - 70].

With respect to female sex hormones, several clinical and experimental studies emphasize their importance as potential modulators of the inflammatory response in females, respectively they show that: hormonally active women are protected from cardiovascular disorders and have a lower incidence of sepsis, pneumonia, and organ failure [71 - 76]; females rats in proestrus or treated with E2 showed reduced pulmonary injury and inflammation in ischemia-reperfusion [77] and in hemorrhagic shock models [78]. Furthermore, E2 treatment has anti-inflammatory effects similar to those of GCs, reducing the expression of transcription factors involved in the inflammatory response and decreasing neutrophil recruitment by reducing the production of interleukins, such as IL-8, chemokines and adhesion molecules [79 - 80].

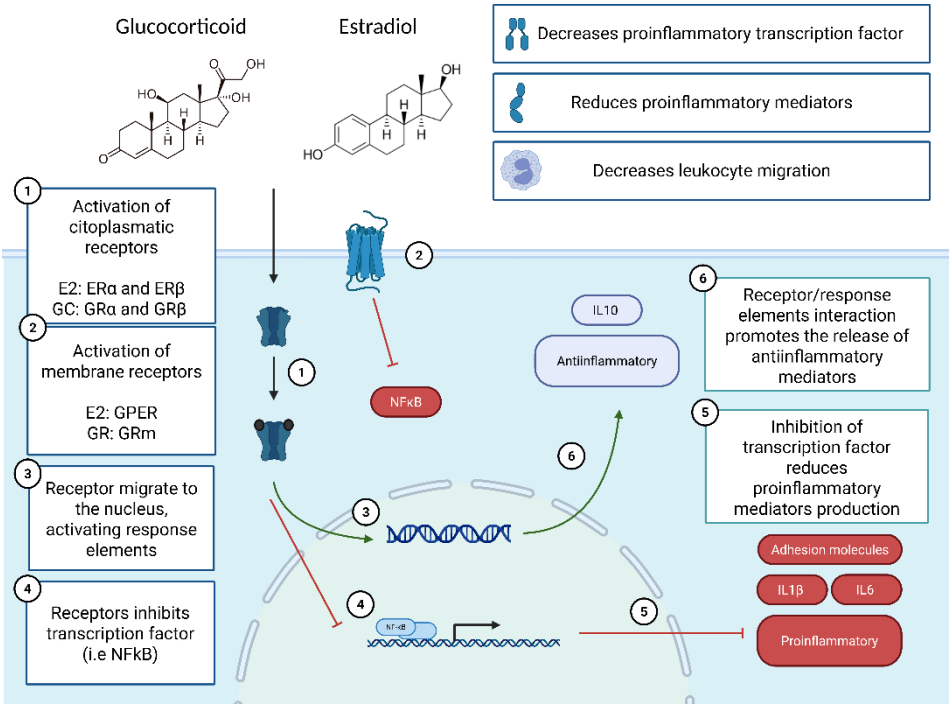

Figure 2 - Genomic and nongenomic pathways associated with the anti-inflammatory effects of 17β-estradiol and glucocorticoids. E2, 17β-estradiol; GC, glucocorticoid; ER, estradiol receptor; GPER, G protein-coupled estrogen receptor 1; GR, glucocorticoid receptor; GRm, glucocorticoid membrane receptor; IL, interleukin; NF-kB, nuclear factor kappa B. Created in BioRender.com.

Additionally, the data point to the interaction between female sex hormones and the HPA axis. GC inhibits the secretion of gonadotropins and gonadotropin-releasing hormone (GnRH). Consequently, gonadal hormones exert negative feedback on the HPA axis in an attempt to prevent or delay the effects of corticosteroids on the reproductive system [81, 82].

The influence of sex hormones leads to a different response between sexes in the HPA axis when exposed to inflammatory factors [82]. Female rats, compared to males, exhibit higher corticosterone release, both

basally and during stress [83 - 86]. Studies by Atkinson & Waddell (1997) evaluated corticosterone levels in female rats and observed variations in plasma concentration during the estrous cycle, with females in proestrus (higher estrogen levels) having corticosterone levels twice as high as those in diestrus (lower estradiol levels). These findings show that the female stress response is closely tied to the concentration of sex hormones, particularly E2. E2 also regulates the HPA axis by modifying GR receptors. E2 alters the ability of these receptors to self-regulation, blocking negative feedback and thereby increasing glucocorticoid release [87, 88]. Additionally, ERs are present in the brain region responsible for controlling the HPA axis, influencing the synthesis and release of adrenocorticotropic hormone (ACTH) and GC metabolism [82].

Both E2 and GC receptors are found in various immune and nonimmune cells. In immune cells, both hormones modulate cellular development and function, either in a convergent or nonconvergent manner, although there are no in-depth studies on how these respective receptors interact in this system [88]. However, studies with various other cell types have shown that there is interaction between ERs and GRs, highlighting mechanisms related to the respective receptors [89 - 92]. Genes involved in the inflammatory process are targeted by both E2 and GC [93, 94]. E2 and GCs interact with key transcription factors related to the inflammatory process, such as AP-1, Sp1, Stat1, and NFκB [95 – 99]. Studies by Edgar et al. (2013) reported that these hormones synergistically modulate the inflammatory response in the

microvasculature, forming a complex that binds to NFκB, inhibiting the transcription of various inflammatory mediators. The use of an E2 antagonist can block the action of GCs on inflammation-related genes regulated by both the ER and the GR [94]. An experimental study of pulmonary inflammation in female rats treated with dexamethasone and tamoxifen (an estradiol antagonist) revealed that blocking the receptor compromised the action of dexamethasone, suggesting a dependence on ER activation for GC action, highlighting that the joint action of these hormones is important for the proper regulation of their anti-inflammatory properties [101].

Moreover, *ex vivo* machine perfusion (EVMP) arouses in the last decades and is currently used as a tool to treat organ outside of the donor before transplantation. As BD management is very divergent between center and new systemic treatments focused on the donor could lead to ethical discussion before approval, EVMP may present itself as an alternative strategy.

EVMP first occurred in the 60's, with the goal of reducing ischemia and reperfusion injury, which is a result of static cold storage. Currently, the use of more modern perfusion solutions enables prolonged preservation times, allowing for better organ assessment and the use of treatment strategies without the limitation of systemic side effects [102]. In the lung, the Toronto protocol allows for prolonged normothermic perfusion times, and treatment strategies aiming to improve organ quality have been tested with positive results [103 - 107]. For kidneys, several strategies, ranging from pharmacological agents to gene and cell therapy, have

been studied [108 - 112]. However, unlike the lungs and liver, little is known about the real metabolic necessity of the kidneys or what represents good kidney function during normothermic machine perfusion. Therefore, more studies are necessary before this technique can be widely implemented in the clinic.

Scope of the thesis

Therefore, the aim of this study was to evaluate the therapeutic potential of the combination of E2 and methylprednisolone (MP) in ameliorating BD-induced inflammation when administered in the donor or during *ex vivo* machine perfusion.

Considering the positive results of hormonal treatment in donors [61 - 63], we investigated the effects of the proposed treatment when administered to female animals after fast induction of BD, with a focus on the lungs **(Chapter 2)** and kidneys **(Chapter 3)**.

Moreover, the inflammatory response differs between males and females after fast induction of BD [30]. Rebolledo et al. (2016) and van Zanden et al. (2020) reported, in an experimental model of slow induction of BD, that the abdominal and thoracic organs are differently affected in male animals. In that sense, in **Chapter 4,** we evaluated pulmonary and renal injury and inflammation in male and female animals via a slow induction model.

Finally, for a more tailored and organ-focused approach, we evaluated how the combined treatment of E2 and MP affects graft quality during machine perfusion. For this purpose, male and female organs were subjected to *ex vivo* lung perfusion **(Chapter 5)** and isolated kidney perfusion **(Chapter 6)**.

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Chapter 2

17β-estradiol and methylprednisolone association as a therapeutic option to modulate lung inflammation in brain-dead female rats

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Abstract

Brain death (BD) is known to compromise graft quality by causing hemodynamic, metabolic, and hormonal changes. The abrupt reduction of female sex hormones after BD was associated with increased lung inflammation. The use of both corticoids and estradiol independently has presented positive results in modulating BD-induced inflammatory response. However, studies have shown that for females the presence of both estrogen and corticoids is necessary to ensure adequate immune response. In that sense, this study aims to investigate how the association of methylprednisolone (MP) and estradiol (E2) could modulate the lung inflammation triggered by BD in female rats. Female Wistar rats (8 weeks) were divided into four groups: sham (animals submitted to the surgical process, without induction of BD), BD (animals submitted to BD), MP/E2 (animals submitted to BD that received MP and E2 treatment 3h after BD induction) and MP (animals submitted to BD that received MP treatment 3h after BD induction). Hemodynamics, systemic and local quantification of IL-6, IL-1β, VEGF, and TNF-α, leukocyte infiltration to the lung parenchyma and airways, and adhesion molecule expression were analyzed. After treatment, MP/E2 association was able to reinstate mean arterial pressure to levels close to Sham animals ($p<0.05$). BD increased leukocyte infiltration to the airways and MP/E2 was able to reduce the number of cells ($p=0.0139$). Also, the associated treatment modulated the vasculature by reducing the expression of VEGF ($p=0.0616$) and maintaining eNOS levels ($p=0.004$) in lung tissue. Data presented in this study show that the association between corticoids and estradiol could represent a better treatment strategy for lung inflammation in the female BD donor by presenting a positive effect in the hemodynamic management of the donor, as well as by reducing infiltrated leukocyte to the airways and release of inflammatory markers in the short and long term.

Background

Lung transplantation remains the main option for treating end-stage lung diseases. Even though several surgical teams struggle to reduce the number of patients on the waiting list by seeking strategies to improve lung transplantation, the number of patients with chronic lung diseases continues to rise. In this scenario, the gap between organ necessity and transplants performed will remain a matter of great concern. The majority of organs are procured from brain-dead donors. During the onset of brain death (BD), the loss of the hypothalamic-pituitary axis, and the consequent reduction of several hormones, as well as systemic inflammation and hemodynamic instability have detrimental effects on graft quality [1,2]. Even though there is no consensus regarding the severity of the endocrine compromise in humans; experimental studies in BD models have demonstrated loss of the anterior and posterior pituitary function [3,4]. Clinical studies with hormonal resuscitation, mainly thyroid hormones, vasopressin, and corticoids, have shown positive effects in ameliorating the physiological imbalance after the permanent loss of brain function [5,6].

In addition, previous evidence indicates that males and females respond differently to the aftermath of BD [8]. In experimental models, BD in females is followed by the acute reduction of estradiol (E2) and corticosterone with higher inflammation [9]. Treatment of donors with either estradiol or corticoids alone has shown positive effects in experimental and clinical studies of BD [10-16]. However, in females, adequate stress response appears to be linked to the presence of both

estradiol and corticoids. In rats, elevated levels of corticosterone were observed during periods of higher estrogen concentration and E2 seems to interfere with glucocorticoid release by modulating the autoregulatory capacity of glucocorticoid receptors (GR) [17]. In addition, some studies indicate that estradiol receptors (ER) and GR interact with each other [18- 20], and could have co-dependent anti-inflammatory actions [21-23]. Thus, the sudden lack of these hormones could compromise the female response to BD. We, therefore, aim to investigate the therapeutic potential of E2 and methylprednisolone association in ameliorating the detrimental effects of BD, focused on the pulmonary inflammatory response in female rats submitted to BD induction.

Methods

Animals

This study used 52 female Wistar rats (7-8 weeks). The animals were kept at 23 ± 2 °C, 12 h of light and dark periods, without restrictions on water and food intake. Guidelines for animal humane care were in accordance with the "Principles of Laboratory Animal Care" written by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" published by the Institute of Laboratory Animal Resources from National Institute of Health (NIH Publication No 86-23, revised 1996). Ethical approval for animal experiments was granted by the Faculdade de Medicina da

Universidade de São Paulo Ethic Committee for Research Projects (SDC n 1257/2019).

Study groups

To assure peak estradiol concentration before surgery, animals in the estrus and proestrus phases of the estrous cycle were selected and randomized into four different groups: Sham: rats subjected only to cranial trepanation; BD: rats submitted to brain death; MP: rats to brain death, which received continuous treatment with methylprednisolone after 3h of BD confirmation; MP/E2: rats submitted to brain death, which received a continuous infusion of estradiol and methylprednisolone after 3h of BD confirmation, (Figure 1).

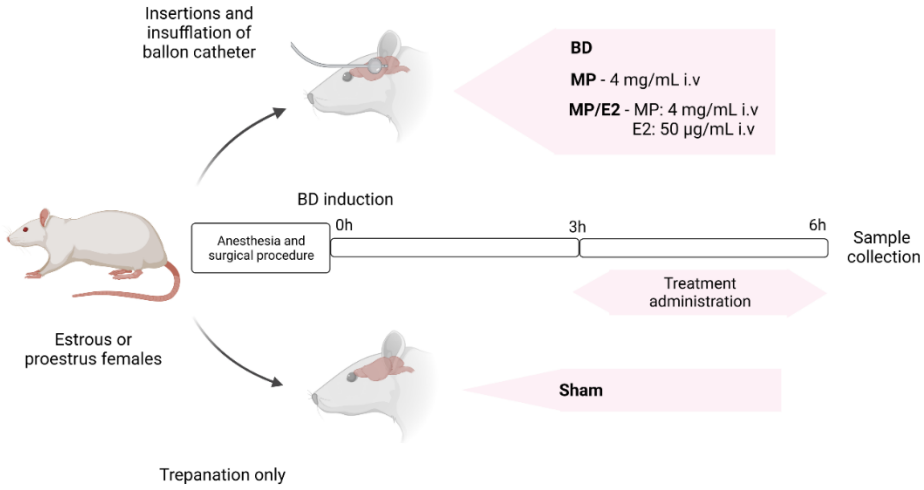

Figure 1 - Experimental design of BD induction and treatment administration. BD, brain death. E2, 17β-estradiol. MP, methylprednisolone.

Anesthesia and induction of brain death

Animals were put under anesthesia with a mixture of isoflurane (5%) and oxygen in a closed chamber, submitted to orotracheal intubation (jelco 16G), and connected to a rodent ventilator (FiO2 of 100%, tidal volume of 10 ml/kg, and frequency of 70 cycles/minute). Anesthesia was maintained with 2% isoflurane. Animals were placed on a surgical platform with local heating (37 °C) and, after the incision in the anterior cervical region, the right carotid artery was cannulated and connected to a pressure transducer to obtain mean arterial pressure (MAP) values. The right internal jugular vein was also cannulated and connected to an infusion pump for volume replacement and treatment administration. Exposure of the skull cap and total perforation with a spherical drill coupled with a surgical motor in the left parietal region was performed for insertion of a Fogarty® 4F catheter.

Brain death was induced by rapid infusion of 400 µL of saline solution into the Fogarty® 4F catheter and was confirmed by the hypertensive peak, absence of reflexes, bilateral mydriasis, and apnea. Once brain death was confirmed, anesthesia was discontinued, volume replacement was initiated, and mechanical ventilation was maintained for 6h. Sham animals were kept under anesthesia with isoflurane (2%) until the end of experiments.

Treatment

In the initial 3 h, all animals received a continuous infusion of saline solution (NaCl 0.9%, 2 ml/h). After 3h of BD confirmation, the MP

group received continuous infusion (2 ml/h) of methylprednisolone alone (4 mg/mL, i.v). The MP/E2 group received continuous infusion (2 ml/h) of 17β-estradiol (50 µg/mL, i.v. - Sigma-Aldrich®, USA) and methylprednisolone (4 mg/mL, i.v – Solu-Medrol®, Pfizer, USA). Sham and BD groups received an equivalent dose of 17β-estradiol dilution vehicle (cyclodextrin) (Sigma-Aldrich®, USA) in saline solution (NaCl 0.9%, 2 ml/h).

Determination of hormones serum levels

Blood samples were collected at the end of the sixth hour from the abdominal aorta. Quantification of circulating concentrations of 17β-estradiol and corticosterone was performed using ELISA kits (Cayman Chemical Company, USA) in accordance with the manufacturer's recommended protocol.

Total and differential cell count on bronchoalveolar lavage

After euthanasia, the bronchoalveolar space was washed with DMEM (5 mL) through the orotracheal cannula. Bronchoalveolar lavage fluid (BAL) was centrifuged (200×g, 15° C. for 10 minutes) and the cell pellet was resuspended in PBS (1 ml). 20 uL of the resulting cell suspensions were used for analyses with an automated hematology analyzer (Mindray BC 2800 Vet, China).

Isolated tissue culture (explant)

After the desired time elapsed after BD (6 h), lung tissue fragments were incubated in 4-well plates and maintained in a humid atmosphere for

24h with 95% O2 and 5% CO2 at 37ºC in DMEM culture medium (Dulbecco's Modified Eagle's Medium, Vitrocell Embriolife, Brazil). The culture medium was collected and stored at -80ºC until analyses and lung fragments were placed to dry in an incubator at 37ºC and were later weighed.

Homogenization of lung tissue

Lung fragments were weighed and dissociated in PBS (4 µL/g) with GentleMACS Dissociator (Miltenyi Biotec, Germany). The homogenate samples were stored at -80 º C until analyses.

Determination of inflammatory mediators' concentration in serum, lung tissue homogenate, and lung culture samples

To determine the concentration of inflammatory mediators in serum (IL-1β, IL-6, VEGF, and TNF-α), lung homogenates supernatants (IL-6, IL-1β, VEGF and TNF-α), and in lung explant media (IL-6, IL-1β, VEGF and TNF-α) ELISA commercial kits (Duo Set, R & D System®, USA) were used in accordance to the manufacturer's instructions. Optical density was obtained by spectrophotometry (SpectraMax® PLUS Microplate Reader, Molecular Devices, USA). Concentration values were presented as pg/ml for serum, as pg/mg of total protein level for lung homogenates, and as pg/ml/mg of dry weight for explant.

Real-time PCR for gene expression analysis of IL-1β, IL-6, VEGF, TNF-α, eNOS, iNOS, and ICAM-1

Gene expression was quantified by using real-time PCR in a Step One Plus® device (Applied Biosystem, USA). RNA extraction from tissues (lung) was performed using a commercial mirVana™ miRNA isolation Kit (Ambion®-Thermo Fisher Scientific, USA), following the manufacturer's protocol. The cDNA was transcribed (High capacity reverse transcriptase kit, Applied Biosystem, USA) and the real-time PCR reaction was performed. The primers used were Taqman (Applied Biosystem, USA) for GAPDH, β-actin, iNOS, eNOS, VEGF, and ICAM-1 and SYBR®Green (Applied Biosystems) for β-actin, IL-1β, IL-6 and TNF-α (Table 1): Cycling conditions were as follow: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 sec 95 °C and 1 min at 60 °C.

Table 1 – RT-PCR primers used for analysis.

Real-time PCR Taqman		
GAPDH	Rn01775763_g1	
β-actin	Rn00667869_m1*	
iNOS	Rn00561646_m1*	
eNOS	Rn02132634_s1*	
VEGF	Rn 01511601_m1	
ICAM-1	Rn005642227_m1*	
Real-time PCR SYBR®Green		
β-actin	RN b-act fw	5´-GGAAATCGTGCGTGACATTAAA-3´
	RN b-act rv	5´-GCGGCAGGGCCATCTC-3´
IL-1β	RN IL-1B fw	5´-CAGCAATGGTCGGGACATAGTT-3´
	RN IL-1B rv	5´-GCATTAGGAATAGTGCAGCCATCT-3´
TNF-α	TB TNF- α fw	5´-AGGCTGTCGCTACATCACTGAA-3´
	RN TNF- α rv	5´-TGACCCGTAGGGCGATTACA-3´
IL-6	RN IL-6 fw	5´-CAACTTCCAATGCTCTCCTAATG-3´
	RN IL-6 rv	5´-TTCAAGTGCTTTCAAGAGTTGGAT-3´

RT-PCR, real-time polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; VEGF, vascular endothelial growth factor; IL, interleukin; TNF- α, tumor necrosis factor-alpha.

Nitrates and nitrites (NOx–) quantification in serum, tissue homogenate, and explant

Lung tissue homogenate, explant, and serum samples were incubated with nitrate reductase (Sigma-Aldrich®, USA) for 2 h at 37°C for the reduction of nitrate (NO3-) into nitrite (NO2-). After reduction, nitrite detection was performed by incubating the samples with Griess reagent, producing a colorimetric reaction with a wavelength reading of 595 nm. The concentration values were obtained against a NaNO2 standard curve (5-60μM). Values are presented as mM/mL in serum and homogenate samples and as nM/ml/mg of dry weight in the explant.

Immunohistochemistry of MPO, ICAM-1, eNOS, and iNOS

The left pulmonary lobe was insufflated with Tissue-Tek® O.C.T. Compound (© Sakura Finetek, USA) through the left bronchus and snapped frozen in a nitrogen-hexane solution. Cryosections (10 μm) were fixated on a glass slide for 10 min in cold acetone. Blockage with endogenous peroxidase (H_2O_2, 2%) was performed. Albumin-rich solution was used for blocking non-specific sites.

Before staining, cryosections were incubated with primary antibodies at TBS-T/BSA overnight at 4°C. Primary antibodies (Boster, 1:100) were used for MPO and ICAM-1, and primary antibodies (Abcam, 1:100) for eNOS and iNOS immunodetection. Sections were then

incubated in HRP-conjugated secondary antibodies and later in a peroxidase substrate. 10 images per section were acquired using a DS-Ri1 digital camera connected to an image acquisition system. Analyses were performed using NIS-Element-BD (Nikon) software. MPO and iNOS results are presented as stained cells per mm2. ICAM-1 and eNOS results are presented as stained area per total area, and VCAM-1 is presented as stained area per vessel area.

Analysis of results

The results are expressed as mean ± standard error of the mean (SEM) or as median and interquartile interval. Statistical analyses were conducted using GraphPad Prism Software v.9.1.0. The data were analyzed for distribution with a normality test and submitted to analysis by Kruskal-Wallis followed by the post-hoc test of two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli, always compared to the BD group. MAP mixed effect analysis was performed followed by post-hoc test of the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli.

Euthanasia

After 6 hours, animals submitted to BD were exsanguinated through the abdominal aorta. Sham animals were euthanized by exsanguination under anesthesia. Animals were disposed of according to current standards for incineration.

Results

Hormonal profile

Data on serum concentration of estradiol and corticosterone showed that both hormones were reduced in the BD group in comparison to Sham animals. Elevated levels of corticosterone were present in both MP/E2 and MP-treated groups, while estradiol increase was only observed in the MP/E2 group (Figure 2).

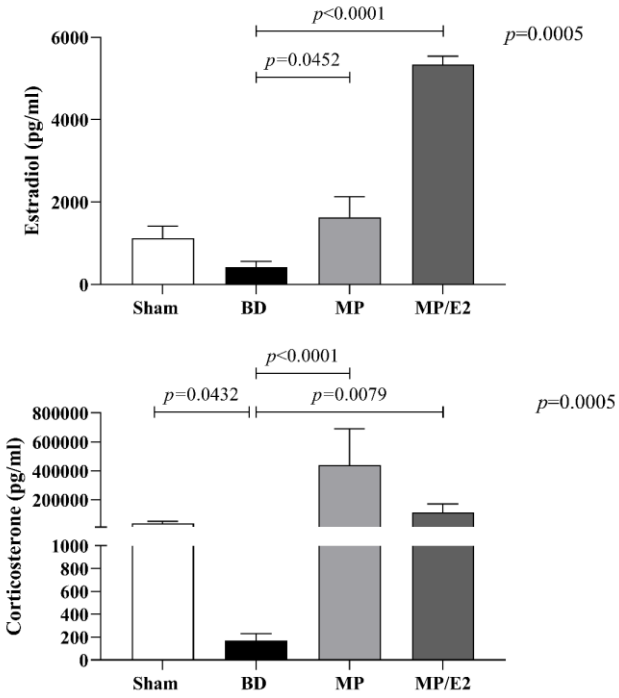

Figure 2 - Serum estradiol (A) and corticosterone (B) concentrations. Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 8 animals. (A) $p^{(Kruskal\ Wallis)}=0.0005$, (B) $p^{(Kruskal\ Wallis)}=0.0005$.

Mean arterial pressure (MAP)

Sham animals presented stable MAP during the 6h of experiments. BD resulted in a transient hypertensive crisis accompanied by a period of hypotension and, lastly, normalization of MAP. No significant difference was observed in the group treated with MP. On the other hand, the MP/E2 group, in comparison to the BD group, presented a significant augmentation of MAP after 4h of BD (Figure 3).

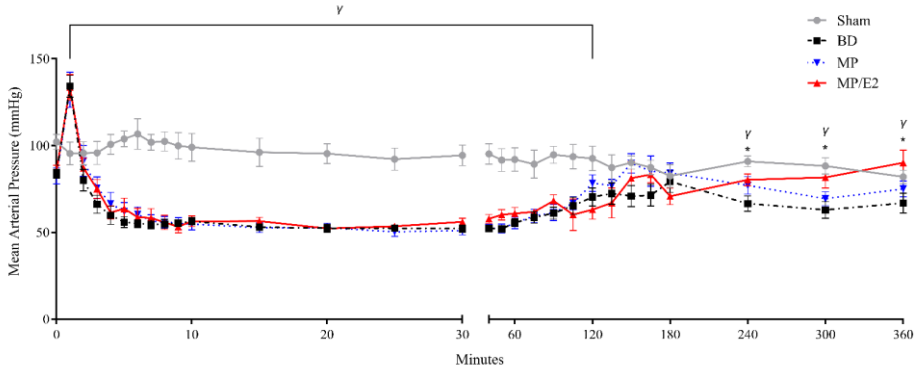

Figure 3 - Mean arterial pressure measurements. Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 8 animals. $^{\gamma *}p^{(\text{Mixed effect})}<0.05$ in relation to the BD group.

Serum quantification of inflammatory markers

To evaluate systemic inflammation, several markers were quantified in serum samples. Significant lower levels of IL-6 were observed in both treated groups in comparison to BD. Regarding VEGF, there was a reduction in the BD group compared to Sham, and even lower levels were found with the associated treatment (MP/E2) in comparison to

BD. None of the other markers analyzed presented significant differences among the groups (Table 2).

Table 2 - Quantification of inflammatory mediators (pg/mL) in the serum of rats submitted to BD. Sham, false-operated rats; BD, rats submitted to brain death; MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD and MP, rats treated with methylprednisolone after 3h of BD confirmation.

pg/mL	Sham	BD	MP/E2	MP	*P (Kruskal-Wallis)*
IL-1β	387.1±127.4	505.2±95.32	327.7±97.75	273.9±21.17	0.4939
IL-6	281.3±88.28	937.1±302.3	44.47±17.64*	55.68±22.45*	0.0003
VEGF	36.59±9.27*	7.948±2.99	1.571±0.07*	5.742±1.706	0.0006
TNF-α	48.67±17.88	40.45±14.87	39.57±9.954	33.90±8.69	0.9301
CINC-1	41.40±9.127	23.96±3.838	27.40±5.260	24.86±4.913	0.3761
NO_x^-	98.55±24.93	186.2±62.50	125.5±17.28	93.27±5.949	0.1582

Data expressed as mean ± SEM from 6-8 animals per group. *p<0.05 in relation to the BD group. IL. interleukin; VEGF. vascular endothelial growth factor; TNF- α. tumor necrosis factor alpha; CINC. cytokine-induced neutrophil chemoattractant; NO. nitric oxide.

Pulmonary inflammation

To evaluate pulmonary inflammation IL-1β, IL-6, TNF-α, and VEGF were quantified in lung tissue and explant.

IL-1β

After BD, IL-1β was increased in lung homogenate and both MP and MP/E2 groups presented lower values. Also, both treatments were effective in reducing gene expression. Whereas in the explant, IL-1β was only reduced in the MP/E2 group (Figure 4).

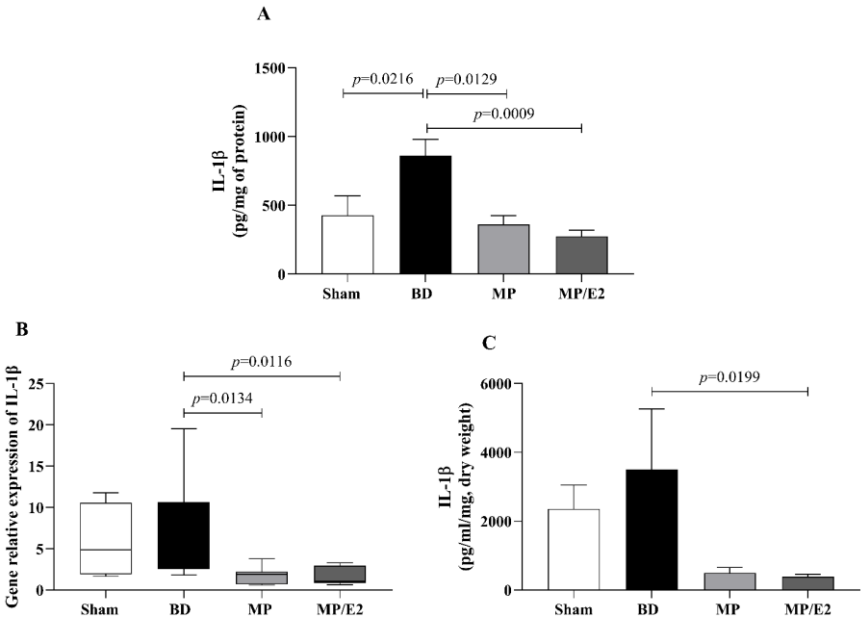

Figure 4 - Quantification of IL-1β in lung homogenate (A) and explant (C) and gene expression in lung tissue (B). Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 8 animals (A) (C). Data expressed as median and 95th percentile from 4-8 animals (B). (A) $p^{(Kruskal\ Wallis)}=0.0057$, (B) $p^{(Kruskal\ Wallis)}=0.0202$, (C) $p^{(Kruskal\ Wallis)}=0.0295$.

IL-6

IL-6 was significantly increased in lung homogenate after BD and both the associated and the isolated treatments reduced protein expression in homogenate and explant. However, treatments had no differences in gene expression. There was no difference between Sham and BD groups in gene expression and explant (Figure 5).

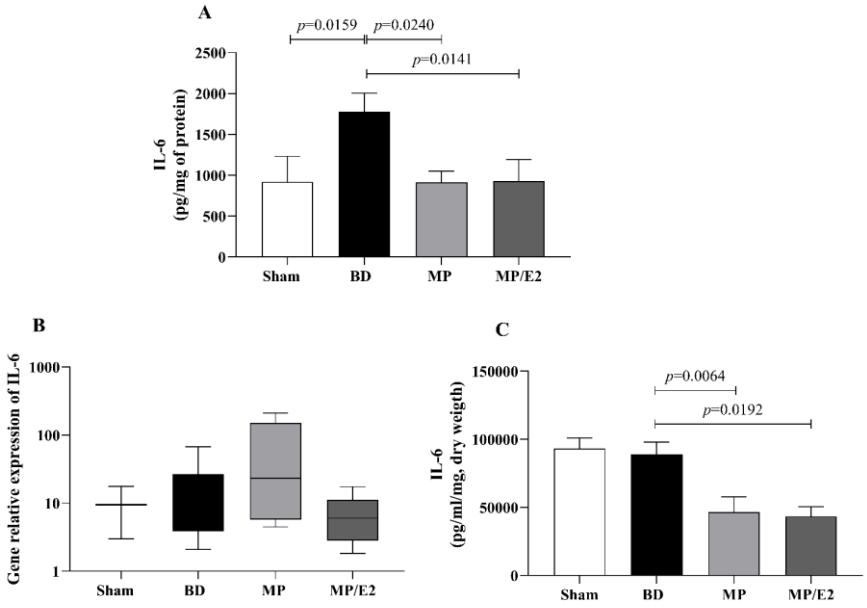

Figure 5 - Quantification of IL-6 in lung homogenate (A) and explant (C) and gene expression in lung tissue (B). Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 8 animals (A) (C). Data expressed as median and 95th percentile from 4-8 animals (B). (A) $p^{(Kruskal\ Wallis)}=0.0288$, (B) $p^{(Kruskal\ Wallis)}=0.2864$, (C) $p^{(Kruskal\ Wallis)}=0.0027$.

TNF-α

BD increased both gene and protein expression in lung tissue, with no change in the explant. Moreover, MP/E2 treatment reduced gene expression of TNF-α and both MP and MP/E2 significantly reduced this cytokine in explant (Figure 6).

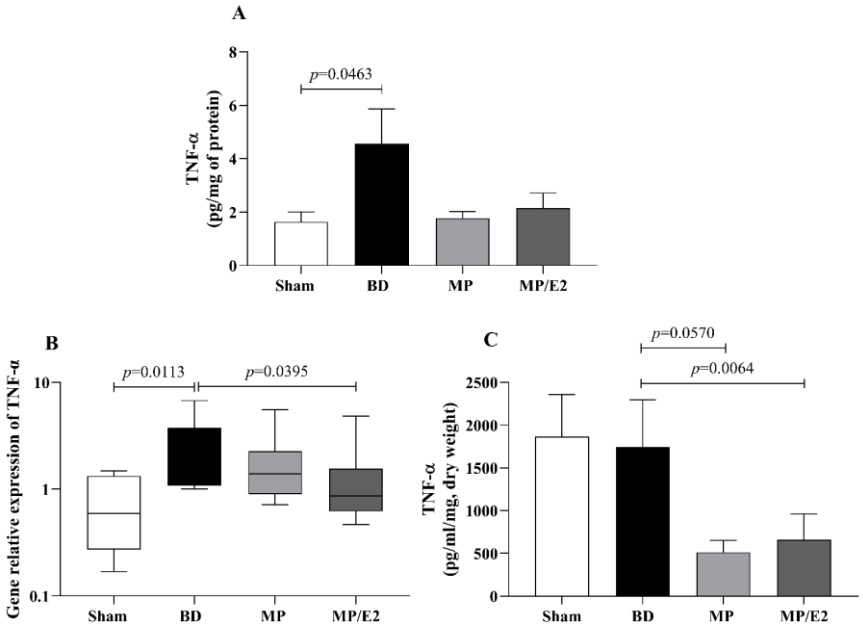

Figure 6 - Quantification of TNF-α in lung homogenate (A) and explant (C) and gene expression in lung tissue (B). Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 8 animals. Data expressed as median and 95th percentile from 5-8 animals (B). (A) $p^{(Kruskal\ Wallis)}=0.1992$, (B) $p^{(Kruskal\ Wallis)}=0.0466$, (C) $p^{(Kruskal\ Wallis)}=0.0369$.

VEGF

In regards to VEGF, the MP/E2 group presented a reduction in gene expression. Also, in explant analyses, overall lower values were observed in the MP/E2 group in comparison to others (Figure 7).

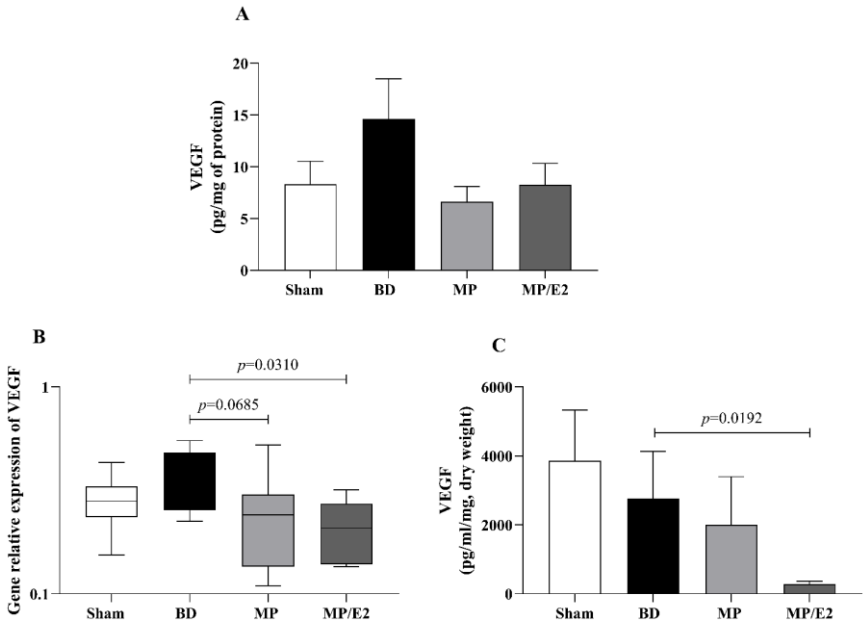

Figure 7 -Quantification of VEGF in lung homogenate (A) and explant (B) and gene expression in lung tissue (C). Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 8 animals (A) (C). Data expressed as median and 95th percentile from 6-8 animals (B). (A) $p^{(Kruskal\ Wallis)}=0.4636$, (B) $p^{(Kruskal\ Wallis)}=0.1275$, (C) $p^{(Kruskal\ Wallis)}=0.0616$.

Leukocyte infiltrates

To evaluate leukocyte migration from the microcirculation to the lung parenchyma and airways, we quantified total and differential cell counts in BAL. Additionally, in the lung parenchyma, MPO activity and protein expression were evaluated. Quantification of CINC-1 levels in lung homogenate and explant was also performed. In parallel, gene and protein expression of ICAM were analyzed.

Bronchoalveolar lavage infiltrate

There was an increase of total infiltrated leukocytes to the alveoli in the BD group and a reduction in the MP/E2 group. Concerning the differential analyses, lymphocytes were increased after BD compared with Sham, with no change in the treatment. Moreover, regarding granulocytes, there was a reduction only in the MP/E2 group (Figure 8).

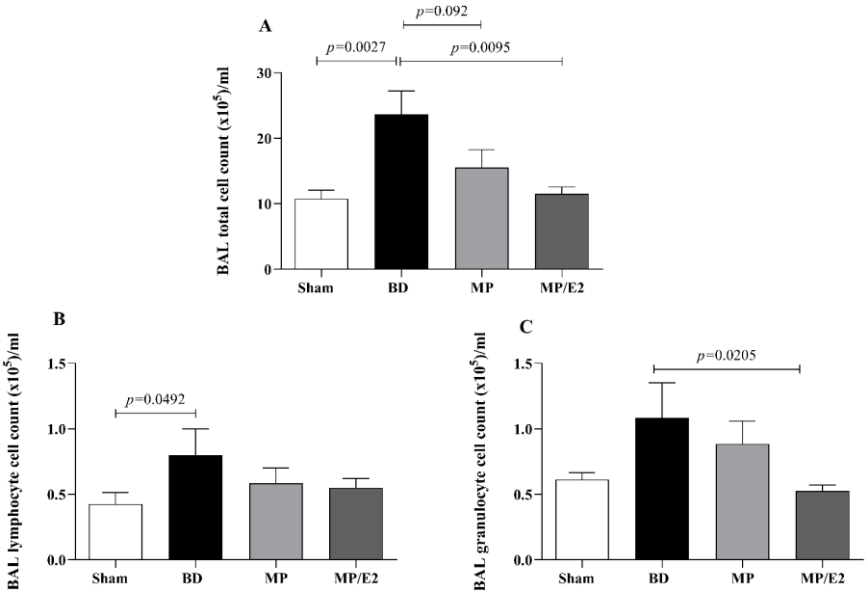

Figure 8 - Total (A) and differential (B) (C) number of cells present in bronchoalveolar lavage. Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 8 animals. (A) $p^{(Kruskal\ Wallis)}=0.0139$, (B) $p^{(Kruskal\ Wallis)}=0.2327$, (C) $p^{(Kruskal\ Wallis)}=0.1015$.

Myeloperoxidase (MPO)

Regarding protein expression of MPO, there was an increase of stained cells in the BD group in comparison to Sham and a decrease in the MP group. There were no significant differences among the groups concerning enzymatic activity (Figure 9).

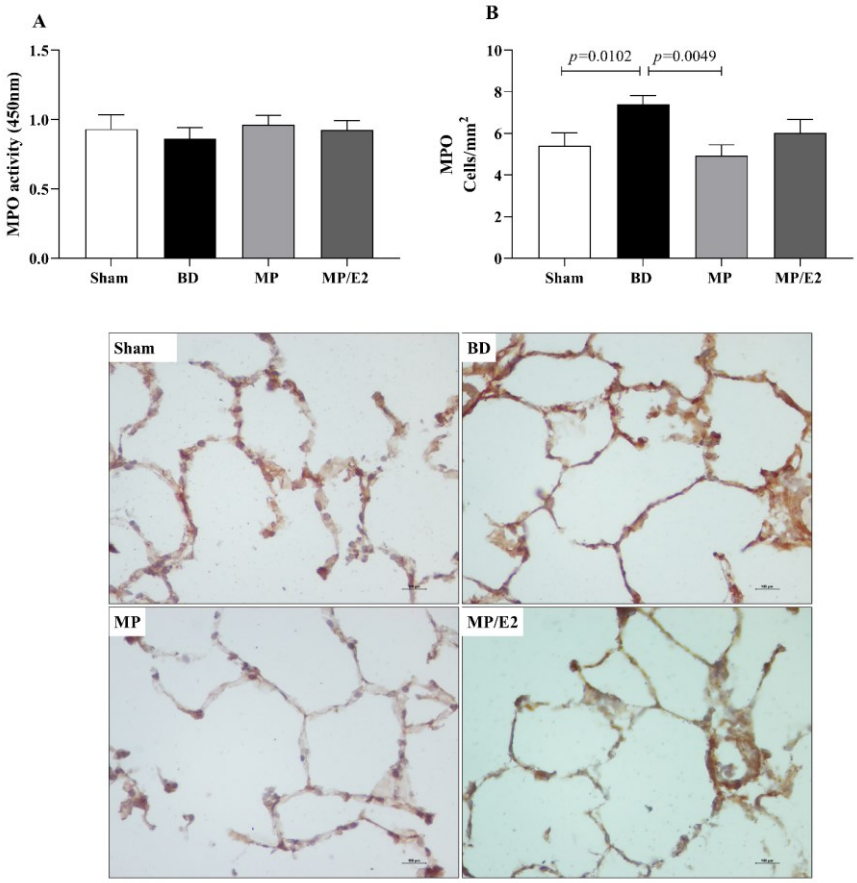

Figure 9 - Enzymatic activity (A) and protein expression (immunohistochemistry) (B) of myeloperoxidase (MPO) in lung tissue. Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 5-8 animals. 1 section per animal and 10 areas per section were analyzed. The photomicrographs (x20) are representative of protein expression on each group. (A) $p^{(Kruskal\ Wallis)}=0.3400$; (B) $p^{(Kruskal\ Wallis)}=0.0191$.

CINC-1, ICAM-1 and VCAM-1

To further analyze leukocyte chemotaxis, early and late release of CINC-1 were quantified in lung homogenate and explant (24h after BD), no difference was observed in lung homogenate, however, both treatments were able to reduce CINC-1 in explant samples (Figure 10). Gene and protein expression of the adhesion molecules ICAM-1 (Figure 11) and VCAM-1 (Figure 12) were also evaluated in lung tissue, but no significant differences were found in both ICAM-1 and VCAM-1 analyses.

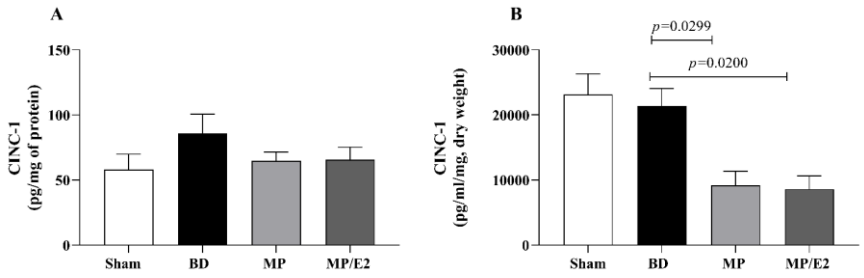

Figure 10 - Quantification of CINC-1 in lung homogenate (A) and explant (B). Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 5-8 animals per group (A) (B). (A) $p^{(Kruskal\text{-}Wallis)}=0.6090$, (B) $p^{(Kruskal\ Wallis)}=0.0075$.

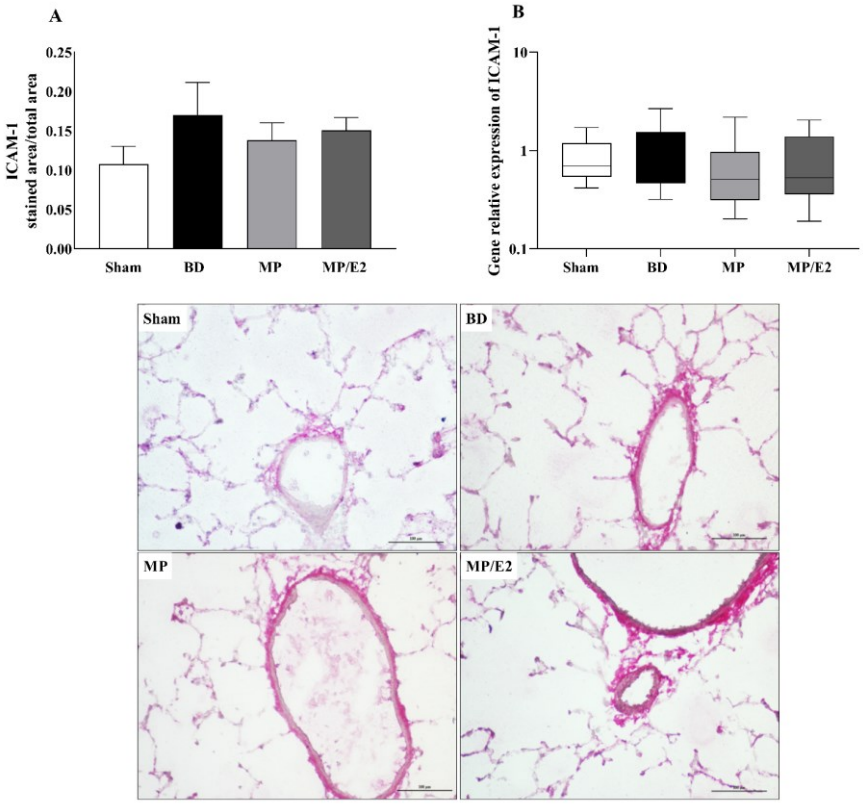

Figure 11 - Protein (A) and gene (B) expression of ICAM-1. Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 5-8 animals per group (A). Data expressed as median and 95th percentile from 6-8 animals (B). 1 section per animal and 10 areas per section were analyzed. The photomicrographs (x20) are representative of protein expression on each group. (A) $p^{(Kruskal\ Wallis)}=0.6009$; (B) $p^{(Kruskal\ Wallis)}=0.7960$.

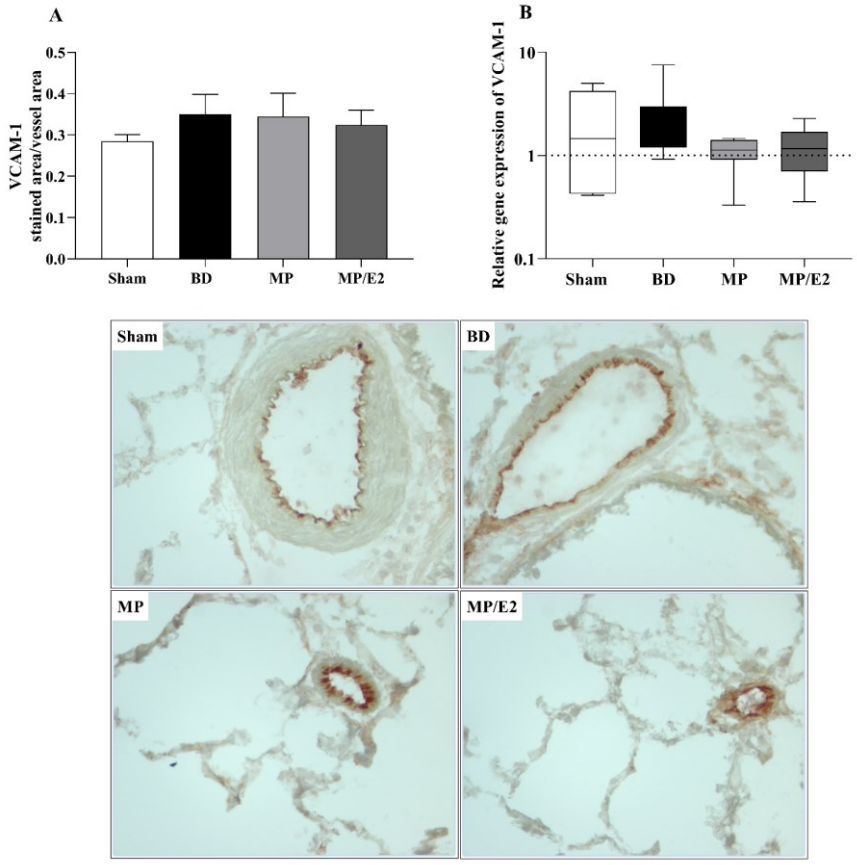

Figure 12 - Protein (A) (immunohistochemistry) and gene (B) expression of VCAM-1. Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 5-8 animals per group (A). Data expressed as median and 95th percentile from 6-8 animals (B). 1 section per animal and 10 areas per section were analyzed. The photomicrographs (x20) are representative of protein expression on each group. (A) $p^{(\text{Kruskal Wallis})}=0.7298$; (B) $p^{(\text{Kruskal Wallis})}=0.3855$.

Analyzes of inducible and endothelial nitric oxide synthase (iNOS and eNOS) protein and gene expression:

Regarding protein, there is a decrease in the expression of eNOS in the MP group compared to BD. In contrast, there is an increase of eNOS in the MP group in gene expression (Figure 13). In iNOS analyses, there was an increase in the BD group and a reduction only in the MP-treated group in protein expression. Regarding gene expression, although there is no difference between the Sham and BD groups, there is an increase in iNOS in the MP group and a reduction after both treatments (Figure 14).

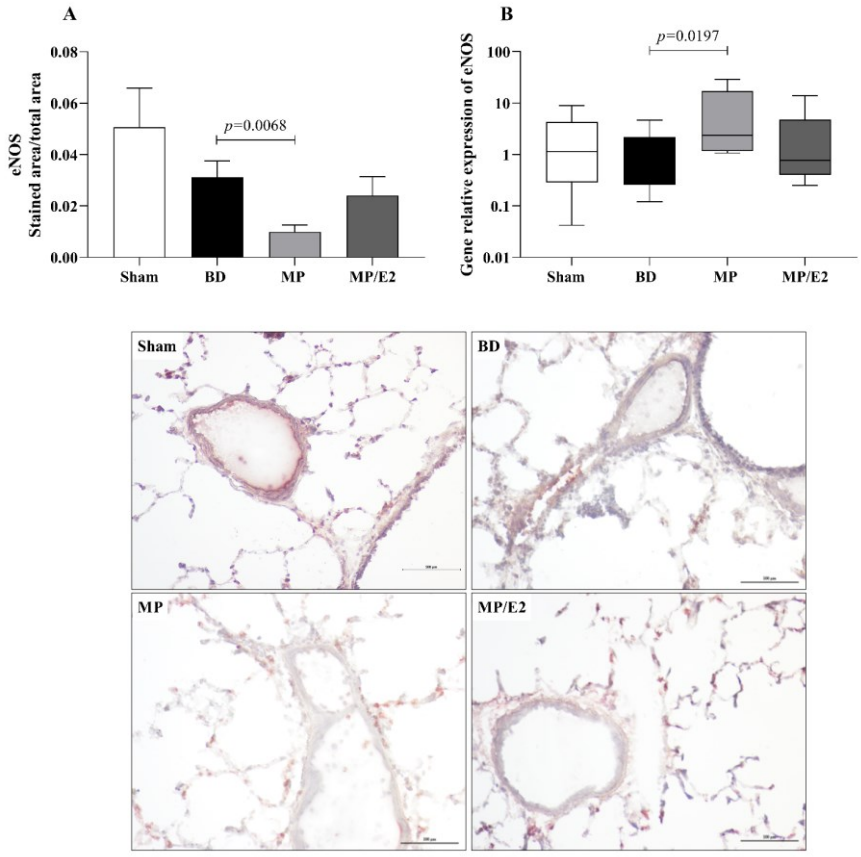

Figure 13 – Protein (A) and gene expression of eNOS (B). Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 5 animals per group (A). Data expressed as median and 95th percentile from 6-8 animals (B). 1 section per animal and 10 areas per section were analyzed. The photomicrographs (x20) are representative of protein expression on each group. (A) $p^{(Kruskal\text{-}Wallis)}=0.0040$, (B) $p^{(Kruskal\ Wallis)}=0.0962$.

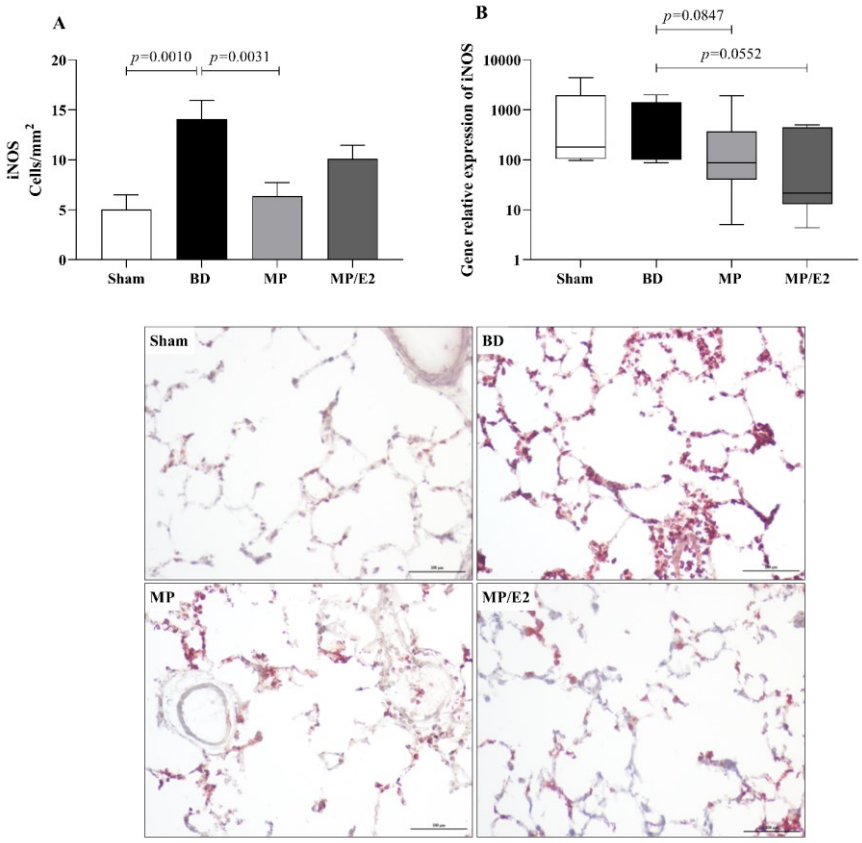

Figure 14 – Protein (A) and gene expression of iNOS (B)**.** Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 5 animals per group (A). Data expressed as median and 95th percentile from 6-8 animals (B). 1 section per animal and 10 areas per section were analyzed. The photomicrographs (x20) are representative of protein expression on each group. (A) $p^{(Kruskal\ Wallis)}=0.0019$; (B) $p^{(Kruskal\ Wallis)}=0.0950$.

Quantification of NOx–

To indirectly determine nitric oxide's presence, nitrites and nitrate were quantified by quantification of NOx– in lung homogenate and explant samples. In the explant, there was a reduction of NOx– in the MP/E2 group in comparison to BD and no changes were observed in the tissue homogenates (Figure 15).

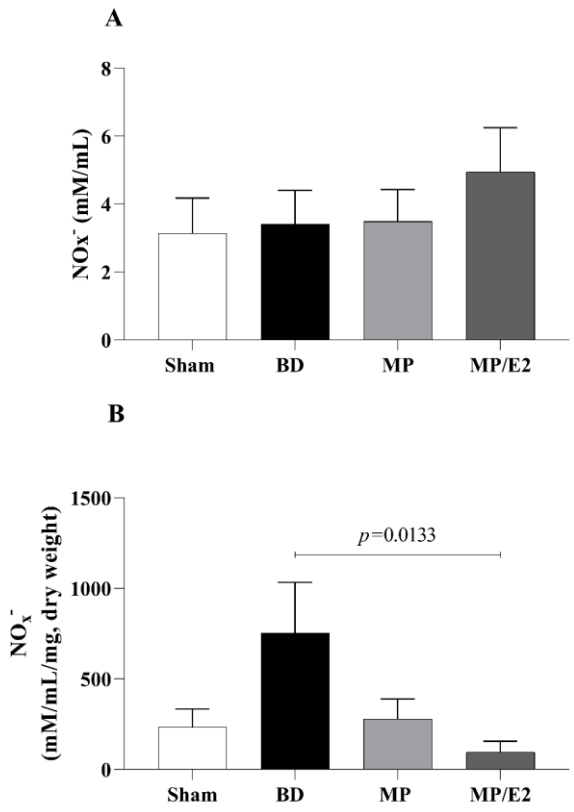

Figure 15 - Quantification of nitric oxide metabolites in homogenate (A) and explant (B). Sham, false-operated rats; BD, rats submitted to brain death; MP, rats treated with methylprednisolone (MP) after 3h of confirmation of BD and MP/E2, rats treated with 17β-estradiol (E2) and methylprednisolone after 3h of confirmation of BD. Data expressed as mean ± SEM from 6-8 animals per group. (A) $p^{(Kruskal\text{-}Wallis)} = 0.9138$, (B) $p^{(Kruskal\ Wallis)} = 0.0845$.

Discussion

This study investigated if E2 and MP association had positive effects in modulating inflammation in females after BD, and we observed that the hormone combination was able to positively regulate inflammation, especially on leukocyte infiltration and endothelial health. Relevant aspects of systemic and pulmonary evaluation were analyzed, such as systemic and local quantification of inflammatory mediators, leukocyte infiltration to the lung parenchyma and airways, as well as adhesion molecule expression. With our data, we observed that the treatments were able to significantly increase corticosterone and estradiol, to levels higher than non-BD controls.

Our model is based on the fast induction of BD and after traumatic brain injury, increased intracranial pressure leads to herniation of the brain stem. We observed a rapid increase in mean arterial blood pressure just after balloon insufflation, followed by a period of hypotension. Several guidelines propose the use of catecholamines in the hemodynamic management of the donor. However, studies suggest that the use of norepinephrine can be detrimental to the organs, causing increased pulmonary permeability. The use of corticoid supplementation has shown positive effects in decreasing the donor's need for catecholamines [24]. Our results, however, show that administration of methylprednisolone and E2 combined was able to reinstate MAP levels close to Sham animals one hour after the start of treatment, while methylprednisolone alone did not present the same effect. Additionally, recent work in a sepsis model showed that

treatment of female animals with E2 increased the expression of corticoid receptor α (GRα) in vascular smooth cells. This receptor was associated with glucocorticoid activation of vascular activity and the upregulation of this receptor by E2, enhanced corticoid-positive action in vascular dysfunction [25].

Another known physiological imbalance triggered by BD is inflammation. High IL-6 levels were correlated with early allograft dysfunction after transplantation, while lower levels were associated with improved graft survival [26]. In our model, BD indeed increased IL-6 levels in the serum, which was reduced by both treatments; the same reduction was observed in the long-term analysis of IL-6 explant levels, indicating that after transplant, IL-6 levels could be controlled by both treatments. Corticoids have notorious anti-inflammatory properties by inhibiting several pro-inflammatory cytokines, including IL-6 [27]. Moreover, high concentrations of E2 also suppress IL-6 expression by down-regulating NK-κB [28].

Lungs are most vulnerable to the detrimental effects of BD, reflecting the low transplant rate for this organ. The sympathetic storm leads to the disruption of endothelial cells and the alveolar barrier [24], and, acute systemic inflammation leads to the infiltration of activated neutrophils to the lungs, leading to tissue injury [29, 32]. Previous studies from our group have shown that BD increases inflammatory markers in lung tissue in the short and long-term and that E2 presents anti-inflammatory properties that could attenuate lung injury [14, 15]. The same behavior can be observed in this study after 6h of BD by the

increased levels of inflammatory markers in lung homogenate and lung culture samples 24 hours after the experiment. Both treatments were effective in reducing protein and gene expression at the moment of organ procurement and 24 hours later. These results indicate that the treatment of the donor, with MP alone or with MP combined with E2, could have a positive effect on the graft in the short and long term.

Moreover, to further evaluate the inflammatory response in the lungs, we investigated the leukocyte infiltration to the parenchyma and airway. Donor leukocyte in the lung is directly involved in acute rejection in the recipient. The migration of donor infiltrate cells to recipient lymph nodes leads to the activation of naïve T cells, resulting in allograph rejection [30]. MPO results show that there was an increase of infiltrate neutrophils in the lung parenchyma and that the treatment with MP showed a lower number of neutrophils. However, no differences were observed among the groups regarding the activity of those cells. Even though no changes were found in adhesion molecules, a higher number of leukocytes were present in bronchoalveolar lavage samples, primarily of granulocytes, and the associated treatment was able to reduce their number in the airways. In the lungs, cytokines and chemokines are released from damaged epithelial cells, as well as resident macrophages after injury. Leukocyte migration to the lung parenchyma follows a specific chain of events that are believed to be independent of adhesion molecules, such as ICAM-1 and VCAM-1. High ICAM-1 levels are expressed in lung vasculature in a steady state and cell migration may be dependent more of chemokine gradients. Indeed,

in a model of LPS challenge in mice, blockage of ICAM-1 did not affect neutrophil recruitment to the lung parenchyma or bronchoalveolar space [31, 32]. Activated neutrophils present a slower transit time in the lung vasculature, which is believed to stimulate neutrophil migration through the endothelial cell junctions. Once in the lung parenchyma, neutrophils are attracted to the airways and secrete proteases, like metalloproteinase-9 (MMP-9), to migrate through the lung interstitium [33, 34]. Here, we show that treatment with MP alone did not prevent the migration of cells from the lung parenchyma to the bronchoalveolar space. Previous studies have shown that estradiol treatment was able to reduce cell migration to the airways, by reducing chemokines such as MIP-1, MIP-2 and CINC-1, along with reduction in MMP-9 activity [14], and depletion of donor cells in lungs have been shown to improve transplant results [35]. Previous and current results suggest an estradiol-dependent mechanism in modulating neutrophil activation in the lung parenchyma of females. These point to the use of estradiol in the management of female BD donors as a therapeutic option to improve transplant outcomes, by reducing leukocyte trafficking to the lung and thus modulating the recipient immunogenic response and allograft rejection.

Additionally, to evaluate the effect of both treatments in the endothelium, we analyzed protein and genomic expression of eNOS and iNOS, NO levels in lung homogenate and lung culture, as well as systemic and local VEGF concentrations. NO is a soluble gas with strong vasodilatory properties that acts in maintaining the homeostasis

of the vascular bed. eNOS and iNOS are the main ones responsible for NO production in the vasculature. iNOS expression is mediated by cytokines, mainly IL-1β, TNF-α, and IFN-γ, and a high concentration of iNOS-derived NO is involved in the immune response and inflammation. eNOS is constitutively expressed in endothelial cells and is related to the maintenance of vascular tone by releasing nanomolar amounts of NO [36, 37]. Previous studies have shown that females after BD present higher expression of eNOS compared to males [38], which was associated with high estradiol levels before BD induction [12]. E2 is known to upregulate eNOS expression by both genomic and non-genomic pathways. E2 binding to ERβ was associated with increased expression of eNOS mRNA, while activation of ERα led to an acute increase in eNOS activity [39, 40]. Also, estradiol treatment after BD in both males and females has been shown to modulate eNOS expression [12, 14, 15] and was associated with increased flow in the mesenteric microcirculation [41]. Our results corroborate those findings by showing that the MP/E2 group was able to prevent further eNOS decrease, while MP alone presents significantly lower values in comparison to BD. Moreover, gene expression of iNOS presented lower values in both treated groups, however, NO quantification in explant samples after 24h suggested that the associated treatment has a long-term effect in reducing NO release.

Likewise, in VEGF analyses, we observed that, overall, all groups that underwent BD induction presented lower levels of VEGF in comparison to Sham. Higher expression of VEGF in Sham animals could be related

to the maintenance of the anesthetic state with isoflurane during the 6 hours of experiment, as isoflurane exposure has been shown to increase VEGF mRNA expression, even in levels as low as 2% [42]. Regarding the treatment groups, MP/E2 treatment was able to reduce both systemic and local expression of VEGF. VEGF actions are related to enhanced permeability, increased leukocyte migration, and activation of angiogenic processes [43]. Corticoids are widely used in different diseases to reduce VEGF levels [44, 45], however, E2 is a known inducer of VEGF mRNA expression [46]. Thus, our results show that MP and E2 association has a positive effect compared to MP alone, suggesting a synergic effect of both hormones in modulating vascular permeability.

This investigation has certain limitations. The time point after 6 hours could limit the analyses of later outcomes. However, explant results provide us with an overview of the lung inflammatory profile 24 hours after procurement. Moreover, we chose to administer a continuous infusion of both hormones after 3 hours of BD. The increased concentration of both hormones for this period could potentially reduce gene and protein expression of ER and GPER (E2 rapid response receptor) and GR, interfering with the receptor response.

In conclusion, this study brings new insights into the role of sex hormones in the management of the BD donor. Showing that E2 association with already well-known anti-inflammatory drugs, like methylprednisolone, could have potentially positive effects on the inflammatory process triggered by BD in females, by modulating the

hemodynamics balance, as well as leukocyte infiltration and maintenance of endothelial and vascular homeostasis.

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Chapter 3

Association of 17β-estradiol and methylprednisolone protects female kidneys from brain death induced inflammation

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Abstract

Background: Brain dead donors are still the main source of organs from transplantation. Brain death (BD) compromises organ quality, and acute reduction in female sex hormones leads to increased inflammation in females. 17β-estradiol (E2) and corticoids have dependent anti-inflammatory effects. Therefore, we aimed to evaluate the effects of the combination of E2 and methylprednisolone (MP) on kidney inflammation after BD. **Methods:** Female Wistar rats were randomly assigned to four experimental groups: control (sham), BD without treatment (BD), BD treated with methylprednisolone after 3 h of BD (4 mg/ml, 2 ml/h-MP) and BD treated with estradiol and methylprednisolone after 3 h of BD (50 μg/ml and 4 mg/ml, 2 ml/h-MP/E2). IL-1β, IL-6, IL-10, TNF-α and VEGF-α were measured in the serum and kidney tissue. The serum concentrations of urea and creatinine were also evaluated. Morphological analyses were performed. **Results:** Both treatments reduced the expression of the analyzed markers in the kidney tissue. Leukocyte mobilization to the renal parenchyma was greater in the BD group compared to sham, and the MP/E2 group presented fewer infiltrated cells. In kidney function, no change in urea or creatinine was observed between the sham and BD groups. Morphological analyses revealed increased necrosis in the proximal tubules after BD and a reduction in the MP/E2 group. **Conclusion:** Our data revealed a positive effect of the combined administration of E2 and MP on renal inflammation triggered by BD. These results point to the therapeutic potential of the combination of both hormones with the aim of improving graft quality.

Chapter 4

Sex differences in kidney and lungs status in an animal model of brain death

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Abstract

Background: Sex dimorphism influences a variety of diseases, driven by genetic and hormonal differences. In transplantation, sex-mismatched procedures correlate with poorer outcomes. Brain death (BD), often caused by trauma or stroke, induces systemic changes that impact organ function. Previous research has indicated that females exhibit heightened inflammatory responses to BD and that organ damage varies with the speed of BD induction. This study aimed to investigate the differential effects of slow BD induction on lung and kidney responses in male and female rats. **Methods:** Males and female rats were subjected to slow induction of BD and observed under ventilation for 4 h. Noradrenaline was administered for ionotropic support. Blood gas samples were taken at 0 h and 4 h. At the end of the experiment, blood and urine samples were collected, as were kidney and lung tissue samples. IL-1β was measured in the plasma, lung homogenate and lung culture. IL-6 was quantified in the plasma, lung culture and kidney homogenate. Gene expression of both interleukins was also analyzed. Leukocyte infiltration/activation in the tissue was evaluated by immunohistochemistry of MPO and iNOS. Biochemical analyses of LDH and creatinine were performed in the plasma. Urine samples were used to quantify creatinine, urea, Na+, and K+ levels. Naïve animals of each sex were used as controls. **Results:** Compared to males, BD-female animals required larger amounts of noradrenaline to maintain normotensive values. With respect to the hormonal profile, males presented reduced testosterone levels after 4 h. Females presented reduced progesterone, whereas estradiol levels were similar at the initial and final time points. Compared with control animals, both BD groups presented increased plasma IL-1β and IL-6 levels after BD. In the blood gas analyses, both males and females presented reduced pO_2 after BD, with females presenting even lower values than males at 4 h. In lung tissue, males presented increased expression of IL-1β, whereas this cytokine was elevated in females in lung culture. Females also presented increased infiltration/activation of neutrophils and macrophages. In the kidney, males presented increased plasma creatinine, increased expression of apoptosis markers and increased leukocyte migration to renal tissue than females. **Conclusions:** In conclusion, we observed an organ- and sex-dependent response to the slow induction of BD. These results suggest that management strategies should consider the sex of the donor to achieve the best treatment, to improve graft quality.

Background

Biological sex has been widely shown to impact disease onset and progression, with males being more susceptible to infectious diseases, whereas females present more susceptibility to autoimmune disorders [1, 2]. These disparities are associated with differences in the innate immune response, which are strongly influenced not only by genetic and epigenetic differences between males and females but also by sex hormones [3].

In the transplantation field, sex also plays an important role in transplant outcomes. Clinical studies have highlighted how sex-mismatched transplantation is associated with a poor post-transplant prognosis, especially in the lungs [4] and kidneys [5]. The majority of organs allocated for transplantation are from donation after brain death (BD). BD is characterized by increased intracranial pressure, leading to herniation of the brain stem, usually as a consequence of trauma or cerebrovascular accidents, resulting in several systemic alterations [6].

In an experimental model of fast induction of BD simulating cranial trauma, our group has previously shown that, compared with males, females present an increased inflammatory response associated with the loss of sex hormones, affecting specially the heart and the lungs [7-10]. Additionally, studies from Rebolledo et al. (2016) and van Zanden et al. (2020) in male animals revealed how the etiology of BD affects thoracic and abdominal organs differently and concluded that kidneys presented increased damage after slow induction of BD, while the reverse occurred

to the lungs. In that sense, considering that males and females have divergent responses to the fast induction of BD and that the slow onset affects specific organs in a different manner, the present study aimed to investigate how the slow induction of BD affects males and female rats, with a focus on the lungs and kidneys.

Methods

Animals

Female and male Wistar rats (8–12 weeks old) from Envigo (The Netherlands) were maintained at 23±2°C, with a 12 h light and dark cycle and food and water ad libitum. The animals received care under the Principles of Laboratory Animal Care (NIH Publication No. 86--23, revised 1985) and the Dutch Law on Experimental Animals Care. This work received approval by the Institutional Animal Care and Use Committee of the University of Groningen.

Animals were randomized into 4 groups:

Female naïve (n=4) = control female animals that did not undergo any surgical procedure;

Female BD (n=8) = female animals that underwent BD induction;

Male naïve (n=4) = control male animals that did not undergo any surgical procedure;

Male BD (n=8) = male animals that underwent BD induction.

Estrous cycle identification

The female animals were used in the estrous and proestrous phases of the estrous cycle (heat period). The cellular profile was assessed via vaginal lavage with a Pasteur pipette filled with 10 µl of saline solution (NaCl 0,9%) and stained with 10 µl of crystal violet (5%). The phase of the cycle was identified via optical microscopy.

Brain death induction

All animals were anesthetized with a mixture of 5% isoflurane and maintained with 2% isoflurane. The temperature was monitored with a rectal probe and maintained at 37°C via a heating mat. The jugular vein was cannulated for fluid administration and any necessary influx of vasoactive drugs for hemodynamic stabilization. The carotid artery was cannulated for blood sampling and blood pressure measurements. A tracheostomy was performed, and the animals were connected to a small animal ventilator (Harvard Apparatus, model 683; Holliston, MA, USA) at a frequency of 70 breaths/min and a tidal volume of 10 mL/kg. For BD induction, a Fogarty® 4F catheter was inserted intracranially and slowly inflated over a span of 30 min. BD was confirmed by bilateral mydriasis and apnea. After BD confirmation, anesthesia was stopped, and fluid administration was initiated (saline solution, NaCl 0.9%, 2 mL/h) for the remaining 4 h. When necessary, noradrenaline was administered for hemodynamic stabilization. Blood samples were collected at the beginning and at the end of the 4 h period. Nonmanipulated (naïve) animals were used as controls.

Around 5 minutes before the end of the experiment, the animals received intravenous injection of a muscle relaxer (suxamethoniumchloride; 0.04 mg/100 g body weight) and heparin (1 ml; 250 U/ml). After 4 h, animals were exsanguinated, and a whole-body flush (maximum pressure of 30 mmHg) was performed with 40 ml of cold saline. Blood, urine and organs were collected.

Lung tissue culture (explant)

After BD, the lung fragments were collected and incubated in Dulbecco's modified Eagle's medium (DMEM) in a humid atmosphere with 5% CO_2 at 37°C for 24 h. The supernatants were collected and stored for further analyses.

Sex hormone determination

Blood samples were collected before and 4 h after BD induction. The quantification of estradiol, progesterone and testosterone was performed using ELISA kits (Cayman Chemical Company, USA) following the manufacturer's instructions.

Blood gas analysis

Arterial blood samples obtained from the carotid artery before BD induction and 4 h after BD induction were used for gas analysis. Blood gas was measured via an ABL90 FLEX blood gas analyzer (Radiometer, the Netherlands), and pCO_2, pO_2 and lactate were recorded.

Biochemical analysis

LDH and creatinine were measured in the plasma. The levels of creatinine, Na^+, K^+ and urea in the urine were measured. Measurements were performed in accordance with the Clinical Laboratory, University Medical Center Groningen, following standard biochemical methods.

IL-1β and IL-6 quantification

IL-1β was measured in the plasma, lung homogenate and explants. IL-6 was measured in the plasma, lung explants and kidney homogenate. Quantifications were performed using DuoSet ELISA commercial kits (R&D Systems, USA) in accordance with the manufacturer´s specifications.

Immunohistochemistry analyses

After 4 h of BD, the lungs and kidneys were collected and subsequently embedded in paraffin. Paraffin sections (4 μm) were prepared for staining. Deparaffinization was performed with xylene and ethanol. For antigen retrieval, the slides were immersed for 3 h at 60°C in EDTA (1 mM), pH 8. The following primary antibodies were used for the lungs: MPO (1:50 - PA1054– Boster, USA), iNOS (1:100 -AB3523– Abcam, UK), and eNOS (1:100 - AO1604-2– Boster, USA). ICAM-1 (1:50 - PB9018– Boster, USA) and VCAM (1:100 - AO119-2– Boster, USA). Kidney markers were MPO (1:100, PA1054– Boster, USA), MMP-9 (1:100, PB9668– Boster, USA), eNOS (1:100, AO1604-2– Boster, USA), iNOS (1:100, AB3523– Abcam, UK) and caspase-3 (1:100, AB4051– Abcam, UK). The sections were incubated with primary antibodies overnight at 4°C. The sections were then incubated with a secondary HRP-conjugated antibody (1:200 – Boster - BA1054) at 37°C for 1h30 to 2h, and later with a peroxidase substrate. Hematoxylin was used for counterstaining. NIS-Element-BD (Nikon, Japan) software was used for the analyses. MPO, lung iNOS and caspase-3 are expressed as the

number of cells per mm2. ImageJ software was used for cell quantification. eNOS, ICAM-1 and VCAM-1 are expressed as the stained area per vessel area. Kidney iNOS and MMP-9 are expressed as the stained area per total area.

Gene expression

RNA was extracted from the kidney and lung using TRIzol reagent (Invitrogen). The yield of extracted RNA was analyzed with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, USA), and the quality was assessed via RNA electrophoresis. The extracted RNA was reverse transcribed via random hexamer primers (Thermo-Fisher, USA) at 37°C for 50 min. Real-time quantitative polymerase chain reaction (qPCR) was conducted using specific primers from SYBR Green (Applied Biosystems, the Netherlands) (Table 1) and a Quant Studio 7 Flex qPCR machine (Applied Biosystems, the Netherlands). The cycle configuration was: 1 cycle of 10 min at 95°C and 40 consecutive cycles of 15 s at 95°C and 1 min at 60°C.

Table 1 – RT-PCR SYBR Green primers.

Primer	Forward sequence	Reverse sequence
β-actin	5-GGAAATCGTGCGTGACATTAAA-3	5-GCGGCAGTGGCCATCTC-3
eNOS	5-AGTCCTCACCGCCTTTTCCA-3	5-GCACGCGGTGAACCTCC-3
IL-6	5-CCAACTTCCAATGCTCTCCTAATG-3	5-TTCAAGTGCTTTCAAGAGTTGGAT-3
Caspase-3	5-GCATGCCAGAAGATACCAGTGG-3	5-AGTTTCAGCATGGCGCAAA-3
BCL-2	5-CTGGGATGCCTTTGTGGAA-3	5-TCAGAGACAGCCAGGAGAAATCA-3
KIM-1	5-AGAGAGAGCAGGACACAGGCTTT-3	5-ACCCGTGGTAGTCCCAAACA-3
IL-1β	5-CAGCAATGGTCGGGACATAGTT-3	5-GCATTAGGAATAGTGCAGCCATCT-3

RT-PCR (real time polymerase chain reaction); eNOS (endothelial nitric oxide synthase); IL-6 (interleukin 6); BCL-2 (B cell lymphoma 2); KIM-1 (kidney injury marker 1); IL-1β (interleukin 1 beta).

Statistical analysis

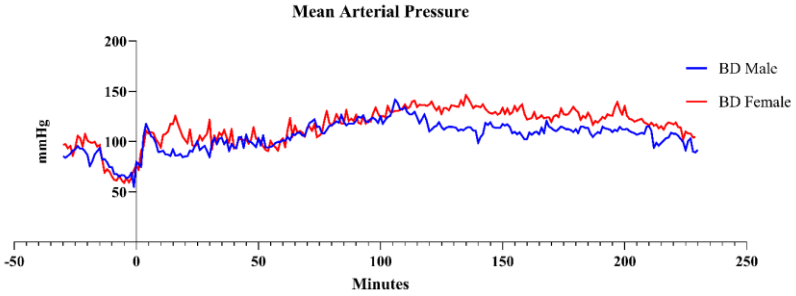
The data are expressed as the mean ± standard error of the mean (SEM) or as the median and the maximum and minimum. The data were analyzed with GraphPad Prism Version 10.3.1. For mean arterial pressure, noradrenaline, hormone quantification, gene expression, urinary creatinine, urea, K^+ and Na^+ were compared using Mann-Whitney test. For all other graphs, groups were compared using two-way ANOVA followed by the post hoc test of the two-stage linear step-up procedure of Benjamin, Krieger and Yekutieli.

Results

Hemodynamic parameters

After balloon catheter insertion, insufflation started once all the animals were stable, with a mean arterial pressure (MAP) of 80 mmHg, and lasted for 30 min (data presented before the Y axis) (Figure 1). During this period, an increase to 100 mmHg was observed in the first 10 min of induction, followed by a decrease to approximately 50 mmHg and a reestablishment of the MAP to 80 mmHg.

After BD confirmation, the MAP was kept stable above 80 mmHg by the administration of intravenous noradrenaline, resulting in no difference between males and females. However, we observed that females required higher volumes of noradrenaline to reach the desired MAP.



	BD Male	BD Female	*P*
Noradrenaline (0.1 mg/mL)	0.0625 ± 0.09161	0.3625 ± 0.2504	**0.0188**

Figure 1 - Mean arterial pressure measurements and volume of noradrenaline (0.01 mg/mL) administered to animals during 4 h of BD. BD female, female rats subjected to brain death (n=8); BD male, male rats submitted to brain death (n=8). MAP data are expressed as mean only and noradrenaline values represent the means and standard errors of the means (SEMs).

Hormonal profile

Estradiol, progesterone and testosterone

In females, there was no change in estradiol plasma levels at the initial (0 h) or final (4 h) measurements (A), whereas progesterone was significantly lower after 4 h of BD than at the initial values (B). In males, plasma levels of testosterone (C) were also lower at the final time point (4 h) in comparison to the initial time point (0 h) (Figure 2).

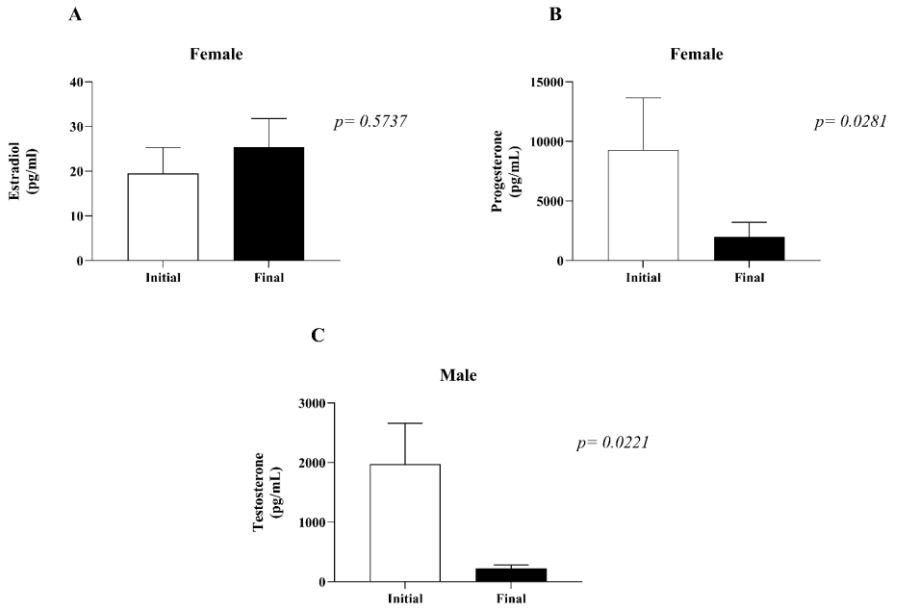


Figure 2 - Plasma quantification of estradiol (A) and progesterone (B) in females and testosterone in males (C). Plasma samples were collected before and 4 h after BD induction. The values represent the means and standard errors of the means (SEMs) of 8 animals per group.

Plasma measurements of IL-1β, IL-6, LDH and lactate

To evaluate the systemic inflammatory profile, the plasma levels of IL-1β and IL-6 were quantified. There was a significant increase in the

concentrations of both cytokines in males after BD compared to naïve. Compared with naïve females, females also presented higher values of these cytokines after BD. The plasma concentrations of LDH and lactate were also evaluated. No difference was observed in LDH, whereas lactate was increased at 4 h after BD induction compared with the initial values in males and females. In regards to sex difference, females presented higher concentrations of lactate than males did at the final measurement point (Table 2).

Table 2 – Systemic measurement of IL-1β, IL-6, LDH and lactate. Blood samples were collected at the beginning (0 h) and at the end (4 h) of the BD period. Naïve, nonmanipulated animals (n=4); BD, rats subjected to brain death (n=8).

Plasma					
	Male		Female		
	Naïve	BD	Naïve	BD	P
IL-1β (pg/mL)	11.22±0.2	36.04±7.1*	11.43±1.3	21.14±3.0	$P_{BD}=0.0063$ $P_{sex}=0.2090$ $P_{interac}=0.1969$
IL-6 (pg/mL)	9.47±0.0	2060±634.9*	9.47±0.0	1521±516.6	$P_{BD}=0.0070$ $P_{sex}=0.6549$ $P_{interac}=0.6546$
LDH (U/L)	265.3±26.4	568.2±148.7	241.0±68.5	339.6±90.5	$P_{BD}=0.1649$ $P_{sex}=0.3746$ $P_{interac}=0.4698$
Blood					
	Male		Female		
	Initial	Final	Initial	Final	P
Lactate	1.462±0.1	2.608±0.5 αβ	1.421±0.1	4.400±0.5 α	$P_{BD}<0.0001$ $P_{sex}=0.0383$ $P_{interac}=0.0306$

The values represent the means and standard errors of the means (SEMs). * $p<0.05$ compared with the naïve group. α $p<0.05$ compared with the initial values. β $p<0.05$

compared with females. IL-1β (interleukin-1β); IL-6 (interleukin-6); LDH (lactate dehydrogenase).

Blood gas analyses

Blood gas measurements before and after BD were evaluated. Both males and females presented a reduction in pO_2 and pCO_2 after 4 h of BD. However, females presented even lower levels of pO2 in comparison to males, suggesting a worsening of lung function (Table 3).

Table 3 – Blood gas measurements. Blood samples were collected at the beginning (0 h) and at the end (4 h) of the BD period. BD female, female rats subjected to brain death (N=8); BD male, male rats submitted to brain death (n=8).

(mmHg)		Male	Female	*P*
pO_2	0 h	453.31± 24.30	502.07±9.01	P_{time}<0.0001
	4 h	306.96±37.80$^{\alpha\beta}$	198.18±26.18$^{\alpha}$	P_{sex}=0.2653
				$P_{interac}$=0.0059
pCO_2	0 h	60.73±6.6	58.92±4.14	P_{time}<0.0001
	4 h	30.60±5.2$^{\alpha\beta}$	26.01±2.41$^{\alpha}$	P_{sex}=0.5158
				$P_{interac}$=0.7773

The values represent the means and standard errors of the means (SEMs). $^{\alpha}$ p<0.05 compared with the initial values. $^{\beta}$ p<0.05 compared with females. pO_2 (partial pressure of O_2); pCO_2 (partial pressure of CO_2).

Quantification of interleukins in the lung homogenate and explants

In the lung homogenate (A), there was an increase in IL-1β in both males and females compared with that in naïve animals, whereas males presented significantly higher values than females after BD. According to the results of the PCR analyses (B) and explants (C), females presented slightly higher concentrations and increased gene expression

of IL-1β than males (Figure 3). No significant difference in the level of IL-6 was observed among the groups (Figure 4).

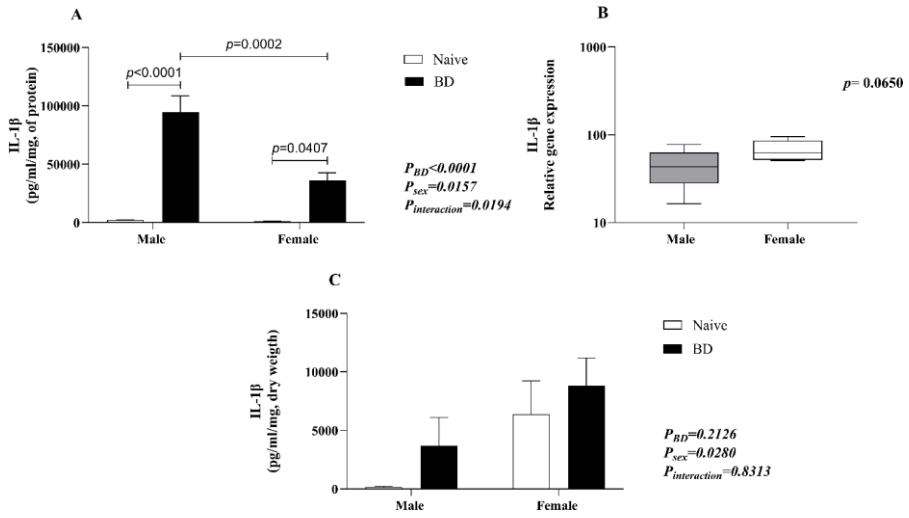


Figure 3 - Quantification of IL-1β in lung homogenates (A) and explants (C) and gene expression (B). Naïve, nonmanipulated animals (n=4); BD, rats subjected to brain death (n=8). The values represent the means and standard errors of the means (SEMs) (A) (C) and median and the maximum and minimum (B). IL-1β (interleukin-1β).

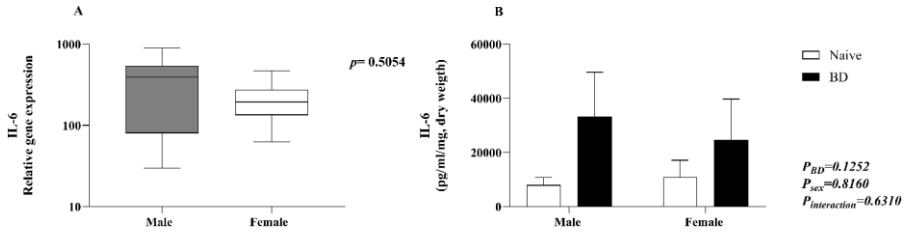


Figure 4 – Gene expression (A) and concentration of IL-6 (B) in lung explants. Naïve, nonmanipulated animals (n=4); BD, rats subjected to brain death (n=8). The values represent the means and standard errors of the means (SEMs) (B) and median and the maximum and minimum (A). IL-6 (interleukin-6).

Leukocyte infiltration

MPO

To evaluate leukocyte infiltration into lung tissue, the protein expression of myeloperoxidase (MPO) was assessed. There was an increase in the number of infiltrated cells in both the male and female BD groups compared with the naïve groups. Concerning sex difference, females presented even higher values than males did after BD (Figure 5).

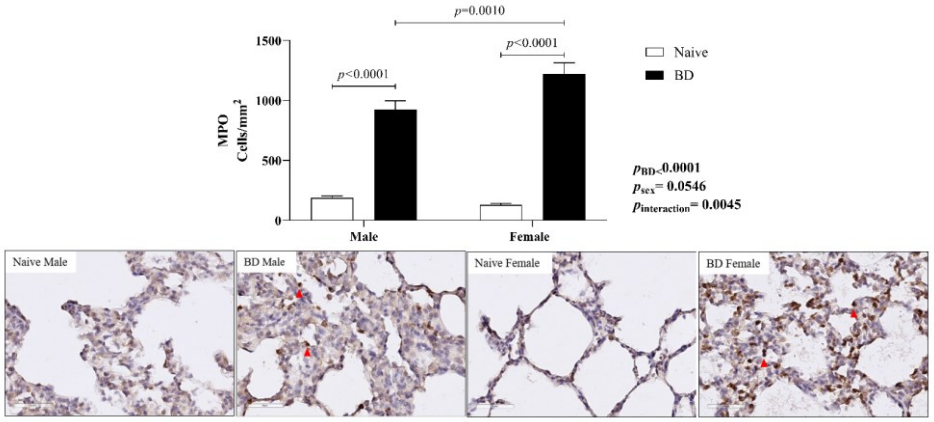


Figure 5 – Protein expression of MPO in lung tissue (red arrow). Naïve, nonmanipulated animals (n=4); BD, rats subjected to brain death (n=8). The values represent the means and standard errors of the means (SEMs). MPO (myeloperoxidase). Representative photomicrographs (x40) of each group.

Adhesion molecules

Additionally, the protein expression of adhesion molecules was evaluated. Both VCAM-1 and ICAM-1 were highly expressed in BD female animals than in the respective naïve animals, which did not occur in male rats. No difference was observed between the sexes (Figure 6).

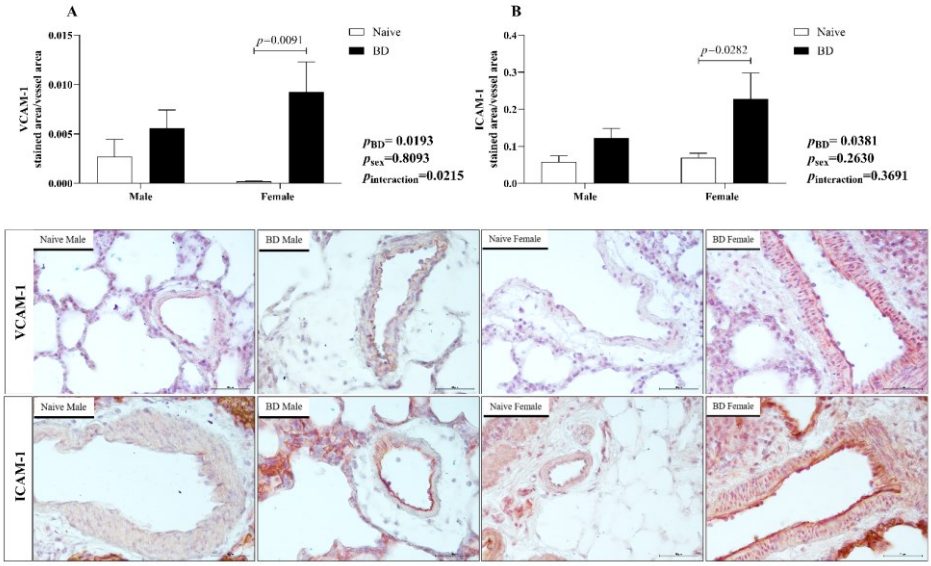


Figure 6 – Protein expression of VCAM-1 and ICAM-1 in lung tissue. Naïve, nonmanipulated animals (n=4); BD, rats subjected to brain death (n=8). The values represent the means and standard errors of the means (SEMs). Representative photomicrographs (x20) of each group. VCAM-1 (vascular cell adhesion molecule); ICAM-1 (intercellular adhesion molecule).

Expression of nitric oxide synthases in lung tissue

iNOS

Inducible nitric oxide synthase (iNOS) was also evaluated in the lung. There was an increase in the number of stained cells in the BD female group compared with those in the female naïve and BD male groups (Figure 7).

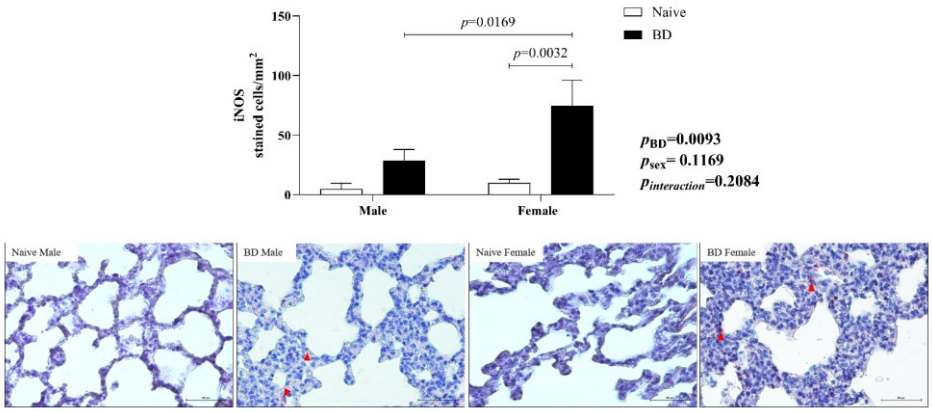


Figure 7 – Protein expression of iNOS in lung tissue (red arrow). Naïve, nonmanipulated animals (n=4); BD, rats subjected to brain death (n=8). The values represent the means and standard errors of the means (SEMs). Representative photomicrographs (x20) of each group. iNOS (inducible nitric oxide synthase).

eNOS

With respect to the protein expression of endothelial nitric oxide synthase (eNOS) in lung tissue (A), both males and females presented reduced expression after BD in relation to the respective naïve animals. Relative gene expression was also evaluated (B), and no difference was observed between the groups (Figure 8).

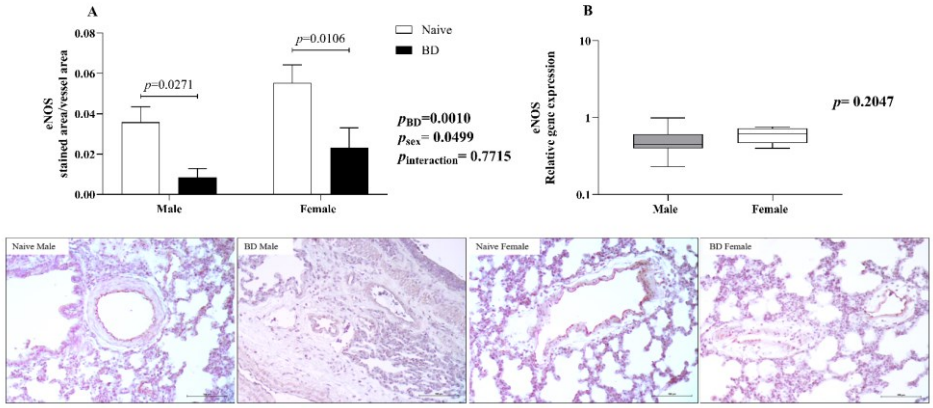


Figure 8 – Protein expression of eNOS in lung tissue (A) and gene expression (B). Naïve, nonmanipulated animals (n=4); BD, rats subjected to brain death (n=8). The values represent the means and standard errors of the means (SEMs) (A) and median and the maximum and minimum (B). Representative photomicrographs (x40) of each group. eNOS (endothelial nitric oxide synthase).

Kidney function

In order to evaluate kidney function, plasma and urinary concentrations of creatinine were quantified. Urinary concentrations of urea, Na+ and K+ were also analyzed (Table 4). In the plasma, males presented higher concentrations of creatinine after BD in comparison to male naïve and BD female animals. The same could be observed in the urine, with males presenting increased values in comparison to females. With respect to the other parameters, K+ was higher in the males than in the females and no difference were observed in relation to urea and Na+.

Table 4 - Plasmatic values of creatinine. Urinary values of creatinine, urea, Na^+ and K^+. Naïve, nonmanipulated animals (n=4); BD, rats subjected to brain death (n=8).

Plasma					
	Male		Female		
(μmol/L)	Naïve	BD	Naïve	BD	P
Creatinine	18.66±0.8	62.50±14.4*β	18.75±1.1	32.12±3.6	$P_{BD}=0.0239$ $P_{sex}=0.2092$ $P_{interac}=0.2068$
Urine					
(mmol/L)	Male		Female		P
Creatinine	4.16±0.74		2.16±0.42		$P=0.0247$
Urea	183.9±25.3		151.8±19.07		$P=0.3282$
Na^+	25.88±3.71		26.88±4.34		$P=0.9608$
K^+	75.81±10.83		41.56±9.30		$P=0.0499$

The values represent the means and standard errors of the means (SEMs). * $p<0.05$ compared with the naïve group. β $p<0.05$ compared with females. Na^+ (sodium); K^+ (potassium).

Quantification of IL-6 in kidney homogenate

IL-6 was quantified in kidney homogenates. No difference was observed between the naïve and BD groups in either sex (A). There were no differences in the gene expression of IL-6 (B) between males and females (Figure 9).

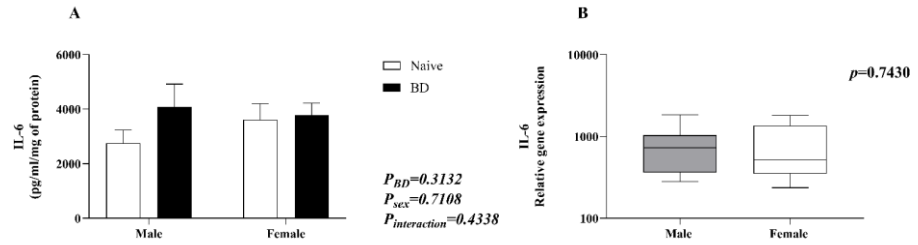


Figure 9 - Quantification of IL-6 in kidney homogenates (A) and gene expression (B). Naïve, nonmanipulated animals (n=4); BD, rats subjected to brain death (n=8). The values represent the means and standard errors of the means (SEMs) and median and the maximum and minimum (B). IL-6 (interleukin-6).

Protein expression of MPO and MMP - 9 in kidney tissue

In the myeloperoxidase (MPO) analysis (A), there was an increase in the number of migrated cells in both males and females after BD compared with that in naïve individuals. In terms of sex difference, males presented greater leukocyte infiltration than females. Metalloproteinase-9 (MMP-9) was also evaluated (B), and there was increased protein expression in BD females compared with BD males (Figure 10).

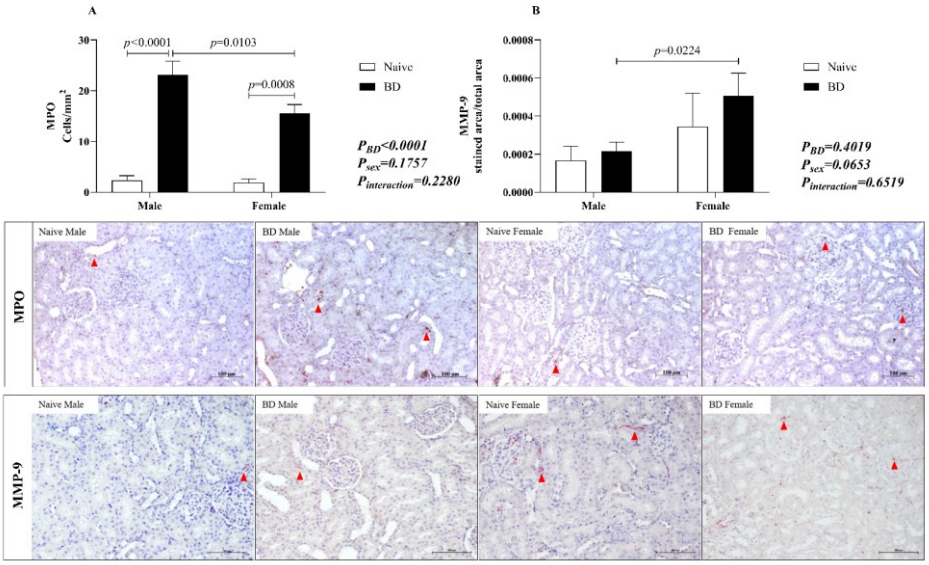


Figure 10 – Protein expression of MPO and MMP-9 in kidney tissue (red arrow). Naïve, nonmanipulated animals (n=4); BD, rats subjected to brain death (n=8). The values represent the means and standard errors of the means (SEMs). Representative photomicrographs (x40) of each group. MPO (myeloperoxidase); MMP-9 (metalloproteinase-9).

Expression of nitric oxide synthases in kidney tissue

iNOS

To further analyze renal inflammation, the protein expression of iNOS was analyzed. Compared with females, males presented greater expression of iNOS after BD (Figure 11).

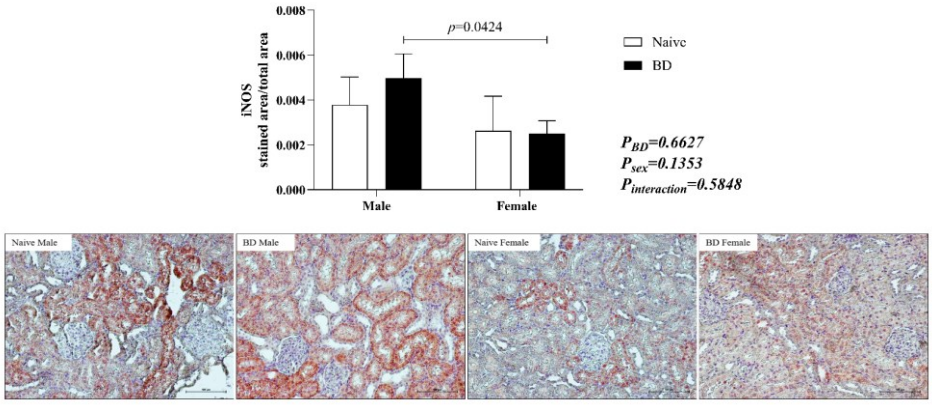


Figure 11 – Protein expression of iNOS in kidney tissue. Naïve, nonmanipulated animals (n=4); BD, rats subjected to brain death (n=8). The values represent the means and standard errors of the means (SEMs). Representative photomicrographs (x40) of each group. iNOS (inducible nitric oxide synthase).

Apoptosis markers

Finally, to analyze kidney apoptosis, the protein and gene expression of caspase-3 was evaluated. After BD, males presented a greater number of stained cells than females (A). However, in gene expression females presented higher expression than males (B) (Figure 12).

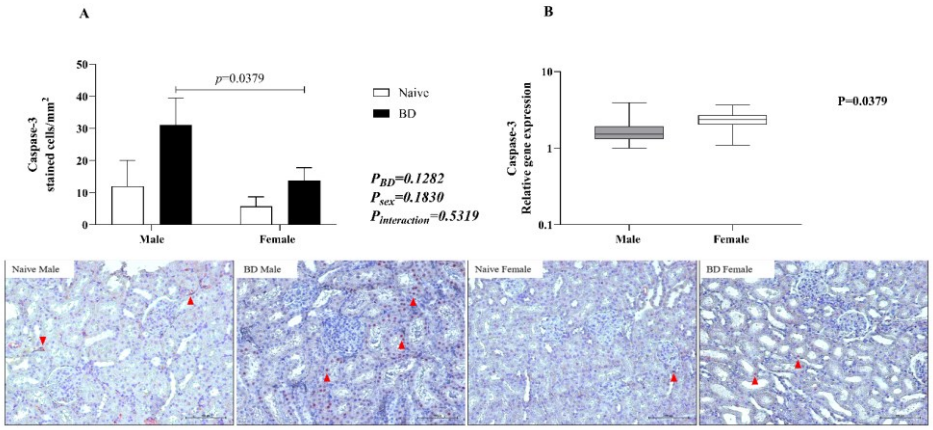


Figure 12 – Protein (A) (red arrow) and gene (B) expression of caspase-3 in kidney tissue. Naïve, nonmanipulated animals (n=4); BD, rats subjected to brain death (n=8). The values represent the means and standard errors of the means (SEMs) (A) and median and the maximum and minimum (B). Representative photomicrographs (x40) of each group.

Discussion

Previous investigations from our group in fast induction model of BD have shown greater impairment of microcirculation in males, marked by increased platelet aggregation, and a more relevant inflammatory response in females with pronounced cell infiltration to the organs [7, 8, 13]. The present study highlights how the slow induction of BD may affect males and females in different manners and how BD-induced sex dimorphism can be organ dependent. Our results indicate increased pulmonary injury in females, with more prominent cell infiltration and reduced blood pO_2. In the kidneys, however, males presented greater leukocyte infiltration and cell apoptosis, accompanied by higher plasma levels of creatinine.

Several studies have used experimental models of BD [14-17], which primarily consist of the insertion and insufflation of a balloon catheter inside the skull, generating an increase in intracranial pressure and herniation of the brain stem followed by a hypotensive phase. Two distinct BD models are described in the literature: the fast and the slow induction. Fast induction is used to simulate acute trauma to the brain, with rapid expansion of the balloon. In this model, an increase in arterial pressure is observed within the first 60 seconds. The slow induction mimics a hemorrhagic stroke via gradual insufflation of the balloon, resulting in a slow increase in arterial pressure and maintaining some hemodynamic stability 18. Studies from Rebolledo et al. (2016) and van Zanden et al. (2020) compared the impact of slow and fast induction of BD in male rats. Both studies revealed a rapid increase in

the mean arterial pressure (MAP) in the fast model, which was not observed in the slow-onset model. Additionally, animals subjected to fast insufflation of the catheter required more inotropic support.

In fact, our group has previously shown that the fast induction of BD leads to an acute increase in the MAP, usually in the first minute, followed rapidly by a hypotensive phase [13, 19, 20]. In studies directly comparing males and females in the fast model, without hemodynamic control, no differences in hemodynamic behavior were observed between the sexes [8]. In the present study, we used a slow induction model in which the intracranial balloon was gradually insufflated for 30 minutes. Unlike fast induction, our model does not show an acute increase in MAP, and the hypotensive phase can be observed in the last 10 minutes before BD confirmation. This pattern was similar in both groups subjected to BD, regardless of sex. After BD induction, noradrenaline was administered when the MAP reached less than 80 mmHg, and both males and females presented similar MAP patterns during the 4 h of BD. However, female animals require higher levels of noradrenaline to achieve normotensive values, suggesting that after slow induction, females are more hemodynamically unstable than males.

Another relevant point in the pathophysiology of BD is the loss of the hypothalamus–hypophysis axis, which compromises the donor endocrine system. Clinical studies have highlighted the reduction in T3, T4, insulin and glucocorticoids, and experimental studies have demonstrated a reduction in female sex hormones [7, 21]. In the literature,

little is known about sex hormones in brain dead males. However, Amado et al. (1995) reported a reduction in testosterone in brain-dead male patients. Our results indicate an acute reduction in testosterone in males after 4 hours of BD compared with the initial values. In females, we previously showed that both progesterone and estradiol were reduced 3 hours after fast induction of BD [7]. In the present study, we also observed a significant reduction in progesterone in the females; however, the estradiol concentrations after 4 hours remained similar to the initial values. This behavior in the concentration of estradiol could be due to a greater stress response of the females to the slow induction of BD, as the 30 min between catheter insertion and BD confirmation could represent a window in which the hormone is acutely released into the bloodstream. In this context, before the surgical procedure, females were selected on the phase of the estrous cycle that present peak levels of estradiol. Indeed, studies have shown that females present a greater stress response during phases of the estrous cycle with high estradiol concentrations [23]. Moreover, differences in estradiol and progesterone concentrations may also be related to the metabolism of both hormones. Progesterone is strongly susceptible to enzymatic reduction and is rapidly transformed into subproducts that take part in the synthesis of other steroid hormones [24], whereas estradiol is converted from testosterone and estrone much later in the pathway [25].

Consistently, several studies have highlighted the increase in inflammatory mediators after BD and its association with a poor prognosis after transplantation [26, 27]. More recently, Belhaj et al. (2022),

in a porcine model, reported that increased serum levels of IL-1β and IL-6 after BD were associated with increased renal injury. Our results revealed a similar scenario, with increased plasma concentrations of IL-1β and IL-6 after BD groups compared with the respective controls. Furthermore, compared with the initial measurements, both males and females presented reduced pO_2 and pCO_2 at 4 hours, with a more relevant decrease in both gases in the females. Ricardo-da-Silva et al. (2024), in the same experimental model, also reported reduced pO_2 in females after BD; however, that difference was not maintained during ex vivo lung perfusion. With respect to LDH, no difference was observed, but females presented higher levels of lactate at the final measurement point compared to males. We suggest that in females, increased lactate could be a result of noradrenaline-derived vasoconstriction and reduced pO_2, leading to hypoxia in peripheral tissue. Lactate is a known byproduct of glycolysis in an anaerobic environment due to insufficient oxygen delivery [29, 30], and compared with other catecholamines, norepinephrine administration is associated with increased serum lactate levels [31]. Moreover, lactate may also result from epinephrine-induced aerobic glycolysis via stimulation of Na^+, K^+ - ATPase activity [32].

The systemic imbalance triggered by BD also locally compromises graft function. In the lungs, changes in vascular resistance and MAP lead to neurogenic pulmonary edema and inflammation [33]. Studies from Breithaupt-Faloppa et al. (2016) and Simão et al. (2016) have shown that, after fast induced BD, females present more exacerbated

pulmonary inflammation than males do, which is associated with a rapid decrease in estradiol. Estradiol is an important regulator of the female immune response and is related to an increased cell-mediated immune response [34, 35] and reduced release of proinflammatory mediators, such as IL-6, IL-1β and TNF-α [36]. Indeed, our results showed an increased neutrophil infiltration into the lungs of female BD animals compared to males, as shown by higher MPO expression after BD. We also quantified macrophages activation by counting iNOS-marked cells. Similar to neutrophils, females presented an increased number of activated cells compared with female controls and BD males. Leukocyte infiltration was accompanied by increased expression of adhesion molecules ICAM-1 and VCAM-1 in females. These findings suggest that females present a more exacerbated cellular immune response in the lungs after BD, independent of induction time. In lung homogenate, we observed lower concentration of IL-1β in females, which could be associated with the maintenance of estradiol levels after BD. Indeed, estradiol has been shown to modulate neutrophil recruitment to the site of ongoing inflammation [37], but also promote its anti-inflammatory activity [34]. In lung explant, however, IL-1β quantification revealed higher values in females than in males after 24 h. Moreover, previous studies in the same model comparing males and females, followed by 4 h of *ex vivo* lung perfusion, has shown increased levels of IL-1β in the perfusate of female's lungs [38]. Such scenarios could be a result of the lack of estradiol in the *ex vivo* system or culture medium, triggering a proinflammatory response in the infiltrate

immune cells. Indeed, low levels of estradiol have been previously associated with increased release of IL-1β [39]. Additionally, several clinical studies have highlighted the worst patient and graft survival after donation between female donors and male recipients, whereas sex-matched transplant recipients presented superior outcomes [40, 41]. These clinical results reiterate our findings that female lungs presented inferior quality, highlighted by decreased pO_2 and increased leukocyte infiltrate, and that these grafts may perform worse in environments with low or non-existing estradiol.

Lastly, endothelial NOS expression was also analyzed in lung tissue, and both sexes presented lower eNOS levels after BD induction than their respective naïve groups. These results corroborated previous findings showing that, in the lung, both males and females presented reduced expression of eNOS after fast induction of BD [10, 42].

With respect to renal damage after slow induction of BD, our results indicate a worsening of kidney function in males, marked by increased plasma and urinary creatinine levels, cell apoptosis and increased infiltration of immune cells. It has been previously shown that inflammation is closely linked to mechanisms that lead to coagulation derangement through thrombin and fibrin formation, especially those related to tissue factor release [43]. IL-6 has also been shown to play a role in the initiation of the coagulation pathway [44]. Coagulopathy is a known consequence of BD and is especially characterized by temporary hypercoagulation [45]. In an experimental model, Correia et al. (2020) described sex differences in the coagulation process after BD, with

males presenting greater platelet aggregation, increased clot firmness and reduced microvascular perfusion than females. Indeed, in the same model of slow induction of BD, Armstrong Jr et al. (2023) reported increased expression of eNOS in females compared with males, suggesting better maintenance of flow in females. eNOS is expressed in the endothelium and promotes vasodilation, and estradiol is associated with increased expression and activity of eNOS [46, 47]. In the kidney, microcirculation is a key point in the development of acute kidney injury (AKI), and hypoperfusion due to microthrombi formation could lead to disruption of cellular homeostasis, culminating in cell death [48]. In the present study, BD males presented increased protein expression of the apoptosis marker caspase-3, whereas gene expression was greater in females. Armstrong Jr et al. (2023) also reported a pro-apoptotic state in male kidneys before machine perfusion. Caspase-3 expression has been linked to the early onset of AKI [49], and our results suggest that such injury may occur earlier in males and later in females. In this context, clinical studies have highlighted a greater risk of AKI in male BD donors before transplantation [50]. Apoptosis is also known to potentiate inflammation, especially by promoting leukocyte recruitment through the release of chemokines in the form of apoptotic extracellular vesicles [51]. Indeed, our results revealed increased leukocyte infiltration in both sexes after BD, with more pronounced neutrophil and macrophage mobilization in males, as indicated by increased expression of MPO and iNOS. Finally, regarding MMP-9, no difference was observed between the control and BD groups in either sex, whereas

BD females presented increased protein expression compared with BD males. These results suggest that increased expression of MMP-9 is related to female sex and not to BD induction. Studies have shown that the activation of estradiol receptors is related to increased MMP-9 expression [52].

Perspective and significance

Finally, the present study provides an understanding of how the slow induction of BD affects males and females in a divergent manner. Unlike fast induction, our model does not show a reduction in estradiol in females, modifying the female response to the systemic and local imbalance triggered by BD. More importantly, our results have shown that tissue damage is sex dependent and that each organ is differently affected in males and females. These insights provide new perspectives on how donor treatment and management strategies should be organ focused and consider the cause of BD, as well as the sex of the donor.

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Chapter 5

Hormonal treatment during ex vivo lung perfusion ameliorates brain death induced inflammation

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Abstract

Background: Lung transplantation remains the primary option to treat end-stage lung disease, and treatments aiming to improve graft quality are necessary. *Ex vivo* lung perfusion (EVLP) is a strategy that allows organ to be assessed and reconditioned before transplantation. Treatment of the donor with a combination of 17β-estradiol (E2) and methylprednisolone (MP) has shown to improve lung quality after brain death (BD). All considered, this study aimed to investigate E2 and MP association during EVLP. **Methods:** Males and females Wistar rats underwent BD induction and were maintained for 4h. Naive animals were used as control. After BD, the pulmonary artery was cannulated, the heart-lung block was collected, submitted to cold ischemia (1 h) and then placed in an EVLP system (4 h). Perfusion solution was home-made STEEN added or not with the treatment (T: MP, 40 mg; E2: 5 μg/mL). Groups were defined as male and female rats, divided as follows: BD (without perfusion), EVLP (without treatment), EVLP+Treat (with treatment). **Results:** Male EVLP+Treat present increased dynamic and static compliance, increased paO_2 and reduced elastance. Treated males also presented reduced iNOS and MPO and increased perfusion flow. Both female perfused groups presented reduced MPO and adhesion molecules. Female EVLP+Treat also presented increased flow. No difference in lung function was observed in female. **Conclusions:** Our results point to a positive effect in the combined use of E2 and MP during EVLP by improving lung function and decreasing inflammation, especially in males.

Chapter 6

Males and females respond differently to treatment during isolated kidney perfusion: combined effects of glucocorticoid and estradiol

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Abstract

Background: Kidney perfusion is a toll that allows organs to be assessed before transplantation. After brain death (BD), hormonal dysfuction compromise graft quality. Hormonal treatment in the donor has shown positive outcomes and treatment during *ex vivo* perfusion may be advantageous. The combination of 17β- estradiol (E2) and methylprednisolone (MP) was able to modulate inflammation in the donor. Therefore, this study aims to evaluate treatment with E2 and MP during isolated perfusion of kidneys in brain-dead male and female rats. **Methods:** Females and males Wistar rats were submitted to BD and maintained for 4h. In the same animal, the right kidney (RK – no IPK) was removed and stored, while the left kidney (LK – with IPK) had the ureter and the renal artery cannulated, and flushed with 5 ml of cold saline. The LK was then taken directly to the IPK system for 90 minutes. Experimental groups were performed in both male and female: IPK (without treatment) and IPK+Treat (MP and E2 added to the perfusate). Perfusion was performed with a constant pressure of 100 mmHg, using William's Medium E supplemented with HEPES, creatinine and albumin as perfusate. Perfusate and urine were collected and flow measurements were recorded. After IPK, the LK was stored. **Results:** IL-6 was reduced in all perfused groups, regardless of treatment. In female IPK+Treat, there was a reduction in perfusion flow, followed by reduced creatinine clearance and Na^+ excretion. No difference was observed in males in regards to treatment. Conclusion: The combined treatment of E2 and MP during isolated kidney perfusion compromised kidney function in females. In males, no detrimental effects were observed. This results shows a sex-dependent action of the proposed treatment.

Chapter 7

Summary

General discussion

Future perspectives

Summary

The shortage of suitable organs for transplantation and the exponential increase in patients on the waiting list demands improvements in current guidelines for the management of brain-dead donors, as well as new treatment strategies with the purpose of ameliorating the impacts of brain death (BD) in the graft and improving its quality. Understanding the type of donor, especially the sex and etiology of BD, may provide new information on how each donor, or even an organ, responds differently to the systemic imbalance triggered by BD. Overall, this thesis evaluated the impact of a new treatment option by evaluating the combined effects of 17β-estradiol (E2) and methylprednisolone (MP) on graft quality for transplantation. Throughout the chapters, we evaluated the effects of the proposed treatment in the lungs (**chapter 2**) and kidneys (**chapter 3**) when administered to female animals after the induction of BD. In both chapters, we observed a positive effect of the treatment, specially by reducing leukocyte infiltration to the airways and the renal parenchima. Later, we evaluated how the slow induction of BD affects males and females (**chapter 4**), and observed that lung and kidney injury vary between the sexes, with female lungs presenting a more exarcebated inflammation, while the males presented worst renal function an increased apoptosis. Finally, we treated lungs (**chapter 5**) and kidneys (**chapter 6**) from rats of both sexes during *ex vivo* machine perfusion with E2 and MP. In male lungs, treatment was able to improve lung function, especially by improving compliance, while in

females, treatment decreased pulmonary inflammation. Regarding the kidneys, treatment was detrimental to females by reducing perfusion flow leading to worst renal function. No difference was observed in male.

General discussion

In the transplantation field, sex plays an important role. The pharmacodynamics and pharmacokinetics of immunosuppressive drugs differ between male and female recipients [1 - 4]. Compared with male recipients, female recipients appear to present lower graft failure rates; however, this may vary depending on the sex of the donor and the age of the recipient. Younger females (<44 years old) present increased graft failure, which diminishes as age increases [5]. Further research is still needed to better understand these sex-related differences, but sex hormones seem to be a factor [6].

Indeed, our group has shown that an acute reduction in E2 is associated with an increased inflammatory response in females, and E2 treatment improved the lungs, hearts and kidneys [7 - 9]. In the clinic, hormonal resuscitation is widely used during donor management [10], improving hemodynamic stability. The use of methylprednisolone is associated with increased organ retrieval by reducing inflammation and edema and improving oxygenation [11 - 14].

In females, however, an adequate immunological response to inflammation seems to be related to both estrogen and glucocorticoid actions. Studies by Cvoro et al. (2011) investigated the crosstalk

between glucocorticoid (GR) and estrogen (ER) receptors in several proinflammatory genes. Their results revealed that each hormone individually was able to repress only three classes of genes, while the combination of E2 and dexamethasone repressed most of them. The combined administration of E2 and dexamethasone also reduces T lymphocyte-mediated hypersensitivity [16]. This evidence highlights the enhanced anti-inflammatory action of the combination of E2 and glucocorticoids (GCs).

In **chapter 2,** we observed a reduction in both E2 and corticosterone after the induction of BD, as previously shown in this model [17]. MP and E2 treatment restored normotensive mean arterial pressure values after BD and reduced systemic IL-6 and VEGF concentrations, which was not achieved by MP alone. More importantly, MP/E2 was able to modulate neutrophil activation and migration to airways.

Modulation of immune cells is one of the main actions of GCs. In the peripheral blood, GCs increase the neutrophil count by promoting entry into the blood circulation, reducing apoptosis and stimulating hematopoiesis in the bone marrow. Despite increasing the neutrophil count in the bloodstream, GCs modulate cell migration to tissues by reducing the expression of integrins and adhesion molecules, as well as reducing the release of cytokines and chemokines [18 - 20]. Our results revealed no difference in adhesion molecule expression; however, in the lung, ICAM-1 is normally expressed even in the absence of proinflammatory stimuli [21].

Additionally, E2 has been shown to have anti-inflammatory effects by modulating leukocyte recruitment, especially through the reduction of chemokines and cytokines. E2 reduced monocyte chemoattractant protein-1 (MCP-1) mRNA levels after lipopolysaccharide (LPS) stimulation. Additionally, the levels of MCP-1 and macrophage inflammatory protein-2 (MIP-2) are reduced after E2 administration in an autoimmune disease model [22, 23]. Indeed, as mentioned previously, Cvoro et al. (2011) investigated the combined effects of E2 and GCs on proinflammatory genes. Their studies revealed a reduction in the levels of the chemokines macrophage inflammatory protein-1 (MIP-1) and IL-8 only in cells that received both E2 and dexamethasone.

Considering the beneficial effects of the associated treatment in the lungs, in **chapter 3,** we evaluated its impact on renal tissue. Local release of inflammatory mediators was reduced by both treatments, and, similar to the lungs, only the combination of both hormones regulated leukocyte infiltration into the parenchyma.

E2 actions are regulated mainly by the activation of the ER. The ER is widely expressed in several tissues, not only reproductive organs. ERβ was found to be expressed in all lung samples from healthy male and female patients [24]. In the kidney, higher ERα was observed [25]. In a study analyzing approximately 10,000 genes, Jelinsky et al. (2003) reported that the kidney contains the third largest number of genes regulated by E2, after only the uterus and pituitary. As several organs are targeted by estrogens, we suggest that treatment of the donor has protective effects on the kidneys and lungs of female animals, which are

related to the anti-inflammatory capacity of E2, especially in terms of leukocyte recruitment.

In **chapters 2** and **3**, we used a model of fast induction of BD. As we have previously shown in this model, E2 and corticosterone are acutely reduced 3 h after the onset of BD [17], we aimed to treat the donor directly. In **chapters 4**, **5** and **6,** we used a slow induction model. Unlike fast induction, where sex differences in the pathophysiology of BD have been reported in the literature [17], few studies on sex dimorphism after slow induction are available.

In **chapter 4,** we focused on describing the differences between males and females, once again targeting the lungs and kidneys. Systemically, we observed a reduction in testosterone in males. In females, progesterone levels also decreased; however, unlike during fast induction, the estradiol concentration remained similar at the initial and final time points. We attributed this lack of change in estradiol levels to a possible acute release of the hormone in response to the stress of slow catheter insufflation. Regarding the organs, we observed a worsening of renal function in the males. We associated this scenario with increased apoptosis due to hypoperfusion of the microvasculature. Even though we did not evaluate the coagulatory status of rats after slow BD, it was previously reported in a model of fast BD induction that males presented greater hypercoagulation than females, leading to reduced flow [28, 29]. Moreover, in lungs, females presented reduced lung function, accompanied by increased inflammation, especially due to increased leukocyte infiltration into the pulmonary parenchyma.

Although E2 has anti-inflammatory effects, these effects seem to be dose dependent. In several studies, Straub et al. (2007) reported that the anti-inflammatory effects of E2 seem to be related to increased E2 concentrations, which are usually close to pregnancy levels. In **chapters 2** and **3,** we used supraphysiologic doses of E2 to treat the animals after BD. In **chapter 4**, even though no reduction in E2 was observed, reported E2 concentrations were not capable of ameliorating leukocyte migration, even though IL-1β was reduced in the lung homogenate.

Furthermore, in **chapters 5** and **6**, we used the growing technique of organ perfusion to evaluate the therapeutic potential of the MP and E2 combination in an *ex vivo* environment. As the protocols for the management of donors after BD are still widely discussed and very much dependent on hospital-specific guidelines, therapeutic options focused on treatment during machine perfusion may result in less ethical discussion and present a greater chance of being accepted by the medical community.

In **chapter 5,** we perfused the lungs in an *ex vivo* lung perfusion (EVLP) system. Despite differences between the sexes at the end of BD, all the lungs presented similar pO_2 values after 15 min of perfusion. Treatment improved lung function in males and reduced lactate levels and the number of MPO- and iNOS-marked cells. Unlike nonperfused lungs, female lungs perfused without treatment presented increased protein and gene expression of IL-1β, similar to previous findings in the same model that also compared males and females [30]. Compared with those in the untreated group, the gene expression of IL-1β in the lungs of the

treated group was lower. Additionally, lung function remained the same during the 4 h in both female groups.

In **chapter 6,** we used kidney perfusion to evaluate how treatment affects organs in an isolated environment. In contrast to previous findings from **chapter 3,** where treatment of the donor improved kidney quality, the administration of MP and E2 in an *ex vivo* environment compromised kidney function in females, especially by reducing perfusion flow. We associated these results with possible cross-activation of mineralocorticoid receptors (MRs) by MPs, as females are more predisposed to vascular damage upon MR activation [31 - 33]. In males, no substantial differences were observed between treated and nontreated kidneys, indicating that there were no detrimental effects.

Conclusion

Overall, we observed how distinct treatment approaches affect organs differently before transplantation. In addition to the different protocols of administration (directly to the donor or in an isolated *ex vivo* system), the effectiveness of the treatment seems to be organ- and sex dependent. When it was administered to brain-dead female donors, treatment improved both the lungs and kidneys. In an *ex vivo* set-up, treatment was positive for both male and female lungs, whereas in the kidneys, it was deleterious to female kidneys.

Our results suggest that treatment of the donor during the BD period is more beneficial than treatment during *ex vivo* perfusion, especially for females. Although *ex vivo* perfusion seeks to reach a near physiological

environment, it does not fully replicate the complexity of a living organism. Perhaps other systemic components not present in an isolated perfusion are important for the proper control of the inflammatory response and return to homeostasis in both sexes. Specifically, in females, the immunological response is tightly controlled by sex hormones, and despite treatment with E2 and MP, other components not considered in this study may be crucial for an appropriate response.

Although the importance of sex differences has been highlighted, the literature still lacks studies focused on this subject in the transplantation field. Typically, donor management protocols are standardized with no consideration of donor characteristics such as sex and age. In this thesis, we provide new insights into how a better understanding of sex differences could impact transplantation, especially with the development of personalized management strategies, with the aim of addressing the necessity of each donor and organ.

Future perspective

Investigations of the pathway through which E2 modulates systemic inflammation are lacking. As mentioned previously, organs present different levels of ER expression. Both genomic and nongenomic actions are responsible for the effects of E2. ERα is highly expressed in endothelial cells, and its activation is linked to E2 vascular actions, i.e., vasodilation and endothelial NO production [34]. Erβ is also responsible

for NO production [35] and is found mainly in cells of the immune system. The E2 membrane receptor GPER is also expressed in immune cells and is capable of promoting both rapid and genomic responses [36]. In that sense, therapies using agonists aimed at identifying E2 pathways may be of interest. Moreover, targeted therapy using E2 agonists may provide more tailored treatments focused on the necessity of each organ.

Furthermore, several studies suggest that donor ageing is an independent risk factor for transplantation outcomes. Increased donor age negatively affects patient and graft survival in several organs. For each organ type, a different age range is considered the start of the negative effects [37]. Older people present a state of chronic low-grade inflammation, marked by endothelial dysfunction, culminating in vascular oxidative stress, resistant hypertension and inflammatory polarization [38]. Experimental studies performed by Reutzel-Selke et al. (2007) have shown that organs from older donors are more immunogenic, presenting more infiltrated cells, cytokine release and activation of immune cells in the recipient. In women, postmenopause hormonal changes can affect the mild proinflammatory state observed in older people. No studies have focused on BD repercussions in older males and females, especially focused on understanding how the chronic lack of hormones affects the donor immune system, thus opening a new area of interest.

Finally, research focused on understanding the repercussions of sex differences and pretransplant treatment strategies in the long term is

also necessary. Studies have described and improved transplantation techniques in small animals [40]. In the literature, there is evidence of a relationship between donor and recipient sex and transplantation outcomes [41]. In that sense, it would be interesting to further investigate how donor sex, age and treatment options may affect the recipient and better correlate with the clinic.

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Chapter 8

Appendices

Samenvatting, algemene discussie en toekomstige perspectieven.

Het tekort aan geschikte organen voor transplantatie en de exponentiële toename van patiënten op de wachtlijst vereist verbeteringen in de huidige richtlijnen voor het beheer van hersendode donoren, evenals nieuwe behandelingsstrategieën met als doel de impact van hersendood (BD) op het transplantaat te verlichten en de kwaliteit ervan te verbeteren. Het begrijpen van het type donor, met name het geslacht en de oorzaak van de BD, kan nieuwe informatie opleveren over hoe elke donor, of zelfs een orgaan, verschillend reageert op de systemische onbalans die door BD wordt veroorzaakt. Over het algemeen evalueerde deze thesis de impact van een nieuwe behandelingsoptie door de gecombineerde effecten van 17β-oestradiol (E2) en methylprednisolon (MP) op de kwaliteit van het transplantaat voor transplantatie te onderzoeken. In de verschillende hoofdstukken hebben we de effecten van de voorgestelde behandeling in de longen (**hoofdstuk 2**) en de nieren (**hoofdstuk 3**) geëvalueerd wanneer deze werd toegediend aan vrouwelijke dieren na de inductie van BD. In beide hoofdstukken observeerden we een positief effect van de behandeling, vooral door het verminderen van leukocyteninfiltratie naar de luchtwegen en het nierparenchym. Later evalueerden we hoe de langzame inductie van BD mannen en vrouwen beïnvloedt (**hoofdstuk 4**), en observeerden we dat long- en nierbeschadiging varieert tussen de geslachten, waarbij vrouwelijke longen een meer verergerde ontsteking vertoonden, terwijl de mannelijke dieren slechtere nierfunctie en verhoogde apoptose vertoonden. Tot slot behandelden we longen

(**hoofdstuk 5**) en nieren (**hoofdstuk 6**) van ratten van beide geslachten tijdens ex vivo machineperfusie met E2 en MP. In mannelijke longen was de behandeling in staat om de longfunctie te verbeteren, vooral door de compliance te verbeteren, terwijl de behandeling bij vrouwen de longontsteking verminderde. Wat betreft de nieren, was de behandeling nadelig voor vrouwen door het verminderen van de perfusieflow, wat leidde tot een slechtere nierfunctie. Er werd geen verschil waargenomen bij mannelijke ratten.

Algemene discussie

Op het gebied van transplantatie speelt sekse een belangrijke rol. De farmacodynamiek en farmacokinetiek van immunosuppressieve geneesmiddelen verschillen tussen mannelijke en vrouwelijke ontvangers [1 - 4]. In vergelijking met mannelijke ontvangers lijken vrouwelijke ontvangers een lager percentage mislukte transplantaten te hebben; dit kan echter variëren afhankelijk van het geslacht van de donor en de leeftijd van de ontvanger. Jongere vrouwen (<44 jaar) vertonen een verhoogd percentage mislukte transplantaten, dat afneemt naarmate de leeftijd toeneemt [5]. Verder onderzoek is nog steeds nodig om deze seksegerelateerde verschillen beter te begrijpen, maar geslachtshormonen lijken een factor te zijn [6].

Onze groep heeft inderdaad aangetoond dat een acute verlaging van E2 geassocieerd is met een verhoogde ontstekingsreactie bij vrouwen, en behandeling met E2 verbeterde de longen, harten en nieren [7 - 9]. In de kliniek wordt hormonale resuscitatie veel gebruikt tijdens

donorbehandeling [10], waardoor de hemodynamische stabiliteit verbetert. Het gebruik van methylprednisolon wordt in verband gebracht met het verkrijgen van meer organen door het verminderen van ontsteking en oedeem en het verbeteren van de oxygenatie [11 - 14].

Bij vrouwen lijkt een adequate immunologische respons op ontstekingen echter gerelateerd te zijn aan zowel oestrogeen- als glucocorticoïdwerking. Studies van Cvoro et al. (2011) onderzochten de wisselwerking tussen glucocorticoïde (GR) en oestrogeen (ER) receptoren in verschillende ontstekingsbevorderende genen. Hun resultaten toonden aan dat elk hormoon afzonderlijk in staat was om slechts drie genenklassen te onderdrukken, terwijl de combinatie van E2 en dexamethason de meeste genen onderdrukte. De gecombineerde toediening van E2 en dexamethason vermindert ook de T-lymfocyt-gemedieerde overgevoeligheid [16]. Dit bewijs benadrukt de versterkte ontstekingsremmende werking van de combinatie van E2 en glucocorticoïden (GC's).

In **hoofdstuk 2** zagen we een vermindering van zowel E2 als corticosteron na de inductie van BD, zoals eerder aangetoond in dit model [17]. Behandeling met MP en E2 herstelde de normotensieve gemiddelde arteriële drukwaarden na BD en verlaagde de systemische IL-6 en VEGF concentraties, wat niet werd bereikt door MP alleen. Belangrijker nog, MP/E2 was in staat om neutrofiele activatie en migratie naar de luchtwegen te moduleren.

Modulatie van immuuncellen is een van de belangrijkste werkingen van GC. In het perifere bloed verhogen GC het aantal neutrofielen door de

toetreding tot de bloedsomloop te bevorderen, apoptose te verminderen en hematopoëse in het bloedmerg te stimuleren. Ondanks het feit dat het aantal neutrofielen in de bloedbaan verhoogt, moduleren GC de celmigratie naar weefsels door de expressie van integrinen en adhesiemoleculen te verminderen, evenals de afgifte van cytokinen en chemokinen [18 - 20]. Onze resultaten toonden geen verschil in de expressie van adhesiemoleculen; in de longen komt ICAM-1 echter normaal tot expressie, zelfs in afwezigheid van proinflammatoire stimuli [21].

Daarnaast is aangetoond dat E2 ontstekingsremmende effecten heeft door de rekrutering van leukocyten te moduleren, met name door de reductie van chemokinen en cytokinen. E2 verlaagde de mRNA-niveaus van monocyte chemoattractant protein-1 (MCP-1) na stimulatie door lipopolysaccharide (LPS). Daarnaast worden de niveaus van MCP-1 en macrofaag ontstekingseiwit-2 (MIP-2) verlaagd na toediening van E2 in een auto-immuunziektemodel [22, 23]. Zoals eerder vermeld, onderzochten Cvoro et al. (2011) de gecombineerde effecten van E2 en GC op ontstekingsbevorderende genen. Hun onderzoek toonde een verlaging van de niveaus van de chemokines macrofaag ontstekingseiwit-1 (MCP-1) en IL-8 alleen in cellen die zowel E2 als dexamethason kregen. Gezien de gunstige effecten van de geassocieerde behandeling in de longen, evalueerden we in **hoofdstuk 3** de invloed ervan op het nierweefsel. Lokale afgifte van ontstekingsmediatoren werd door beide behandelingen verminderd en, vergelijkbaar met de longen, reguleerde alleen de combinatie van beide hormonen de infiltratie van leukocyten in het parenchym.

De werking van E2 wordt voornamelijk gereguleerd door de activering van het ER. Het ER komt op grote schaal tot expressie in verschillende weefsels, niet alleen in voortplantingsorganen. ERβ bleek tot expressie te komen in alle longmonsters van gezonde mannelijke en vrouwelijke patiënten [24]. In de nieren werd een hogere ERβ waargenomen [25]. In een onderzoek waarin ongeveer 10.000 genen werden geanalyseerd, rapporteerden Jelinsky et al. (2003) dat de nier het op twee na grootste aantal genen bevat dat door E2 wordt gereguleerd, na alleen de baarmoeder en de hypofyse. Aangezien oestrogenen gericht zijn op verschillende organen, suggereren wij dat behandeling van de donor beschermende effecten heeft op de nieren en longen van vrouwelijke dieren, die verband houden met de ontstekingsremmende capaciteit van E2, vooral wat betreft de rekrutering van leukocyten.

In **hoofdstuk 2** en **3** gebruikten we een model van snelle inductie van BD. Omdat we eerder in dit model hebben aangetoond dat E2 en corticosteron acuut verlaagd zijn 3 uur na het begin van BD [17], wilden we de donor direct behandelen. In de **hoofdstukken 4**, **5** en **6** gebruikten we een traag inductiemodel. In tegenstelling tot snelle inductie, waar geslachtsverschillen in de pathofysiologie van BD werden gerapporteerd in de literatuur [17], zijn er weinig studies beschikbaar over geslachtsdimorfisme na trage inductie.

In **hoofdstuk 4** richtten we ons op het beschrijven van de verschillen tussen mannen en vrouwen, waarbij we ons opnieuw richtten op de longen en nieren. Systemisch zagen we een afname van testosteron bij mannen. Bij vrouwen daalden de progesteronspiegels ook, maar in

tegenstelling tot tijdens de snelle inductie bleef de oestradiolconcentratie gelijk op de begin- en eindtijdstippen. We schreven dit gebrek aan verandering in de oestradiolspiegels toe aan een mogelijke acute afgifte van het hormoon als reactie op de stress van langzame katheterinsufflatie. Wat betreft de organen zagen we een verslechtering van de nierfunctie bij de mannen. We brachten dit scenario in verband met verhoogde apoptose als gevolg van hypoperfusie van de microvasculatuur. Hoewel we de stollingsstatus van ratten na trage BD niet evalueerden, werd eerder gerapporteerd in een model van inductie van snelle BD dat mannetjes een grotere hypercoagulatie vertoonden dan vrouwtjes, wat leidde tot een verminderde doorstroming [28, 29]. Bovendien vertoonden vrouwtjes in de longen een verminderde longfunctie die gepaard ging met toegenomen ontsteking, vooral door toegenomen leukocyteninfiltratie in het longparenchym.

Hoewel E2 ontstekingsremmende effecten heeft, lijken deze effecten dosisafhankelijk te zijn. In verschillende studies rapporteerden Straub et al. (2007) dat de ontstekingsremmende effecten van E2 gerelateerd lijken te zijn aan verhoogde E2-concentraties, die gewoonlijk dicht bij zwangerschapsniveaus liggen. In **hoofdstuk 2** en **3** gebruikten we suprafysiologische doses E2 om de dieren na BD te behandelen. In **hoofdstuk 4** waren de gerapporteerde E2-concentraties niet in staat om de migratie van leukocyten te verbeteren, hoewel IL-1β in het longhomogenaat was verminderd.

Bovendien gebruikten we in **hoofdstuk 5** en **6** de groeiende techniek van orgaanperfusie om het therapeutische potentieel van de combinatie van MP en E2 in een *ex vivo* omgeving te evalueren. Aangezien de protocollen voor het beheer van donoren na BD nog steeds veel besproken worden en sterk afhankelijk zijn van ziekenhuisspecifieke richtlijnen, kunnen therapeutische opties die gericht zijn op behandeling tijdens machinale perfusie leiden tot minder ethische discussie en een grotere kans hebben om geaccepteerd te worden door de medische gemeenschap.

In **hoofdstuk 5** perfuseerden we de longen in een *ex vivo* longperfusiesysteem (EVLP). Ondanks verschillen tussen de geslachten aan het einde van BD, vertoonden alle longen vergelijkbare pO_2-waarden na 15 minuten perfusie. De behandeling verbeterde de longfunctie bij mannen en verlaagde het lactaatniveau en het aantal MPO- en iNOS-gemarkeerde cellen. In tegenstelling tot niet-geperfuseerde longen vertoonden vrouwelijke longen die zonder behandeling werden geperfuseerd een verhoogde eiwit- en genexpressie van IL-1β, vergelijkbaar met eerdere bevindingen in hetzelfde model waarbij ook mannen en vrouwen werden vergeleken [30]. Vergeleken met die in de onbehandelde groep was de genexpressie van IL-1β in de longen van de behandelde groep lager. Bovendien bleef de longfunctie hetzelfde gedurende de 4 uur in beide vrouwelijke groepen.

In **hoofdstuk 6** gebruikten we nierperfusie om te evalueren hoe behandeling organen beïnvloedt in een geïsoleerde omgeving. In tegenstelling tot eerdere bevindingen uit **hoofdstuk 3,** waar de

behandeling van de donor de kwaliteit van de nieren verbeterde, bracht de toediening van MP en E2 in een *ex vivo* omgeving de nierfunctie in vrouwen in gevaar, met name door het verminderen van de perfusiestroom. We brachten deze resultaten in verband met mogelijke kruisactivering van mineralocorticoïdreceptoren (MR's) door MP's, aangezien vrouwen meer vatbaar zijn voor vasculaire schade bij MR-activering [31 - 33]. Bij mannen werden geen substantiële verschillen waargenomen tussen behandelde en onbehandelde nieren, wat aangeeft dat er geen nadelige effecten waren.

Conclusie

Over het algemeen hebben we gezien hoe verschillende behandelingsbenaderingen organen verschillend beïnvloeden vóór de transplantatie. Naast de verschillende toedieningsprotocollen (direct aan de donor of in een geïsoleerd *ex vivo* systeem) lijkt de effectiviteit van de behandeling orgaan- en geslachtsafhankelijk te zijn. Wanneer het werd toegediend aan hersendode vrouwelijke donoren, verbeterde de behandeling zowel de longen als de nieren. In een *ex vivo* opstelling was de behandeling positief voor zowel mannelijke als vrouwelijke longen, terwijl het in de nieren schadelijk was voor vrouwelijke nieren. Onze resultaten suggereren dat behandeling van de donor tijdens de BD-periode gunstiger is dan behandeling tijdens *ex vivo* perfusie, vooral voor vrouwen. Hoewel *ex vivo* perfusie een bijna fysiologische omgeving probeert te bereiken, bootst het de complexiteit van een levend organisme niet volledig na. Misschien zijn andere systemische componenten die niet aanwezig zijn in een geïsoleerde perfusie

belangrijk voor de juiste controle van de ontstekingsreactie en de terugkeer naar homeostase bij beide geslachten. Met name bij vrouwen wordt de immunologische respons strak geregeld door geslachtshormonen en ondanks behandeling met E2 en MP kunnen andere componenten die niet in deze studie zijn meegenomen cruciaal zijn voor een goede respons.

Hoewel het belang van sekseverschillen is benadrukt, zijn er in de literatuur nog steeds geen studies over dit onderwerp op het gebied van transplantatie. Meestal zijn protocollen voor donorbeheer gestandaardiseerd zonder rekening te houden met donorkarakteristieken zoals geslacht en leeftijd. In dit proefschrift geven we nieuwe inzichten in hoe een beter begrip van sekseverschillen van invloed zou kunnen zijn op transplantatie, in het bijzonder met de ontwikkeling van gepersonaliseerde managementstrategieën, met als doel de noodzaak van elke donor en elk orgaan aan te pakken.

Toekomstperspectief

Onderzoek naar de weg waarlangs E2 systemische ontsteking moduleert ontbreekt. Zoals eerder vermeld vertonen organen verschillende niveaus van ER-expressie. Zowel genomische als niet-genomische acties zijn verantwoordelijk voor de effecten van E2. ERα komt in hoge mate tot expressie in endotheelcellen en de activering ervan is gekoppeld aan de vasculaire werking van E2, d.w.z. vasodilatatie en endotheliale NO-productie [34]. Erβ is ook verantwoordelijk voor NO-productie [35] en wordt voornamelijk gevonden in cellen van het immuunsysteem. De E2-

membraanreceptor GPER komt ook tot expressie in immuuncellen en is in staat om zowel snelle als genomische reacties te bevorderen [36]. In die zin kunnen therapieën met agonisten die gericht zijn op het identificeren van E2 pathways interessant zijn. Bovendien kan gerichte therapie met behulp van E2-agonisten meer op maat gemaakte behandelingen bieden die gericht zijn op de noodzaak van elk orgaan.
Bovendien suggereren verschillende onderzoeken dat het ouder worden van de donor een onafhankelijke risicofactor is voor de transplantatieresultaten. Een hogere leeftijd van de donor heeft een negatief effect op de overleving van patiënten en transplantaten in verschillende organen. Voor elk orgaantype wordt een andere leeftijdsgroep beschouwd als het begin van de negatieve effecten [37]. Ouderen vertonen een toestand van chronische laaggradige ontsteking, gekenmerkt door endotheeldisfunctie, culminerend in vasculaire oxidatieve stress, resistente hypertensie en ontstekingspolarisatie [38]. Experimentele studies uitgevoerd door Reutzel-Selke et al. (2007) hebben aangetoond dat organen van oudere donoren immunogener zijn, met meer geïnfiltreerde cellen, afgifte van cytokinen en activering van immuuncellen in de ontvanger. Bij vrouwen kunnen hormonale veranderingen na de menopauze de milde proinflammatoire toestand die bij ouderen wordt waargenomen, beïnvloeden. Er zijn geen studies die zich hebben gericht op de gevolgen van BD bij oudere mannen en vrouwen, met name gericht op het begrijpen hoe het chronische gebrek aan hormonen het immuunsysteem van de donor beïnvloedt, waardoor een nieuw interessegebied wordt geopend. Tot slot is ook onderzoek

nodig dat zich richt op het begrijpen van de repercussies van sekseverschillen en pretransplantatiebehandelstrategieën op de lange termijn. Studies hebben transplantatietechnieken bij kleine dieren beschreven en verbeterd [40]. In de literatuur is aangetoond dat er een verband bestaat tussen het geslacht van de donor en de ontvanger en de uitkomsten van transplantaties [41]. In die zin zou het interessant zijn om verder te onderzoeken hoe het geslacht van de donor, de leeftijd en de behandelopties de ontvanger kunnen beïnvloeden en beter kunnen correleren met de kliniek.

Resumo, discussão geral e perspectivas futuras

A escassez de órgãos adequados para transplante e o aumento exponencial de pacientes na lista de espera exigem melhorias nas diretrizes atuais para o manejo de doadores com morte encefálica, bem como novas estratégias de tratamento com o objetivo de amenizar os impactos da morte encefálica (ME) no enxerto e melhorar sua qualidade. Compreender o tipo de doador, especialmente o sexo e a etiologia da ME, pode fornecer novas informações sobre como cada doador, ou até mesmo um órgão, responde de maneira diferente ao desequilíbrio sistêmico desencadeado pela ME. No geral, esta tese avaliou o impacto de uma nova opção de tratamento, avaliando os efeitos combinados de 17β-estradiol (E2) e metilprednisolona (MP) na qualidade do enxerto para transplante. Ao longo dos capítulos, avaliamos os efeitos do tratamento proposto nos pulmões (**capítulo 2**) e rins (**capítulo 3**) quando administrados a animais fêmeas após a indução da ME. Em ambos os capítulos, observamos um efeito positivo do tratamento, especialmente pela redução do infiltrado leucocitário nas vias aéreas e no parênquima renal. Posteriormente, avaliamos como a indução lenta da ME afeta machos e fêmeas (**capítulo 4**), e observamos que a lesão nos pulmões e rins varia entre os sexos, com os pulmões femininos apresentando uma inflamação mais exacerbada, enquanto os machos apresentaram pior função renal e aumento da apoptose. Finalmente, tratamos os pulmões (**capítulo 5**) e rins (**capítulo 6**) de ratos de ambos os sexos durante a perfusão *ex vivo* com E2 e MP. Nos pulmões masculinos, o tratamento foi capaz de melhorar a função pulmonar,

especialmente ao melhorar a complacência, enquanto nas fêmeas, o tratamento diminuiu a inflamação pulmonar. Quanto aos rins, o tratamento foi prejudicial para as fêmeas, reduzindo o fluxo de perfusão e levando a uma pior função renal. Nenhuma diferença foi observada nos machos.

Discussão geral

No campo do transplante, o sexo desempenha um papel importante. A farmacodinâmica e a farmacocinética de medicamentos imunossupressores diferem entre dependendo do sexo do receptor [1, 2, 3, 4]. Comparadas a homens, mulheres apresentam taxas mais baixas de falha do enxerto. No entanto, isso pode variar dependendo do sexo do doador e da idade do receptor. Muheres mais jovens (<44 anos) apresentam maior falha do enxerto, o que diminui à medida que a idade aumenta [5]. Mais pesquisas ainda são necessárias para compreender melhor essas diferenças relacionadas ao sexo, mas os hormônios sexuais parecem ser um fator [6].

De fato, nosso grupo demonstrou que uma redução aguda do E2 está associada a uma resposta inflamatória aumentada nas fêmeas, e o tratamento com E2 foi capaz de melhorar órgãos como os pulmões, corações e rins [7, 8, 9]. Na clínica, o tratamento hormonal é amplamente utilizado durante o manejo do doador [10], ajudando a melhorar a hemodinâmica. O uso de metilprednisolona está associado ao aumento da captação de órgãos, reduzindo a inflamação e o edema, levando a melhora da oxigenação [11, 12, 13, 14].

Nas fêmeas, no entanto, uma resposta imunológica adequada à inflamação parece estar relacionada tanto à ação do estrogênio quanto dos glicocorticoides. Estudos de Cvoro et al. (2011) investigaram a interação entre os receptores de glicocorticoides (GR) e estrogênio (ER) em vários genes pró-inflamatórios. Os resultados revelaram que cada hormônio individualmente foi capaz de reprimir apenas três classes de genes, enquanto a combinação de E2 e dexametasona reprimiu a maioria deles. A administração combinada de E2 e dexametasona também reduziu a hipersensibilidade mediada por linfócitos T [16]. Essas evidências destacam a ação anti-inflamatória aumentada da combinação de E2 e glicocorticoides (GCs).

No **capítulo 2**, observamos uma redução tanto do E2 quanto da corticosterona após a indução da ME, como mostrado anteriormente neste modelo [17]. O tratamento com MP e E2 restaurou os valores de pressão arterial média após a ME e reduziu as concentrações sistêmicas de IL-6 e VEGF, o que não foi alcançado pelo MP isoladamente. Mais importante ainda, o tratamento com MP/E2 foi capaz de modular a ativação de neutrófilos e sua migração para as vias aéreas.

A modulação das células imunológicas é uma das principais ações dos glicocorticoides. No sangue periférico, os glicocorticoides aumentam a contagem de neutrófilos, promovendo sua entrada na circulação sanguínea, reduzindo a apoptose e estimulando a hematopoese na medula óssea. Apesar de aumentar a contagem de neutrófilos na corrente sanguínea, os glicocorticoides modulam a migração celular para os tecidos, reduzindo a expressão de integrinas e moléculas de

adesão, bem como diminuindo a liberação de citocinas e quimiocinas [18, 19, 20]. Nossos resultados não revelaram diferença na expressão das moléculas de adesão; no entanto, nos pulmões, a ICAM-1 é normalmente expressa, mesmo na ausência de estímulos pró-inflamatórios [21].

Além disso, foi mostrado que o E2 tem efeitos anti-inflamatórios, modulando o recrutamento de leucócitos, especialmente através da redução de quimiocinas e citocinas. O E2 reduziu os níveis de mRNA da proteína quimiotática de monócitos-1 (MCP-1) após estimulação com lipopolissacarídeo (LPS). Além disso, os níveis de MCP-1 e da proteína inflamatória de macrófagos-2 (MIP-2) são reduzidos após administração de E2 em um modelo de doença autoimune [22, 23]. De fato, como mencionado anteriormente, Cvoro et al. (2011) investigaram os efeitos combinados de E2 e glicocorticoides nos genes pró-inflamatórios. Seus estudos revelaram uma redução nos níveis das quimiocinas MIP-1 e IL-8 apenas nas células que receberam tanto E2 quanto dexametasona.

Considerando os efeitos benéficos do tratamento associado nos pulmões, no **capítulo 3** avaliamos seu impacto no tecido renal. A liberação local de mediadores inflamatórios foi reduzida por ambos os tratamentos e, semelhante aos pulmões, somente a combinação dos dois hormônios regulou a infiltração de leucócitos no parênquima.

As ações do E2 são reguladas principalmente pela ativação do ER. O ER é amplamente expresso em vários tecidos, não apenas nos órgãos reprodutivos. O ERβ foi encontrado expresso em todas as amostras de

pulmão de pacientes saudáveis do sexo masculino e feminino [24]. Nos rins, foi observada maior expressão do ERα. Em um estudo que analisou aproximadamente 10.000 genes, Jelinsky et al. (2003) relataram que o rim contém o terceiro maior número de genes regulados pelo E2, ficando atrás apenas do útero e da hipófise. Como vários órgãos são alvo dos estrogênios, sugerimos que o tratamento do doador tem efeitos protetores nos rins e pulmões de animais fêmeas, que estão relacionados à capacidade anti-inflamatória do E2, especialmente no que se refere ao recrutamento de leucócitos.

Nos **capítulos 2** e **3**, utilizamos um modelo de indução rápida de ME. Como mostramos anteriormente neste modelo, o E2 e a corticosterona são reduzidos de forma aguda 3 horas após o início da ME [17], por isso visamos tratar o doador diretamente. Nos **capítulos 4**, **5** e **6**, utilizamos um modelo de indução lenta. Ao contrário da indução rápida, onde diferenças sexuais na fisiopatologia da ME foram relatadas na literatura [17], poucos estudos sobre dimorfismo sexual após indução lenta estão disponíveis.

No **capítulo 4**, focamos em descrever as diferenças entre machos e fêmeas, mais uma vez abordando os pulmões e rins. Sistematicamente, observamos uma redução da testosterona nos machos. Nas fêmeas, os níveis de progesterona também diminuíram. No entanto, ao contrário da indução rápida, a concentração de estradiol permaneceu semelhante no começo e no final do período de ME. Atribuímos essa falta de mudança nos níveis de estradiol a uma possível liberação aguda do hormônio em resposta ao estresse da insuflação lenta do cateter. Quanto

aos órgãos, observamos uma piora da função renal nos machos. Associamos esse cenário ao aumento do apoptose devido à hipoperfusão da microcirculação. Mesmo não tendo avaliado a hemostasia destes ratos após a ME lenta, foi relatado anteriormente em um modelo de indução rápida de ME que os machos apresentaram maior hipercoagulação do que as fêmeas, levando à redução do fluxo [28, 29]. Além disso, nos pulmões, as fêmeas apresentaram redução na função pulmonar, acompanhada por aumento da inflamação, especialmente devido ao aumento da infiltração de leucócitos no parênquima pulmonar.

Embora o E2 tenha efeitos anti-inflamatórios, esses efeitos parecem ser dependentes da dose. Em vários estudos, Straub et al. (2007) relataram que os efeitos anti-inflamatórios do E2 parecem estar relacionados ao aumento das concentrações de E2, geralmente próximas aos níveis de gravidez. Nos **capítulos 2** e **3**, usamos doses supra fisiológicas de E2 para tratar os animais após a ME. No **capítulo 4**, embora nenhuma redução de E2 tenha sido observada, as concentrações de E2 relatadas não foram capazes de melhorar a migração de leucócitos, embora o IL-1β tenha sido reduzido no homogeneizado pulmonar.

Além disso, nos **capítulos 5** e **6**, usamos a técnica relativamente recente de perfusão de órgãos para avaliar o potencial terapêutico da combinação de MP e E2 em um ambiente *ex vivo*. Como os protocolos para o manejo de doadores após ME ainda são amplamente discutidos e muito dependentes das diretrizes hospitalares específicas, opções terapêuticas focadas no tratamento durante a perfusão de máquina

podem resultar em menos discussões éticas e apresentar uma maior chance de serem aceitas pela comunidade médica.

No **capítulo 5**, perfundimos os pulmões em um sistema de perfusão *ex vivo* (EVLP). Apesar das diferenças entre os sexos no final da ME, todos os pulmões apresentaram valores semelhantes de pO_2 após 15 minutos de perfusão. O tratamento melhorou a função pulmonar nos machos e reduziu os níveis de lactato e o número de células marcadas por MPO e iNOS. Ao contrário dos pulmões não perfundidos, pulmões de fêmeas perfundidos sem tratamento apresentaram aumento na expressão gênica e proteica de IL-1β, semelhante a achados anteriores no mesmo modelo que também compararam machos e fêmeas. Comparados com o grupo não tratado, a expressão gênica de IL-1β nos pulmões do grupo tratado foi menor. Além disso, a função pulmonar permaneceu a mesma durante as 4 horas em ambos os grupos femininos.

No **capítulo 6**, usamos perfusão renal para avaliar como o tratamento afeta os órgãos em um ambiente isolado. Ao contrário dos achados anteriores do **capítulo 3**, onde o tratamento do doador melhorou a qualidade do rim, a administração de MP e E2 em um ambiente *ex vivo* comprometeu a função renal nas fêmeas, especialmente pela redução do fluxo de perfusão. Associamos esses resultados à possível ativação cruzada dos receptores mineralocorticoides (MRs) pelos MP, já que as fêmeas são mais predispostas a danos vasculares com a ativação do MR [31, 32, 33]. Nos machos, não foram observadas diferenças substanciais entre os rins tratados e não tratados, indicando que não houve efeitos prejudiciais.

Conclusão

De maneira geral, observamos como diferentes abordagens de tratamento afetam os órgãos de maneira distinta antes do transplante. Além dos diferentes protocolos de administração (diretamente ao doador ou em um sistema *ex vivo* isolado), a eficácia do tratamento parece ser dependente do órgão e do sexo. Quando foi administrado a doadoras com morte cerebral, o tratamento melhorou tanto os pulmões quanto os rins. Em um ambiente *ex vivo*, o tratamento foi positivo para os pulmões de ambos os sexos, enquanto nos rins, foi prejudicial para os rins femininos.

Nossos resultados sugerem que o tratamento do doador durante o período de ME é mais benéfico do que o tratamento durante a perfusão *ex vivo*, especialmente para as fêmeas. Embora a perfusão *ex vivo* busque atingir um ambiente quase fisiológico, ela não replica completamente a complexidade de um organismo vivo. Talvez outros componentes sistêmicos não presentes na perfusão isolada sejam importantes para o controle adequado da resposta inflamatória e para o retorno à homeostase em ambos os sexos. Especificamente, nas fêmeas, a resposta imunológica é rigidamente controlada pelos hormônios sexuais e, apesar do tratamento com E2 e MP, outros componentes não considerados neste estudo podem ser cruciais para uma resposta adequada.

Embora a importância das diferenças sexuais tenha sido destacada, a literatura ainda carece de estudos focados nesse assunto no campo do transplante. Normalmente, os protocolos de manejo do doador são

padronizados sem considerar as características do doador, como sexo e idade. Nesta tese, fornecemos novos perspectivas sobre como uma melhor compreensão das diferenças sexuais pode impactar o transplante, especialmente com o desenvolvimento de estratégias de manejo personalizadas, com o objetivo de atender à necessidade de cada doador e órgão.

Perspectiva futura

Investigações sobre como o E2 modula a inflamação sistêmica ainda são escassas. Como mencionado anteriormente, os órgãos apresentam diferentes níveis de expressão do ER. As ações genômicas e não genômicas são responsáveis pelos efeitos do E2. O ERα é altamente expresso em células endoteliais, e sua ativação está ligada às ações vasculares do E2, ou seja, vasodilatação e produção de NO endotelial [34]. O ERβ também é responsável pela produção de NO e é encontrado principalmente em células do sistema imunológico [35]. O receptor de membrana de E2, GPER, também é expresso em células imunológicas e é capaz de promover respostas rápidas e genômicas [36]. Nesse sentido, terapias utilizando agonistas voltadas para identificar as vias de E2 podem ser de interesse. Além disso, terapias utilizando agonistas de E2 podem fornecer tratamentos mais personalizados, focados na necessidade de cada órgão.

Além disso, vários estudos sugerem que o envelhecimento do doador é um fator de risco para os resultados do transplante. O aumento da idade do doador afeta negativamente a sobrevida do paciente e do enxerto em

vários órgãos. Para cada tipo de órgão, uma faixa etária diferente é considerada para o início dos efeitos negativos [37]. Pessoas mais velhas apresentam um estado de inflamação crônica de baixo grau, caracterizado por disfunção endotelial, culminando em estresse oxidativo vascular, hipertensão resistente e polarização inflamatória [38]. Estudos experimentais realizados por Reutzel-Selke et al. (2007) mostraram que os órgãos de doadores mais velhos são mais imunogênicos, apresentando mais células infiltradas, maior liberação de citocinas e ativação de células imunológicas no receptor. Nas mulheres, as mudanças hormonais pós-menopausas podem afetar o estado pró-inflamatório observado em pessoas mais velhas. Nenhum estudo focou nas repercussões da ME em homens e mulheres mais velhos, especialmente focados em entender como a falta crônica de hormônios afeta o sistema imunológico do doador, abrindo assim uma nova área de interesse.

Finalmente, também é necessário um estudo focado na compreensão das repercussões das diferenças sexuais e das estratégias de tratamento pré-transplante a longo prazo. Estudos descreveram e melhoraram as técnicas de transplante em pequenos animais [40]. Na literatura, há evidências de uma relação entre o sexo do doador e do receptor e os resultados do transplante [41]. Nesse sentido, seria interessante investigar mais a fundo como o sexo, idade e opções de tratamento do doador podem afetar o receptor e se correlacionar melhor com a clínica.

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List of abbreviations

AB - antibody

ABTO – associação brasileira de transplante de órgãos (Brazilian association for organ transplantation)

ACTH -adrenocorticotropic hormone

AKI – acute kidney injury

ANOVA – analyses of variance

AP-1 – activator protein 1

ARF - acute renal failure

ATP - adenosine triphosphate

BAL – bronchoalveolar lavage

BD – brain death

BSA – bovine serum albumin

Cdyn – dynamic compliance

CEUA – comitê de ética no uso animal (ethics committee for animal use)

CINC-1 - cytokine-induced neutrophil chemoattractant-1

DMEM - Dulbecco's Modified Eagle Medium

E2 - 17β-estradiol

ELISA - enzyme-linked immunosorbent assay

eNOS – endothelial nitric oxide synthase

ER – estrogen receptor

EVLP – *ex vivo* lung perfusion

EVMP – *ex vivo* machine perfusion

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GC - glucocorticoids

GnRH - gonadotropin-releasing hormone

GPER – g protein-coupled estrogen receptor 1

GR – glucocorticoid receptor

HPA - hypothalamic-pituitary-axis

HRP - horseradish peroxidase

ICAM-1 – intercellular adhesion molecule 1

ICP – intracranial pressure

IFN-γ - interferon-gamma

IL - interleukin

iNOS – inducible nitric oxide synthase

IPK – isolated perfused kidney

IR – ischemia reperfusion

K - potassium

LDH - lactate dehydrogenase

MAP – mean arterial pressure

MMP-9 – metalloproteinase 9

MP -methylprednisolone

MPO – mieloperoxidase

MR – mineralocorticoid receptor

mRNA – micro ribonucleic acid

Na - sodium

NF-κB - nuclear factor-kappa B

NIH - national Institutes of health

NKP – normothermic kidney perfusion

NO – nitric oxide

NPE – neurogenic pulmonary edema

PBS - phosphate buffered saline

PEEP – positive end expiratory pressure

PI3K - phosphatidylinositol-3 kinase

PIP – peak inspiratory pressure

qPCR - quantitative polymerase chain reaction

RNA - ribonucleic acid

ROS – reactive oxygen species

RT-PCR – real time polymerase chain reaction

SEM – standard error of the mean

Sp-1 - specificity protein 1

STATL - signal transducer and activator of transcription

T3 – thyroxine 3

T4 – thyroxine 4

TBS-T - tris-buffered saline – tween

Th – T helper

TLR7 - toll-like receptor 7

TNF-α - tumor necrosis factor alpha

UNOS - united network for organ sharing

VCAM-1 - vascular cell adhesion protein 1

VEGF – vascular endothelial growth factor

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About the author

Marina Vidal dos Santos was born on January 10th 1997 in São Paulo, Brazil. She finished high school in 2014 and started her undergraduate studies in biomedical science at the Universidade Cidade de São Paulo in 2015. During her second semester of university, she started her activities as a scientific initiation student at the Laboratório de Cirurgia Torácica e Cardiovascular (LIM- 11) at the Faculty of Medicine from the Universidade de São Paulo, under the supervision of Dr. Ana Cristina Breithaupt Faloppa. In 2017, she was awarded a schorlaship from Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) for her scientific initiation project. In 2019, after her graduation, she started her PhD, also under supervision of Dr. Breithaupt Faloppa, and was awarded again with a FAPESP schorlaship in 2021 for her PhD. In 2020 it was proposed to expand her PhD to a double degree in collaboration with professor Henri Leuvenink. After her qualification exam, she went to the Netherlands in February 2022 to start her two years intership at the Surgical Lab from the University Medical Center Groningen. In January 2024, she returned to São Paulo to finish her studies. The completion of her work is presented in this thesis.