# Lung inflammation after brain death

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# Chapter 1

## General Introduction

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## Brain death and lung grafts

Transplants are the therapy of choice for end-stage lung failure. However, even with the advancements in donor management and organ preservation, there is still a shortage of donors[1]. As the majority of lung grafts originate from brain death (BD) donors[2], it is important to understand its systemic effects (hemodynamic, hormonal, and immunological) and the inflammatory response, which greatly affect the lungs compared to other organs[3,4]. Indeed, there is a low number of multi-organ brain dead donors, and among them, it is estimated that only 15%–20% of potential lungs are considered suitable for transplantation[5].

BD is defined as an irreversible loss of brain and brain stem function, often caused by major hemorrhage, hypoxia, or metabolic dysregulation[6]. Intracranial pressure increases above blood pressure, resulting in subsequent ischemia. To reestablish perfusion, high sympathetic activation occurs with the release of catecholamine, called the 'adrenergic storm', observed by a hypertensive peak followed by a hypotensive period[7,8]. Hypothalamus and pituitary failure create important hormonal and metabolic imbalances. As the primary thermoregulatory control center, hypothalamus loss will cause hypothermia and may aggravate vasomotor tone loss[9]. Hormones, such as the antidiuretic hormone, stored in the posterior pituitary will decrease, frequently producing evident diabetes insipidus in patients and may lead to hypernatremia, hypokalemia, hypocalcemia, and hypomagnesemia. This contributes to the loss of vasomotor tone and endothelial integrity[9,10]. The anterior pituitary may result in low cortisol, insulin, and thyroid hormone levels, which influence the inflammatory process[11,12]. The catecholamine storm leads to extensive peripheral vasoconstriction and organ ischemia; thus, aerobic metabolism will switch to anaerobic conditions[7]. With a decrease in adenosine triphosphate (ATP) and oxygen supply, cells activate proteolytic enzymes and nitric oxide synthase (NOS) and produce reactive oxygen species (ROS), which can cause further cell damage after cold ischemia and reperfusion (I/R) before transplantation[13]. Thus, BD initially affects organ viability through the production of hemodynamic instability, reduced oxygenation, and perfusion impairment.

The lung specifically could develop neurogenic pulmonary edema, after the autonomic storm, as a consequence of the increase in capillary hydrostatic pressure resulting from systemic vasoconstriction and hemorrhage[3,7]. Parallelly, lung tissue will also develop an inflammatory response, with the release of interleukin (IL)-1β, IL-6, and TNF-α[14]. Alveolar macrophages

release IL-1β and tumor necrosis factor (TNF)-α, which are associated with acute lung injury, endothelial cell activation, and increased expression of adhesion molecules, while inducing the production of IL-8 by both endothelial and epithelial cells. As a result, there is increased neutrophil and monocyte infiltration in the lung, which could result in lung injury by the release of ROS and proteolytic enzymes by neutrophils[15-17]. There is evidence of neutrophil infiltration as a risk factor for the development of primary graft failure (PGF)[18,19]. Likewise, complement system activity also contributes to donor lung injury, specifically by the classical and lectin pathways[20]. Other studies have reported a correlation between complement system activity and lung injury or transplant success[21-23].

**Lung transplant and sex differences**

Understanding how BD induces lung injury contributes to donor management and consequently improves graft quality and lung transplant outcomes. Currently, many issues are considered when evaluating a lung donor, such as age, smoking history, pre-existing disease, organ size, active infection, and oxygenation capacity[24]. However, sex hormones are often overlooked when designing treatments for BD patients. Studies have shown worse outcomes in lung grafts from female donors[25,26], which is also observed in other organs such as the heart[27] and kidney[28]. Female donors could also be a risk factor for the development of primary graft failure[29]. However, few studies have evaluated sex as a factor affecting lung transplantation, despite the known differences in the immune response between males and females[30,31]. Studies using a BD model have investigated these differences in the lungs, where they found that female animals have a higher inflammatory response than that of male animals, and this was associated with an acute reduction of female sex hormones (FSH)[32,33].

**Estradiol and lung inflammation**

In general, females present stronger cellular and humoral immune responses to antigenic challenges compared to males, resulting in greater efficacy in eliminating pathogens and vaccination efficacy. At the same time, this makes females more susceptible to autoimmune disease. Sex-related differences in the immune response are regulated by sex hormones, such as estrogen, progesterone, and testosterone. Women specifically modulate their immune response, even within the menstrual cycle, in response to hormonal fluctuations of estradiol, displaying a $T_{helper}1$ ($T_h1$)-cell response and enhanced cell-mediated immunity with a low level of estradiol, and $T_{helper}2$ ($T_h2$)-cell and humoral response with a high level of estradiol[30,31].

Estradiol, estriol, and estrone are three of the main types of estrogens present in the human body, with estradiol present at lower levels. They regulate growth, differentiation, and function

in different tissues through estrogen receptors (ER), are divided into subtypes α and β, and are located in the cell nucleus and cytoplasm. ER-α and ER-β subtypes are found distributed in the organs, with one subtype sometimes having more expression than the other, which may influence estradiol actions on those organs. For example, ER-α is mainly present in the mammary glands, uterus, ovary (thecal cells), male reproductive organs (testes and epididymis), prostate (stroma), bone, kidney, adrenal, and liver. ER-β is mainly found in the prostate (epithelium), bladder, ovaries (granulosa cells), colon, bone marrow, salivary gland, and lung[34,35]. The classical mechanism of action starts with the binding of an activated ER to the estrogen response element (ERE) located in the deoxyribonucleic acid (DNA) sequence, allowing the transcriptional regulation of target genes. There are also ER located in the plasma membrane that indirectly modulate gene expression without binding to DNA; these non-genomic actions are frequently associated with the activation of various protein kinase cascades and the indirect effects of ER on transcription interactions. This occurs through protein-protein interactions with activator protein (AP)-1, specificity protein 1 (SP-1), and nuclear factor (NF)-κB proteins. Additionally, even in the absence of estradiol (E2), other signaling pathways can modify ER through phosphorylation[36,37]. Another receptor is the G protein-coupled estrogen receptor (GPER or GPR30) located in the cell membrane that activates intracellular signaling cascades, producing non-genomic and transcriptional events. Non-genomic actions can occur within seconds or minutes, giving no time for mRNA or protein transcription. The GPER may activate signal transduction pathways of the phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase family (MAPKs), adenylyl cyclase, Src tyrosine kinase, and calcium-calmodulin-dependent kinases, or cause the release of intracellular $Ca^{2+}$ [36,38].

Studies have shown that hormonally active (pre-menopausal) women respond better to severe traumatic injury than men, presenting a higher survival rate and better physiological response to a similar degree of injury[39,40]. Female trauma patients, compared to males of the same age (< 50 years old), had decreased incidence of multiple organ dysfunction syndrome, sepsis, and low cytokine levels[41,42] and presented a significantly higher chance of survival[43,44]. Nonetheless, Eachempati et al.[45] observed that women > 80 years had a higher mortality when compared to men of the same age, indicating that women in menopause are less protected. Likewise, women will develop coronary heart disease later than men, but the risk is significantly higher after menopause as FSH, especially estradiol, are dramatically reduced[46].

Sex dimorphism has also been observed in experimental models of acute injury, such as hemorrhagic shock, sepsis, and ischemia/reperfusion[47-49]. Females in proestrus (high level of

estradiol) have presented resistance to lung injury compared to males in the intestinal I/R model by controlling neutrophil activity[49]. Indeed, neutrophils from females had decreased priming and activation after trauma and hemorrhage models, when compared to males[50]. In a trauma-hemorrhagic shock model, when compared to males, proestrus females had lower gut and lung injuries, with less neutrophil infiltration and inducible nitric oxide syntase (iNOS) activity[48], and higher survival rate to sepsis after trauma-hemorrhage[47]. In addition, organ blood flow showed a consistent increase after volume resuscitation; in contrast, male flow remained similar to the shock period, and this preservation of perfusion possibly explains the greater resistance to organ injury in females[51]. Other studies involving females who underwent ovariectomy and later treated with estradiol demonstrated the positive effects of estradiol on lung injury. In the trauma-hemorrhagic shock model, the ER-β agonist was effective in restricting lung injury, reducing permeability, and reducing neutrophil infiltrate[52]. In an I/R model, estradiol was shown to control nitric oxide (NO) production by increasing endothelial nitric oxide syntase (eNOS) expression and reducing microvascular permeability and release of inflammatory mediators[53,54]. Macrophage immunocompetence was also restored in ovariectomized (OVx) treated with 17β-estradiol (E2), which prevented trauma-induced immunodepression and increased susceptibility to subsequent sepsis[55]. Studies by Hildebrand et al.[56] indicated that both ER-α and ER-β are responsible for restoring macrophage immunoprotection. Partially, through a nongenomic pathway mediated via MAPK, nongenomic pathways also seem to control macrophage cytokine production[57]. In a trauma and hemorrhage model, males are seen to have T-cell effects controlled by ER-α activation, likely mediated via MAPK, NF-κB, and AP-1[58]. Additionally, with estradiol treatment in the same model, ER-β is responsible for the downregulation of iNOS activity in the lungs[59].

We must emphasize that, in contrast to the acute injury models mentioned, in the event of brain death, the hormonal milieu will be unbalanced and estradiol levels will decrease. This modifies the inflammatory response in females, producing a higher lung inflammation, compared to their male counterparts, whereas OVx females, who already have lower levels of FSH when submitted to BD, will have a smaller reduction in estradiol levels and produce inflammation to a degree similar to that in males[32,33]. In a recent study, male rats received a single dose of estradiol, which effectively reduced BD-induced lung injury. The authors identified NOS modulation by estradiol as one of the main mechanisms, decreasing the expression of adhesion molecules, chemokine release, lung hemorrhage, and edema[60]. Although we lack knowledge regarding the effects of estradiol on a female BD donor, the above

data indicate that estradiol could be considered a potential modulator of inflammation after BD in females and a therapeutic option to improve lung quality.

**Lung preservation and e*x vivo* lung perfusion**

Cold static preservation has been the standard for lung graft preservation after retrieval, intending to reduce lung metabolism and cell death by hypothermia. Although cold storage will decrease the organ's metabolic function, it will also impede any evaluation of lung function before transplantation[61,62]. Cold storage ischemia can contribute to primary graft dysfunction (PGD), acute rejection, chronic graft dysfunction, and postoperative mortality[18]. As an alternative, the *ex vivo* lung perfusion (EVLP) method provides a form of assessing and treating lung grafts. More specifically, normothermic perfusion permits lung preservation of homeostasis and metabolic function, preventing injury from prolonged ischemia and increasing lung recovery when compared to cold static preservation[63].

Data show that EVLP increased the number of lung transplants by at least 15%-20% within our limited donor pool[64,65]. Jirsch et al.[66] introduced the idea of EVLP as a means to investigate the quality of lung grafts before transplantation; however, attempts failed because of the inability to preserve the lung air/fluid barrier, causing edema formation and increased pulmonary vascular resistance. Years later, with the intent of evaluating lungs from non-heart-beating donors, a new system for EVLP was developed and successfully evaluated the lungs, although only for a short period of time. In addition, a solution was developed for perfusion (STEEN Solution™, XVIVO Perfusion, Sweden), which maintains homeostasis by preserving the intravascular space and providing the necessary nutrients during perfusion[67]. This allowed the first lung transplantation after EVLP by reconditioning a severely injured lung[68], followed by strategies that permit a 12 h normothermic *ex vivo* perfusion in both experimental and clinical studies[63,69,70].

When subjected to *ex vivo* perfusion, it is possible to control perfusate composition, arterial pressure, temperature, and mechanical ventilation (tidal volume, positive end-expiratory pressure [PEEP], fraction of inspired oxygen). The ability that EVLP provides to assess, recondition, and repair allows for the implementation of specific therapies to treat injured lungs for several hours by controlling dehydration, removing harmful and toxic waste byproducts, and recruiting atelectatic areas, thus resulting in better ventilation/perfusion and improved microcirculation[71].

Studies have shown that EVLP has been successfully applied in the clinic[72-76]. In addition, experimental studies have demonstrated the beneficial effects of EVLP in preventing ischemic damage and accelerating lung repair[6970,77]. A clinical study showed that lung normothermic perfusion reduced apoptosis and iNOS expression, compared to cold ischemic preservation[78]. Transplantable lungs were associated with a high level of eNOS at the start of EVLP[79], which also protects I/R-injured lungs[80]. A study by Andreasson et al.[81] selected IL-1β as a predictive biomarker for transplant success. Additionally, they have shown that blockade of the IL-1β pathway during EVLP reduces endothelial activation and neutrophil adhesion. Machuca et al.[82] suggested the quantification of IL-8, chemokines growth-regulated oncogene alpha (GROα), macrophage inflammatory protein (MIP)-1α, MIP-1β, and granulocyte colony-stimulating factor (G-CSF) in perfusate as possible biomarkers for PGD. IL-8 is the most promising, as it relates to the pathogenesis of acute lung injury. IL-8 in bronchoalveolar lavage and lung biopsies has also been correlated with the development of PGF[19]. By performing EVLP of lungs previously unacceptable to transplant, Sadaria et al.[83] noticed an increase of IL-6, IL-8, monocyte chemoattractant protein (MCP), and G-CSF in the lung biopsies during a 12 h perfusion, resulting in no loss of lung function.

Several studies have attempted to filter leukocytes and/or cytokines circulating in the perfusate to improve lung preservation and quality[84-86]. The removal of these immune cells during EVLP can reduce donor leukocyte transfer into the recipient, reducing allorecognition and T cell priming[86]. Cytokine filtration can also control edema formation and neutrophil activity[85]. Noda et al.[84] correlated circulating leukocytes derived from donor lungs and non-circulating proinflammatory cytokines with impaired quality of lung grafts, suggesting the use of leukocyte filters or therapies that target leukocyte activity. On the other hand, Luc et al.[87] refuted the use of leukocyte filters, since evidence indicates that the removal of leukocyte filters does not harm the lungs. Taken together with data on sex dimorphism in immune response and the impact of BD on lung grafts, it would be of interest to investigate the impact of EVLP in both males and females as a means of treating and improving unacceptable lung grafts.

## Scope of Thesis

This thesis aimed to investigate the effects of sex differences on the pathophysiology and future clinical consequences of BD-induced lung injury. The study highlights the association of estradiol, a female sex hormone, in the higher female response to BD due to the loss of hormonal and immunological control. **Chapter 2** focuses on the investigation of sex differences in the

response to BD effects on coagulation, platelet behavior, and microvascular perfusion, showing the reduction of microcirculation perfusion in males due to microthrombi formation, whereas females preserve microvascular perfusion, producing a higher inflammatory response and NO synthesis. In **Chapter 3**, we show the importance of FSH in BD lung inflammation. Similar to menopausal women, ovariectomized females have physiologically adapted to a lower level of estradiol and, when submitted to BD, present lower lung inflammation than those in non-ovariectomized animals. Since cycling females will have an acute reduction of FSH after BD, the resulting inflammatory response is higher with the release of local and systemic inflammatory mediators and higher lung leukocyte infiltrate. In **Chapter 4**, estradiol was chosen as a treatment for females submitted to BD as a means to investigate the protective effect of this hormone in BD-induced lung injury. Indeed, treatment with estradiol partially controlled the inflammatory response in the lung by decreasing the release of chemokines and adhesion molecules and reducing leukocyte infiltration into the lung. In **Chapter 5,** we focus on the long-lasting effect of estradiol treatment in the female BD model to further investigate the possible inflammatory response of the lung graft after transplantation. Estradiol reduced the long-lasting release of inflammatory mediators and cell apoptosis in the lung tissue, possibly by reducing the activation of cells such as alveolar macrophages and controlling signals that amplify the inflammatory response, such as complement system components. Finally, in **Chapter 6**, we compare lung inflammation and function after BD and EVLP in female and male rats. The data showed that lung inflammation in BD females was more severe than that in male BD patients. This indicates that EVLP does not improve the condition of the female graft lungs, highlighting the importance of therapeutic measures that can improve donors organs before transplantation.

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