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Lung inflammation after brain death

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Chapter 7

Summary, general discussion and future perspectives

Samenvatting, algemene discussie en toekomstperspectivieven

Resumo, discussão geral e perspectivas futuras

Summary and General Discussion

Given the importance of lung transplantation as a lifesaving therapy for patients with end-stage lung disease[1], we continue to look for ways to improve the number and quality of available lungs. Brain dead donors are the major source of lung grafts, although brain death (BD) itself has systemic repercussions that affect organ quality[2-4]. Currently, the lung donor criteria include age, organ size, and oxygenation[5]. However, in this thesis, we highlight sex differences in response to BD and the role of female sex hormones (FSH). Few studies have started to account for the differences between males and females as a parameter of investigation for lung transplantation matching and the greater risk with female-to-male transplants[6,7]. Experimental studies have confirmed the hypothesis that female donors have worse lungs after BD, with more severe lung inflammatory responses compared to males, which is associated with the acute reduction of FSH[8,9]. In **Chapter 2**, we have shown that there is dimorphism in both perfusion and coagulation processes after BD, possibly affecting the inflammation and contributing to the greater response in females and the microcirculatory compromise in males.

Conversely, different experimental models of trauma and inflammation, comparing male and female responses, showed that females have more effective control of the systemic inflammatory response[10-12]. This female protection becomes more apparent when comparing ovariectomized (OVx) and non-OVx animals after ischemia and reperfusion (I/R), with I/R female OVx having greater lung inflammation, compared to non-OVx animals[13]. In addition, estradiol treatment in OVx females effectively protects against lung inflammation and injury[13-16]. In this sense, it is possible to infer that the treatment of female patients with FSH could potentially control BD-induced lung injury. Thus, we focused on the effects of estradiol treatment on females subjected to the BD model. Finally, we investigated different sex responses to BD followed by *ex vivo* perfusion as a means of assessment and treatment of lung grafts. Consequently, we can better understand the role of sex in the process of choosing and managing lungs for transplantation.

Current studies have mainly focused on males despite the literature indicating differences in immune responses[17,18], accompanied by clinical and experimental trauma studies showing distinctions between males and females[10,19,20]. In a BD model, male rats show a compromised microcirculation, both in the mesenteric[21] and lung microvessels[22], while presenting a corticosterone reduction and, consequently, an increase in inflammatory mediators. Most notably, the lungs not only had decreased perfusion, but also increased leukocyte infiltration.

As mentioned, previous studies on sex differences have connected the acute drop of FSH (after 3 h of BD) with greater lung inflammation in females than in males. At the same time, we observed diminished corticosterone levels in both males and females, resulting in BD hypothalamus-pituitary failure[8,23]. In addition, females, contrary to males, maintain the perfusion of microvessels after BD, which is associated with high endothelial nitric oxide synthase (eNOS) expression[23]. In line with these studies, in **Chapter 2,** we aimed to investigate sex differences in the mechanisms involved in microcirculatory alterations after BD. Males present reduced perfusion in microcirculation after BD, and this phenomenon could be associated with intravascular microthrombi formation due to increased platelet aggregation and firmer clotting. On the other hand, we observed perfusion maintenance in females, which was attributed to reduced clot firmness and lysis. Under normal circumstances, females seem to exhibit higher platelet aggregation than males[24,25], and estradiol could be one of the regulating factors of coagulation, platelet function, and activation[26,27]. With the event of BD, resulting in estradiol reduction, its effects on platelet function and activation are lost, which can be seen in our data with increased clotting time. The opposite results were observed in males, with not only faster clotting formation, but also hypercoagulability and thrombus formation. The absence of estradiol in females could explain the observed sex dichotomy. In addition, both ER-α and ER-β have been found to be expressed in platelets, at a similar level in both males and females[9,28]. Even though they are anucleated cells, estrogen can indeed have rapid nongenomic effects in platelets involving ER-β, the main receptor expressed in platelets, and tyrosine kinase Src. Thus, modulating platelet aggregation is prompted by a low level of thrombin, resulting in a robust increase in integrin αIIbβ3 for fibrinogen[30]. We have also considered the role of NO, which could contribute to platelet activity reduction during hypoxia[31]. Females have a higher expression of induced nitric oxide synthase (iNOS) and eNOS after BD, and the greater NO synthesis and vascular tone could explain the different responses in females[8,23]. This phenomenon can be seen in studies with BD male rats, where estradiol treatment can improve lung injury and mesenteric microcirculation perfusion, as well as reduce edema and hemorrhage parameters, possibly by estradiol modulation of NOS[32,33].

In order to evaluate the role of sex hormones in females submitted to BD, **Chapter 3** focused on determining whether the hormones modulate lung inflammation caused by BD in females. Data showed that OVx females, which, similar to menopausal women, have the time to adapt to diminished levels of FSH (estradiol and progesterone), present lower lung inflammation compared to non-ovariectomized animals after BD. In non-OVx females, we

confirmed the acute drop in estradiol and progesterone concentrations after BD induction. In parallel with intense lung inflammation, as shown by the greater leukocyte infiltration in the parenchyma and bronchoalveolar lavage, explained by a higher adhesion molecule expression (intercellular adhesion molecule [ICAM]-1 and vascular adhesion molecules [VCAM]-1), release of inflammatory mediators (granulocyte colony-stimulating factor [G-CSF], vascular endothelial growth factor [VEGF], and cytokine-induced neutrophil chemoattractant [CINC]-1), and iNOS protein and gene expression, in non-OVx females compared to that in OVx females. NO production by iNOS can contribute to systemic inflammation[34]. OVx animals are given an adaptation period (10 days), in which estradiol receptors will reduce in number[35] and in parallel, corticosterone levels will be modified by the end of the adaptation period, which are much lower in Ovx animals than in non-OVx animals. This could be explained by the excitatory role of estradiol in the release of corticosterone by controlling the hypothalamus-pituitary-adrenal axis in basal conditions through ER-α or under stress conditions through both ER-α and ER-β[36,37]. In this sense, OVx rats could be used to better understand menopausal female donors and pre-menopausal women. These results indicate that the role of corticosterone in inflammation is not only crucial to understanding BD inflammation, but also highlights the influence of estradiol after BD.

The data on sex differences and hormonal drop after BD generated the hypothesis that estradiol could be a tool to control lung injury caused by BD and grounded the evaluation of estradiol treatment in female rats subjected to the BD model at two different time points. As a way to better investigate estradiol as a therapeutic option, treatment is administered as soon as BD is confirmed to avoid the drop of estradiol concentrations, followed by a later treatment after 3 h of BD confirmation. As shown in **Chapter 4**, estradiol was able to reduce lung inflammation in BD females, most notably by the 3 h treatment group. Showed by a reduction in leukocyte infiltration in the alveoli and parenchyma. Supported by estradiol control over adhesion molecules (ICAM-1 and VCAM-1) genic and protein expression, along with chemokine release in lung culture (macrophage inflammatory protein [MIP]-1α, MIP-2, and CINC-1), which are responsible for the recruitment of neutrophils and macrophages[38,39]. Serum and gene expression of inflammatory mediators (IL-1β and tumor necrosis factor [TNF]-α) decreased, and metalloproteinase activity was also reduced, indicating the effect of estradiol on inflammatory cell activation and consequently on lung injury.

It is important to mention that estradiol can only partially control edema formation, since microvascular permeability and NOS were not altered by estradiol, despite histopathological

analysis showing a decrease in edema. Despite this, estradiol could be considered a therapeutic option for controlling BD-induced lung inflammation in females, especially by reducing leukocyte infiltration. Neutrophils have been implicated in the development of acute lung injury and the incidence of primary graft failure (PGF), also associated with reactive oxygen species (ROS) production, endothelial activation, and cytokine release[40-42]. Neutrophils, for instance, could be activated by estradiol through ER-β[43] and the same receptor mRNA was also found to increase when lung infiltrate was attenuated by estradiol treatment[44]. Another possibility is that G protein-coupled estrogen receptor (GPER) expressed in neutrophils could limit the entry of calcium into the cell via store-operated calcium entry channels. Both proestrus female rats and males treated with estradiol showed attenuation of neutrophil capacity to participate in inflammation and GPER-signaling mechanisms including the increase of intracellular calcium[45,46].

As the next step in **Chapter 5,** we investigated the long-term influence of estradiol treatment on lung inflammation in females after BD, trying to understand the lung graft immune response after transplantation through longer maintenance of lung viable samples. Our results showed that estradiol had a long-lasting effect on the release of inflammatory mediators (IL-1β and TNF-α), reducing them even 24 h after BD. IL-1β and TNF-α are released early by macrophages, amplifying inflammation by increasing the expression of adhesion molecules and the release of chemokines[3,47]. Subsequently, there is a production of superoxide and proteolytic enzymes, as well as activation of apoptosis[48]. We hypothesize that estradiol reduces macrophage activation, therefore reducing the production of inflammatory mediators, and controlling the inflammatory response in BD. It has been reported that low concentrations of estradiol increase the release of IL-1β and TNF-α by macrophages, whereas a high concentration decreases it. This protective effect at high doses could also influence the development of acute lung injury through inhibition of nuclear factor (NF)-κB[47]. In addition, a study by Acconcia et al.[49] discovered that in cancer cell lines, estradiol could have a dual effect depending on each receptor activation. ER-α rapidly activated multiple signal transduction pathways, such as extracellular-signal-regulated kinase/mitogen-activated protein kinase family (ERK/MAPK) and phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), which promoted the progression of the cell cycle and prevented apoptosis. In contrast, ER-β induced rapid and persistent phosphorylation of p38/MAPK, controlling caspase-3 activation and cleavage, and initiating the apoptotic cycle. Therefore, further studies on estradiol actions in controlling apoptosis in the lung tissue are needed, as the profile of estrogen receptors in the

lung is different, mainly expressing ER-β, and has been attributed to the protective effect of estradiol in the lung [15,44]. Finally, corroborating the anti-inflammatory role of estradiol, we found that the complement system was activated by BD in females until the C3 level with no effect on the membrane attack complex (C5b-9). The literature indicates that compared to males, females have lower expression of terminal complement system components[51,52]. There are studies on the direct role of the complement system, through classical and lectin pathways, in BD-induced lung injury and transplant success[53,54]. Here, we connected estradiol treatment to lung inflammation in BD females with decreased release of inflammatory mediators and reduced activation of the complement system, which possibly modulates neutrophils and macrophages with a lasting protective effect on lung inflammation.

Finally, in **Chapter 6**, we focused on a technique that has gained importance in lung transplantation, *ex vivo* lung perfusion (EVLP). It has been used for assessing function before transplantation, helping clinical decisions on marginal lungs, but it also enables potential treatment and repair of injured lungs[55]. Together with the knowledge that male and female lung inflammation caused by BD differs[8,9], we designed a study to compare lung inflammation in BD male and female rats followed by EVLP. Our data showed that, independent of sex, BD reduced lung function by decreasing oxygenation capacity. Regarding mechanical ventilation parameters, we found that EVLP was maintained over 4 h of the experiment. More importantly, BD females continued to show higher lung inflammation than males after perfusion. The female BD lungs presented higher leukocyte infiltration, from which we found a high neutrophil presence. Additionally, we found that BD females had higher IL-1β concentration in the perfusate after 4 h of EVLP, increased IL-1β released in lung culture medium (24 h), and increased gene expression. Therefore, we suggest that leukocytes in females are more numerous and active than those found in male lungs. An experimental study with non-beating donors has also analyzed the influence of sex on EVLP. They found a sex-linked difference in lung function, where males presented a reduced ability to transport oxygen. Females had protective effects, resulting in better perfusion than males. However, dimorphism has not been observed in the development of pulmonary edema[56]. It is important to highlight that non-heart-beating donors have a similar profile as observed after an ischemia and reperfusion model, in the sense that hormonal levels are not altered in response to circulatory death; therefore, they present no hormonal imbalance and no reductions in estradiol or corticosterone levels. In our EVLP data, we also did not observe any links between sex and edema formation, as assessed by an edema index. However, more accurate methods of microvascular permeability evaluation should be

used to confirm this parameter. With this knowledge, we can reiterate the understanding that the hormonal imbalance produced by BD, with emphasis on the loss of FSH by females, will produce different inflammatory responses in the lung after BD, which EVLP alone could not modify.

Future Perspectives

Overall, this thesis has added to our understanding of sex differences in BD pathophysiology and estradiol modulation in BD-induced lung injury. Opening the path for further studies on donor management and lung quality improvement, taking into account the importance of female sex hormones and their implications on the inflammatory process. It also creates an opportunity for new studies that test estradiol as a viable therapeutic option for donor management.

Donor management aims to preserve and optimize post-transplant lung function by using strategies that focus on optimizing pulmonary and extra-pulmonary physiologic parameters or preservation during procurement and transit[57]. Brain dead donors lung management protocols require hemodynamic, neuroendocrine, and lung-specific approaches, thus treating potential donors with vasopressin, glucocorticoids, thyroid hormones, and insulin[5].

Our data in **Chapters 4** and **5** showed that estradiol treatment in BD females controls inflammation by reducing adhesion molecule expression and cytokine and chemokine release, resulting in lower leukocyte infiltration. However, it was not able to protect the lung from microvascular permeability increase, leading to edema formation. In our studies, we have also raised attention to the acute decrease in corticosterone, accompanied by estradiol reduction after BD. Glucocorticoids are released in the early inflammatory stages and are known to have a synergistic role in controlling inflammation with estradiol[36]. In the event of hypothalamus-pituitary failure in BD, we observed a loss of both hormones. Therefore, it would be beneficial to investigate the combined treatment of corticosterone and estradiol, which could potentially have positive repercussions, not only on lung inflammation but also on edema formation in female donors. A study in BD females evaluating this combination effect on lung injury is ongoing as joint research between laboratories of the University of Groningen and the Universidade de São Paulo. Their preliminary results suggest that methylprednisolone treatment alters lung inflammation in females that underwent BD by reducing cytokine and chemokine release and expression of adhesion molecules, thus positively affecting alveolar leukocyte infiltration. Once this study is completed, these results will increase our knowledge of the repercussions of hormone interactions on the lung caused by BD in females.

Few studies have investigated the pathway by which estradiol and its receptors could produce a protective effect on the lung, and these studies have mainly focused on trauma-hemorrhage shock models. These models show genomic and non-genomic vias of action, where there seems to be a consensus that the protective effects of estradiol in the lung are mainly via ER-β, similar to heart[19,43]. Thus, acknowledging the gap in the literature, it is important to further study estradiol and its effect on lung protection so that specific treatments of BD impact in the lung can be developed. Therefore, experiments that use specific agonists or antagonists of estradiol receptors would be of interest to narrow down how estradiol modulates lung injury in a BD model.

It is also important to consider that estradiol treatment effects on BD donors could positively alter the success of transplantation in a short- or long-term period (acute rejection or primary graft dysfunction), as estradiol has been shown to have a protective effect on lung inflammation (**Chapters 4** and **5**). With this knowledge, we propose an EVLP that uses estradiol as a treatment during perfusion, as a means to improve lung injury and preserve the lungs of females. These could prove to have some protective effects, possibly by reducing neutrophil and macrophage release of inflammatory mediators, proteolytic enzymes, and ROS production. Nevertheless, our results from **Chapter 6** indicate that female lungs had worse inflammatory parameters than male lungs after BD followed by EVLP, pointing to the possibility that EVLP alone cannot improve the condition of the female graft lungs. Emphasizing the necessity of new therapies to reduce lung inflammation before the use of the EVLP machine and consequently improve transplant outcomes. Therefore, it is possible to speculate that estradiol treatment in the EVLP machine may have different results in male and female lungs.

It has even been proposed the use of an adsorbent membrane to remove cytokines has proven to be effective in reducing cytokines in the perfusate, but not effective in reducing neutrophil activity[58]. In that sense, estradiol treatment could be of great use, since it would control the inflammatory process on the BD donor, especially in females with an accentuated inflammatory response, by dampening the inflammatory response before perfusion. There also seem to be controversial studies on the effectiveness of leukocyte filters in EVLP, regarding its saturation and time of reperfusion[59,60]. However, what should be brought to attention is that most studies use lungs after an ischemia period only and without BD, a protocol that does not mimic the clinical setting. This brings the question of what would happen to the leukocytes that migrated to the lung parenchyma and would influence the lung graft during perfusion. Hence,

we hypothesized that leukocyte activity and lung infiltration could be controlled in the DBD before harvesting or placing the lung graft in an *ex vivo* perfusion machine.

In conclusion, this thesis has brought to attention the inherent differences between males and females present in the event of BD, highlighting the role of female sex hormones in the development of lung injury in female donors and pointing to donor sex as an evaluation criterion for donor management. Moreover, this thesis suggests the use of estradiol as a donor therapy once BD is established or during EVLP as a future perspective to improve lung quality for transplantation.

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Chapter 7

Samenvatting en algemene discussie

Gezien het belang van longtransplantaties als levensreddende therapie voor patiënten met longziekte in het eindstadium[1], blijven we zoeken naar manieren om het aantal en de kwaliteit van de beschikbare longen te verbeteren. Hersendode donoren zijn de belangrijkste bron voor longtransplantaties, hoewel hersendood (HD) zelf systemische gevolgen heeft die de orgaankwaliteit beïnvloeden[2-4]. Momenteel zijn de belangrijkste longdonorcriteria leeftijd, orgaangrootte en oxygenatie[5]. In dit proefschrift onderzoeken we sekseverschillen in de respons op HD en de rol van vrouwelijke geslachtshormonen. Er zijn maar weinig studies die onderzoek doen naar de verschillen tussen mannen en vrouwen in longtransplantatie. Het is wel bekend dat transplantaties van vrouw naar man een hoger risico met zich meebrengt[6,7]. Experimentele studies hebben de hypothese dat vrouwelijke donoren slechtere longen hebben na HD bevestigd, met een ernstigere ontstekingsreactie in vergelijking met mannen mogelijk veroorzaakt door de acute vermindering van vrouwelijke geslachtshormonen[8,9]. In **Hoofdstuk 2** hebben we aangetoond dat er dimorfisme is in zowel perfusie- als coagulatieprocessen na HD, wat mogelijk de ontsteking beïnvloedt en bijdraagt aan de grotere ontstekings-respons bij vrouwen en de microcirculatiestoornis bij mannen.

Omgekeerd, verschillende experimentele modellen van trauma en ontsteking, die mannelijke en vrouwelijke dieren vergelijken, tonen aan dat vrouwtjes een verlaagde systemische ontstekingsreactie vertonen[10-12]. Deze vrouwelijke bescherming wordt duidelijker wanneer dieren met ovariëctomie (OVx) en naïeve dieren (zonder OVx) worden vergeleken na ischemie en reperfusie (I/R). De OVx dieren vertonen hierbij een grotere ontsteking dan niet-OVx diren[13]. Bovendien beschermt oestradiolbehandeling bij OVx vrouwtjes effectief tegen longontsteking en letsel[13-16]. In die zin is het mogelijk om te concluderen dat de behandeling van vrouwelijke patiënten met vrouwelijke geslachtshormonen mogelijk door de door HD geïnduceerde longbeschadiging zou kunnen verminderen. Daarom hebben we ons gericht op het effect van behandeling met oestradiol op vrouwtjes die werden onderworpen aan het HD-model. Ten slotte onderzoeken we ook verschillende geslachtsreacties op HD gevolgd door *ex vivo* perfusie, als middel voor beoordeling en behandeling van longtransplantaten. Zodoende kunnen we de rol van de geslachtsvariabele in het proces van het kiezen en beheren van longen voor transplantatie beter begrijpen.

Ondanks literatuur die verschillen in immuunrespons tussen mannetjes en vrouwtjes aangeeft[17,18], aangevuld met klinische en experimentele trauma-onderzoeken[10,19,20], zijn de

meeste onderzoeken voornamelijk gericht op mannetjes. In een HD-model vertonen mannelijke ratten een verslechterde microcirculatie, zowel in de mesenteriale[21] als in de microvaten in de long[22], gecombineerd met een vermindering van corticosteron en dientengevolge een toename van de ontstekingsmediatoren. Het meest opvallend was dat de longen niet alleen een verminderde perfusie hadden, maar ook een verhoogde infiltratie van leukocyten.

Zoals eerder vermeld, hebben eerdere onderzoeken naar sekseverschillen de acute daling van vrouwelijke geslachtshormonen (na 3 uur HD) in verband gebracht met een grotere longontsteking, bij vrouwtjes vergeleken met mannetjes. Tegelijkertijd zien we afnemende corticosteronspiegels bij zowel mannetjes als vrouwen, resulterend in HD hypothalamus-hypofyse falen[8,23]. Bovendien hebben vrouwtjes in tegenstelling tot mannetjes de perfusie van microvaten die behouden blijven na HD, wat geassocieerd was met hoge endotheliale stikstofoxidesynthase (eNOS) uiting[23]. In lijn met deze onderzoeken wilden we in **Hoofdstuk 2** sekseverschillen onderzoeken in de mechanismen die betrokken zijn bij veranderingen in de microcirculatie na HD. Mannetjes vertonen verminderde perfusie in de microcirculatie na HD en hier kunnen we dit fenomeen associëren met intravasculaire microtrombivorming als gevolg van verhoogde bloedplaatjesaggregatie en stolling. Bij vrouwtjes werd de perfusie niet beinvloedt mogelijk toe te schrijven aan verminderde stevigheid van het stolsel en lysis. Onder normale omstandigheden lijken vrouwtjes een hogere bloedplaatjesaggregatie te vertonen dan mannetjes[24,25], en oestradiol zou een van de regulerende factoren kunnen zijn van de stolling. [26,27]. In het geval van HD, resulterend in estradiol-reductie, gaan de effecten op de bloedplaatjesfunctie en -activering verloren, wat te zien is in onze gegevens met de verhoogde stollingstijd. De tegenovergestelde resultaten werden waargenomen bij mannetjes, niet alleen snellere stollingsvorming, maar ook hypercoagulabiliteit en trombusvorming. De afwezigheid van estradiol bij vrouwtjes zou een verklaring kunnen zijn voor de waargenomen geslachtsdichotomie. De oestradiol receptor (ER)-α als ER-β receptoren komen op een vergelijkbaar niveau bij zowel mannetjes als vrouwtjes op bloedplaatjes tot expressie[28,29]. Ondanks het feit dat bloedplaatjes anucleaire cellen zijn kunnen oestrogenen via ER-β, de belangrijkste receptor die op bloedplaatjes wordt gezien tyrosinekinase Src activeren. De modulatie van de bloedplaatjesaggregatie, veroorzaakt door een laag trombinegehalte en resulterend in een robuuste toename van integrine αIIbβ3 voor fibrinogeen is de meest voor de hand liggende verklaring[30]. We hebben ook de rol van NO (nitric oxide) overwogen, die zou kunnen bijdragen aan de vermindering van de activiteit van bloedplaatjes tijdens hypoxie[31]. Vrouwtjes vertonen een hogere expressie van zowel induceerbare stikstofoxidesynthase (iNOS)

als eNOS na HD, en de grotere NO-synthese en vasculaire tonus zouden de verschillende reacties bij vrouwtjes kunnen verklaren[8,23]. Dit fenomeen is te zien in onderzoeken met mannelijke HD-ratten, waar behandeling met estradiol longbeschadiging en mesenteriale microcirculatieperfusie kan verbeteren, en ook oedeem- en bloedingsparameters kan verminderen, mogelijk door estradiol gemedieerde modulatie van NO-synthasen[32,33].

De rol van geslachtshormonen bij vrouwtjes die hersendood waren, was de focus in **Hoofdstuk 3.** Vrouwelijke dieren waarvan de eierstokken verwijderd zijn (OVx), hebben net als vrouwen in de menopauze, de tijd hebben om zich aan te passen aan verminderde niveaus van vrouwelijke geslachtshormonen (estradiol en progesteron) en vertonen een verminderde ontsteking in vergelijking met dieren zonder ovariëctomie na HD. Zoals eerder aangetoond werd bij niet-OVx-vrouwtjes de acute daling van de estradiol- en progesteronconcentraties na HD-inductie gezien. Parallel met meer longontsteking, zoals aangetoond door het grotere infiltraat van leukocyten in het parenchym en bronchoalveolaire lavage. Dit kan verklaard worden door de hogere expressie van adhesiemoleculen (intercellulair adhesiemolecuul [ICAM]-1 en vasculair celadhesiemolecuul [VCAM]-1), de afgifte van ontstekingsmediatoren (granulocyt-kolonie stimulerende factor [G-CSF], vasculaire endotheliale groeifactor [VEGF] en ytokine-geïnduceerde neutrofiele chemoattractant [CINC]-1) en iNOS-eiwit en genexpressie. De productie van stikstofmonoxide door iNOS kan bijdragen aan systemische ontstekingen[34]. De OVx-dieren ondergaan een aanpassingsperiode (10 dagen), waarin de oestradiolreceptoren in aantal zullen verminderen[35], en parallel daaraan zullen de corticosteronspiegels aan het einde van de aanpassingsperiode worden verlaagd. dieren. Het onderliggende mechanisme is dat estradiol een stimulerende rol speelt bij de afgifte van corticosteron, door de hypothalamus-hypofyse-bijnier-as in basale omstandigheden via ER-α of in stressomstandigheden via zowel ER-α als ER-β te reguleren[36,37]. In die zin kunnen OVx-ratten een model kunnen zijn om vrouwelijke donoren in de menopauze en premenopauzale vrouwen beter te begrijpen. Deze resultaten geven aan dat niet alleen de rol van corticosteron bij ontstekingen cruciaal is voor het begrijpen van HD-ontsteking, maar benadrukt ook de invloed van oestradiol na HD.

De gegevens over geslachtsverschillen en hormonale daling na HD leiden tot de hypothese dat estradiol een hormoon zou kunnen zijn om longbeschadiging veroorzaakt door HD onder controle te houden en vormden de basis voor de evaluatie van estradiolbehandeling bij vrouwelijke ratten die op twee verschillende tijdstippen aan het HD-model werden gegeven. Een vroege behandeling zodra HD is bevestigd, waarbij de daling van de estradiolconcentraties

wordt vermeden, en een latere behandeling op 3 uur na HD-bevestiging. Zoals aangetoond in **Hoofdstuk 4**, was estradiol inderdaad in staat om longontsteking bij HD-vrouwtjes te verminderen, met name in de 3u-behandelingsgroep. Ons onderzoek toonde een vermindering van leukocyteninfiltratie in longblaasjes en parenchym. Dit is mogelijk het geval van de door oestradiol beïnvloedbare adhesiemoleculen (ICAM-1 en VCAM-1) Ook in de long kweek werden macrofaag inflammatoire eiwit (MIP)-1α, MIP-2 en CINC-1 eiwitten gevonden die verantwoordelijk zijn voor de rekrutering van neutrofielen en macrofagen[38,39]. Serum- en genexpressie van ontstekingsmediatoren (interleukine [IL]-1β en tumornecrosefactor [TNF]-α) namen af en de metalloproteïnase-activiteit was ook verminderd.

Het is belangrijk te vermelden dat oestradiol de vorming van oedeem slechts gedeeltelijk voorkomen, aangezien de microvasculaire permeabiliteit en NO-synthasen niet werden veranderd door estradiol, ondanks dat de histopathologische analyse een afname van oedeem liet zien. Desondanks kan oestradiol worden beschouwd als een therapeutische optie bij het verminderen van door HD veroorzaakte longontsteking bij vrouwtjes, met name door het verminderen van leukocytinfiltraat. Vooral neutrofielen zijn betrokken bij de ontwikkeling van acute long schade en de incidentie van primair transplantaatfalen (PGF), samengaand met de productie van reactieve zuurstofspecies (ROS), endotheliale activering en cytokineafgifte[40-42]. De neutrofiel activering wordt mogelijk gecontroleerd door estradiol via ER-β[43] door een verhoging van deze receptor-mRNA door behandeling met estradiol[44]. Een andere mogelijkheid is dat de G-eiwit-gekoppelde oestrogeenreceptor (GPER), die tot expressie wordt gebracht in de neutrofielen, de influx van calcium in de cel zou kunnen beperken. Vrouwelijke ratten in pro-oestrus en mannetjes behandeld met estradiol, lieten beide een reductie in neutrofielen gemedieerde ontsteking zien waaronder de GPER-gerelateerde toename van intracellulair calcium[45,46].

Als volgende stap in **Hoofdstuk 5** hebben we de invloed van oestradiolbehandeling op longontsteking van vrouwtjes na HD onderzocht door long explantaten gedurende langere tijd te bestuderen. Onze resultaten toonden aan dat oestradiol een langdurig effect had op de afgifte van ontstekingsmediatoren (IL-1β en TNF-α), waardoor ze zelfs 24 uur na HD werden verminderd. IL-1β en TNF-α worden vroeg na hersendood inductie afgegeven door macrofagen, waardoor de ontsteking wordt versterkt door de verhoogde expressie van adhesiemoleculen en afgifte van chemokinen[3,47]. Dit wordt later gevolgd door een verhoogde productie van superoxide en proteolytische enzymen, evenals activering van apoptose[48]. We veronderstellen dat estradiol de activering van macrofagen vermindert, wat de productie van

ontstekingsmediatoren vermindert en daardoor de HD-ontstekingsreactie zal verlagen. Uit eerdere studies is gebleken dat lage concentraties oestradiol inderdaad de afgifte van IL-1β en TNF-α door macrofagen zullen verhogen, terwijl een hoge concentratie deze kan verminderen. Dit beschermende effect bij hoge doses zou, door remming van nuclearfactor (NF)-KB, ook de ontwikkeling van acute longschade kan beïnvloeden[47]. Daarnaast blijkt uit een studie van Acconcia et al.[49] dat estradiol in kankercellijnen een dubbel effect kan hebben, afhankelijk van elke receptoractivering. ER-α activeerde snel meerdere signaaltransductieroutes, zoals signaal gerelateerde kinase (ERK)/mitogeen-geactiveerde proteïne kinase (MAPK) en fosfatidylinositol 3-kinase (PI3K)/ proteïne kinase B (AKT), die de voortgang van de celcyclus en apoptotische cascadepreventie bevorderden, terwijl ER-β snelle en aanhoudende fosforylering van p38/MAPK veroorzaakte, die de activering en splitsing van caspase-3 regelt. Verdere studies naar de werking van estradiol bij het beheersen van apoptose in het longweefsel zijn nodig, aangezien het profiel van oestrogeenreceptoren in de long voornamelijk bestaat uit ER-β receptoren[15,44].

Een laatste interessante observatie in dit hoofstuk is dat estradiol de HD geinduceerde activatie van het complementsysteem in vrouwen gedeeltelijk kan reduceren op C3-niveau zonder effect op het membrane attack complex (C5b-9). Het is bekend dat vrouwen in vergelijking met mannen een lagere uiting hebben van componenten van het terminale complementsysteem[51,52]. Er zijn onderzoeken naar de directe rol van het complementsysteem, via klassieke en lectine-routes, in door HD geïnduceerd longletsel en transplantatiesucces[54,55]. Een estradiol behandeling bij HD-vrouwen zou dus niet alleen een verlaagde afgifte van ontstekingsmediatoren maar ook een verminderde activering van het complementsysteem tot gevolg hebben met een langdurig beschermend effect op ontsteking in de long.

Als laatste richten we ons in **Hoofdstuk 6** op een techniek die belangrijk is geworden bij longtransplantatie, de ex vivo longperfusie (EVLP). Het wordt gebruikt voor het beoordelen van de functie vóór transplantatie voor het maken van klinische beslissingen over marginale longen, maar het maakt ook mogelijke behandeling van longen mogelijk[55]. Op basis van de kennis dat longontsteking door HD verschilt bij mannen en vrouwen[8,9], hebben we een onderzoek ontworpen om dit te onderzoeken bij mannelijke en vrouwelijke hersendode te bekijken op de EVLP. Onze gegevens toonden aan dat HD, onafhankelijk van geslacht, de oxygenatiecapaciteit vermindert. Met betrekking tot mechanische ventilatieparameters vonden we dat EVLP in het algemeen gedurende de 4 uur van het experiment gehandhaafd bleef. Opvallend was dat longen afkomstig van HD-vrouwelijke dieren na perfusie een hogere

longontsteking vertonen dan mannen. De vrouwelijke HD-longen vertoonden een hoger leukocytinfiltraat van met name neutrofielen. Bovendien vonden we dat HD-vrouwtjes een hogere IL-1β-concentratie in de perfusievloeistof hebben na 4 uur EVLP, afgegeven in longkweekmedium (24 uur). Een experimenteel onderzoek met deceased circulatory death (DCD) donoren heeft ook de invloed van geslacht op de longfunctie in de EVLP geanalyseerd. Hier werd een geslachtsgebonden verschil in longfunctie, waarbij mannen een verminderd vermogen hadden om zuurstof te transporteren. Er werd echter geen dimorfisme waargenomen bij de ontwikkeling van longoedeem[56]. In onze EVLP-gegevens hebben we ook geen verband waargenomen tussen geslacht en oedeemvorming, beoordeeld door een oedeemindex. Er moeten echter meer nauwkeurigere methoden voor de evaluatie van de microvasculaire permeabiliteit worden gebruikt om deze bevindingen te bevestigen. Concluderend kan gesteld worden dat de hormonale onbalans die door HD wordt veroorzaakt diverse ontstekingsreacties in de longen geeft die door gebruik van de EVLP methode alleen niet veranderd kan worden.

Toekomstperspectieven

Dit proefschrift heeft bijgedragen aan ons begrip van sekseverschillen in de HD-pathofysiologie en oestradiolmodulatie van HD-geïnduceerde longbeschadiging. Dat kan als basis dienen voor verder onderzoek naar donormanagement en verbetering van de longkwaliteit, rekening houdend met het belang van vrouwelijke geslachtshormonen en hun implicaties voor het ontstekingsproces. Daarnaast creëert het ook een kans voor nieuwe onderzoeken die estradiol testen als een haalbare therapieoptie voor donormanagement.

Donormanagement is gericht op het behouden en optimaliseren van de longfunctie na transplantatie, door gebruik te maken van strategieën die gericht zijn op het optimaliseren van pulmonale en extrapulmonale fysiologische parameters of conservering tijdens aanschaf en doorvoer[57]. Bij donoren na hersendood zal het longmanagementprotocol hemodynamische, neuro-endocriene en long specifieke benaderingen vereisen, waardoor potentiële donoren worden behandeld met vasopressine, glucocorticoïden, schildklierhormonen en insuline[5].

Onze gegevens in **Hoofdstukken 4** en **5** laten zien dat behandeling met estradiol bij HD-vrouwen ontstekingen reguleert door de uiting van adhesiemoleculen, cytokines en chemokinen te verminderen, wat resulteert in een lager leukocyteninfiltraat. Toch was deze behandeling niet voldoende in staat om de long te beschermen tegen toename van de microvasculaire permeabiliteit, wat leidde tot oedeemvorming. In onze studies hebben we ook de aandacht gevestigd op de acute afname van corticosteron, vergezeld van oestradiolreductie na HD.

Glucocorticoïden komen vrij in vroege ontstekingsstadia en het is bekend dat ze een synergetische rol spelen bij het verminderen van ontstekingen samen met estradiol[36]. Bij een hypothalamus-hypofyse-falen bij HD zien we een verlies van beide hormonen. Daarom zou het nuttig zijn om de gecombineerde behandeling met corticosteron en estradiol te onderzoeken, die mogelijk positieve gevolgen zou kunnen hebben, niet alleen op longontsteking maar ook op oedeemvorming bij vrouwelijke donoren. Een onderzoek bij HD-vrouwen om dit combinatie-effect op longbeschadiging te evalueren, loopt inderdaad als gezamenlijk onderzoek tussen laboratoria van de Rijksuniversiteit Groningen en de Universiteit van São Paulo. De voorlopige resultaten suggereren dat een behandeling met methylprednisolon de longontsteking vermindert bij hersendode vrouwelijke dieren, door de afgifte van cytokinen en chemokinen en de expressie van adhesiemoleculen te verminderen, waardoor de alveolaire infiltratie van leukocyten positief wordt beïnvloed. Als deze studie eenmaal is afgerond, zullen deze resultaten onze kennis van hormoneninteractie in de long bij HD.

Het is belangrijk op te merken dat er maar weinig studies zijn die proberen de route te onderzoeken waarlangs estradiol en zijn receptoren een beschermend effect in de long kunnen produceren. Genomische en niet-genomische routes worden gesuggereerd mar er lijkt consensus te zijn dat de beschermende effecten van estradiol in de long voornamelijk via ER-β zijn, vergelijkbaar met hart[19,43]. Daarom zouden experimenten die gebruik maken van specifieke agonisten of antagonisten van estradiolreceptoren interessant zijn om te bepalen hoe estradiol longbeschadiging moduleert in een HD-model.

De effecten van de behandeling met oestradiol op HD-donoren zou het succes van een transplantatie op korte of lange termijn positief kunnen beïnvloeden (acute afstoting of primaire transplantaatdisfunctie), aangezien estradiol een beschermend effect bleek te hebben op longontsteking (**hoofdstuk 4** en **5**). Met deze kennis zouden we een EVLP kunnen voorstellen die estradiol gebruikt als een behandeling tijdens perfusie, als middel om longbeschadiging te verbeteren en vooral de longen van vrouwen te behouden. Deze zouden enige beschermende effecten kunnen hebben, mogelijk door de afgifte van neutrofielen en macrofagen van ontstekingsmediatoren, proteolytische enzymen en ROS-productie te verminderen. Desalniettemin geven onze resultaten uit **Hoofdstuk 6** aan dat vrouwelijke longen slechtere ontstekingsparameters hadden dan mannelijke longen na HD gevolgd door EVLP, wat wijst op de mogelijkheid dat EVLP alleen de conditie van de vrouwelijke transplantlongen niet kan verbeteren, en benadrukt de noodzaak van nieuwe therapieën om de longontsteking vóór het gebruik van de EVLP-machine en bijgevolg de transplantatieresultaten verbeteren.

Een andere mogelijke therapie is het gebruik van een adsorberend membraan om cytokines te verwijderen. Dit bleek werkzaam bij het verminderen van cytokines in het perfusaat, maar het was niet effectief in het verminderen van de neutrofiele activiteit[58]. Er zijn twijfels naar de effectiviteit van leukocytenfilters bij EVLP, met betrekking tot de verzadiging en het tijdstip van reperfusie[59,60]. Wat echter onder de aandacht moet worden gebracht, is dat de meeste onderzoeken alleen longen gebruiken na een ischemieperiode en zonder HD, een protocol dat de klinische setting niet nabootst. Met de vraag wat er zou gebeuren met de leukocyt die naar het longparenchym migreerde en het longtransplantaat tijdens perfusie zou beïnvloeden. Daarom stellen we voor om de leukocytenactiviteit en longinfiltraat te behandelen in de donor, dus voordat het longtransplantaat wordt geoogst of in een ex vivo perfusiemachine wordt geplaatst.

Concluderend, dit proefschrift heeft de inherente verschillen tussen mannen en vrouwen in het geval van HD onder de aandacht gebracht, waarbij de rol van vrouwelijk geslachtshormoon in de ontwikkeling van longbeschadiging bij vrouwelijke donoren wordt benadrukt, en wijst op het geslacht van de donor als evaluatiecriterium voor effectief donormanagement.

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Resumo e Discussão Geral

Devido a importância do transplante pulmonar como terapia escolhida para pacientes com doença pulmonar em estágio terminal[1], continuamos a buscar maneiras de melhorar o número e a qualidade dos pulmões disponíveis. Os doadores em morte encefálica (ME) são a principal fonte de enxertos pulmonares, embora a própria ME tenha repercussões sistêmicas que afetam a qualidade do órgão[2-4]. Atualmente, os critérios para doadores de pulmão incluem idade, tamanho do órgão e oxigenação[5]. No entanto, nesta tese, destacamos a diferença entre os sexos em resposta à ME e o papel dos hormônios sexuais femininos nessa resposta. Poucos estudos consideram as diferenças entre homens e mulheres como um parâmetro para investigação no pareamento de doadores e receptores no transplante pulmonar e o maior risco associado ao transplante de doadoras mulheres para homens[6,7]. Estudos experimentais confirmaram a hipótese de que doadoras do sexo feminino apresentam pulmões com menor lesão após ME, com resposta inflamatória pulmonar mais grave em comparação a machos, e que está associada à redução aguda dos hormônios sexuais femininos[8,9]. De fato, no **Capítulo 2** mostramos que há dimorfismo tanto nos processos de perfusão quanto de coagulação após ME, possivelmente afetando a inflamação e contribuindo para a maior resposta em fêmeas e o comprometimento da microcirculação em machos.

Por outro lado, diferentes modelos experimentais de trauma e inflamação que comparam as respostas de machos e fêmeas, mostram que fêmeas possuem um controle mais eficaz da resposta inflamatória sistêmica[10-12]. Esta proteção feminina torna-se mais aparente na comparação entre ratas ovariectomizadas (OVx) e não-OVx após isquemia e reperfusão (I/R), sendo que fêmeas OVx em I/R apresentam maior inflamação pulmonar, quando comparado às fêmeas não-OVx[13]. Além disso, o tratamento com estradiol em fêmeas OVx protege efetivamente contra inflamação e lesão pulmonar[13-16]. Nesse sentido, é possível inferir que o tratamento de pacientes do sexo feminino com hormônios sexuais femininos teria potencial de controlar a lesão pulmonar induzida por ME. Assim, focamos no efeito do tratamento com estradiol em fêmeas submetidas ao modelo de ME. Por fim, investigamos também as respostas de diferentes sexos após ME e perfusão *ex vivo*, como forma de avaliação e tratamento de enxertos pulmonares. Consequentemente, podemos entender melhor o papel da variável sexo no processo de escolha e manejo de pulmões para transplante.

Apesar de ser claro na literatura as diferenças relacionadas ao sexo na resposta imune[17,18]. Estudos clínicos e experimentais de trauma mostrando distinções entre machos e fêmeas[10,19,20],

os quais têm como foco principal o sexo masculino. Em modelo de ME ratos machos apresentam o comprometimento da microcirculação, tanto nos microvasos mesentéricos[21] quanto nos pulmonares[22]. Ao mesmo tempo ocorre uma redução da corticosterona, resultado da perda do eixo hipotálamo-hipófise-adrenal e consequentemente, observamos um aumento dos mediadores inflamatórios. Principalmente, os pulmões não só diminuíram a perfusão, mas também o aumento do infiltrado leucocitário.

Conforme mencionado, estudos anteriores sobre diferenças de sexo relacionaram a queda aguda dos hormônios sexuais femininos (após 3h de ME) com uma maior inflamação pulmonar em fêmeas comparado aos machos. Ao mesmo tempo, observamos níveis diminuídos de corticosterona em machos e fêmeas, resultado da falência do eixo hipotálamo-hipofisária por ME[8,23]. Além disso, as fêmeas, ao contrário dos machos, têm a perfusão dos microvasos mantida após a ME, a qual foi associada à alta expressão da sintase de óxido nítrico endotelial (eNOS)[23]. Seguindo a mesma linha desses estudos, no **Capítulo 2**, buscamos investigar as diferenças sexuais nos mecanismos envolvidos nas alterações da microcirculação após o ME. Os machos apresentam redução da perfusão na microcirculação após ME e esse fenômeno pode se associar à formação de microtrombos intravasculares, devido ao aumento da agregação plaquetária e coágulos mais firmes. Por outro lado, observamos a manutenção da perfusão no sexo feminino, o que foi atribuído à redução da firmeza e da lise dos coágulos. Em circunstâncias normais, as fêmeas parecem exibir maior agregação plaquetária do que os machos[24,25], e o estradiol pode ser um dos fatores reguladores da coagulação e da função e ativação plaquetária[26,27]. Com o estabelecimento da ME resultando na redução do estradiol, os efeitos do estradiol sobre a função e ativação plaquetária são perdidos, o que pode ser verificado em nossos dados que mostram o aumento do tempo de coagulação. Os resultados opostos foram observados no sexo masculino, não apenas na formação de coagulação mais rápida, mas também na hipercoagulabilidade e formação de trombo. A ausência de estradiol em mulheres pode ser uma explicação para a dicotomia sexual observada. Além disso, ambos receptor de estradiol (ER)-α e ER-β foram expressos em plaquetas em um nível semelhante em ambos homens e mulheres[9,28]. Mesmo sendo células anucleadas os estrogênios podem apresentar efeitos rápidos não genômicos nas plaquetas, envolvendo ER-β, o principal receptor expresso nas plaquetas e tirosina quinase Src. Modulam assim a agregação plaquetária, induzida por um baixo nível de trombina e resultando em um aumento robusto da integrina αIIbβ3 para o fibrinogênio[30]. Também consideramos o papel do óxido nítrico (NO) que poderia contribuir para a redução da atividade plaquetária durante a hipóxia[31]. Fêmeas apresentam maior expressão da sintase de

óxido nítrico induzida (iNOS) e eNOS após ME, e a maior síntese de NO e tônus vascular poderia explicar as diferentes respostas em fêmeas[8,23]. Esse fenômeno pode ser observado em estudos com ratos machos em ME, já que o tratamento com estradiol melhora a lesão pulmonar e a perfusão da microcirculação mesentérica, reduzindo também os parâmetros de edema e hemorragia, possivelmente pela modulação das NO sintases pelo estradiol[32,33].

Para avaliar o papel dos hormônios sexuais em fêmeas submetidas à ME, no **Capítulo 3,** o foco foi determinar se os hormônios modulam a inflamação pulmonar causada pela ME em fêmeas. Os dados mostraram que fêmeas OVx, assim como mulheres em menopausa, tem tempo para se adaptar às baixas concentrações de hormônios sexuais femininos (estradiol e progesterona), apresentando menor inflamação pulmonar em comparação com animais não-OVx após ME. Confirmamos, a queda aguda das concentrações de estradiol e progesterona após a indução de ME em fêmeas não-OVx e em paralelo foi observada uma intensa inflamação pulmonar, em comparação com fêmeas OVx, isso foi evidenciado pelo maior infiltrado leucocitário no parênquima e lavado broncoalveolar. Resultado da maior expressão de moléculas de adesão (molécula de adesão intercelular [ICAM]-1 e molécula de adesão vascular [VCAM]-1), acompanhada da liberação de mediadores inflamatórios (fator estimulante de colônias granulocíticas [G-CSF], fator de crescimento endotelial vascular [VEGF] e citocina indutora de quimioatração em neutrófilos (CINC)-1) e expressão de iNOS. A produção de NO pela iNOS pode contribuir para a inflamação sistêmica[34]. Os animais OVx passam por um período de adaptação (10 dias), durante o qual os receptores de estradiol diminuem em número[35] e, em paralelo, os níveis de corticosterona são modificados ao final do período de adaptação e encontrados muito abaixo em animais Ovx em comparação com não OVx. Este fenômeno poderia ser explicado uma vez que o estradiol desempenha um papel excitatório na liberação de corticosterona, controlando o eixo hipotálamo-hipófise-adrenal em condições basais pelo ER-α ou em condições de estresse por ER-α e ER-β[36,37]. Nesse sentido, as ratas OVx poderiam ser uma ponte para entender melhor as mulheres doadoras em menopausa e pré-menopausa. Esses resultados indicam que não apenas o papel da corticosterona na inflamação é crucial para a compreensão da inflamação por ME, mas também destaca a influência do estradiol após a ME.

Os dados sobre diferenças sexuais e queda hormonal após ME geraram a hipótese de que o estradiol poderia ser uma ferramenta no controle da lesão pulmonar causada por ME e embasaram a avaliação do tratamento com estradiol em ratas submetidas ao modelo de ME em dois momentos distintos. Um tratamento precoce, iniciado assim que confirmada a ME, evita a

queda das concentrações de estradiol, e um tratamento posterior passadas 3h da confirmação do ME, para melhor investigar o estradiol como opção terapêutica. Como mostrado no **Capítulo 4**, de fato, o estradiol foi capaz de reduzir a inflamação pulmonar em fêmeas em ME, principalmente no grupo de tratamento de 3h. Nosso estudo mostrou a redução do infiltrado leucocitário nos alvéolos e parênquima. Reforçando então o papel do estradiol no controle da expressão gênica e proteica de moléculas de adesão (ICAM-1 e VCAM-1), assim como a liberação de quimiocinas em cultura de pulmão (proteína quimioatraente de monócitos [MIP]-1α, MIP-2 e CINC-1), que são responsáveis pelo recrutamento de neutrófilos e macrófago[38,39]. A expressão sérica e gênica de mediadores inflamatórios (interleucina [IL]-1β e fator de necrose tulmonar [TNF]-α) diminuiu, assim como a atividade da metaloproteinase, indicando o efeito do estradiol na ativação de células inflamatórias e consequentemente na lesão pulmonar.

É importante mencionar que o estradiol pode controlar apenas parcialmente a formação do edema, uma vez que a permeabilidade microvascular e as NO sintases não foram alteradas, mesmo sendo observada uma diminuição do edema na análise histopatológica. Apesar disso, o estradiol pode ser considerado uma opção terapêutica no controle da inflamação pulmonar induzida por ME em fêmeas, especialmente na redução do infiltrado leucocitário. Os neutrófilos, em particular, têm sido implicados no desenvolvimento de lesão pulmonar aguda e na incidência de disfunção primária do enxerto (DPE), juntamente com a produção de espécies reativas do oxigênio (ERO), ativação endotelial e liberação de citocinas[40-42]. Os neutrófilos, por exemplo, poderiam ter sua ativação controlada pelo estradiol por meio do ER-β[43] e o mesmo mRNA do receptor também foi encontrado aumentado quando o infiltrado pulmonar foi atenuado pelo tratamento com estradiol[44]. Outra possibilidade seria o receptor de estrogênio acoplado à proteína G (GPER) expresso nos neutrófilos, pode estar limitando a entrada de cálcio na célula por meio dos canais de entrada de cálcio operados por armazenamento. Uma vez que ratas em proestro e machos tratados com estradiol apresentaram atenuação da capacidade dos neutrófilos de participar da inflamação e dos mecanismos de sinalização GPER incluem o aumento do cálcio intracelular[45,46].

Na etapa seguinte (**Capítulo 5)** investigamos a influência a longo prazo do tratamento com estradiol na inflamação pulmonar de fêmeas após a ME, tentando entender a resposta imune do enxerto pulmonar após o transplante, por meio de uma manutenção mais prolongada de amostras de pulmão viáveis. Nossos resultados mostraram que o estradiol teve um efeito duradouro sobre a liberação de mediadores inflamatórios (IL-1β e TNF-α), reduzindo-os 24 horas após a ME. IL-1β e TNF-α são liberados inicialmente pelos macrófagos, amplificando a

inflamação por meio do aumento da expressão de moléculas de adesão e liberação de quimiocinas[3,47]. Posteriormente, haverá a produção de superóxidos e enzimas proteolíticas, além da ativação de vias de apoptose[48]. Formulamos a hipótese de que o estradiol reduz a ativação de macrófagos, reduzindo então a produção de mediadores inflamatórios e, portanto, controlando a resposta inflamatória gerada pela ME. Foi relatado que baixas concentrações de estradiol realmente aumentam a liberação de IL-1β e TNF-α pelos macrófagos, ao passo que altas concentrações diminuem. Esse efeito protetor das altas doses poderia, por meio da inibição do fator nuclear (NF)-κB, também influenciar o desenvolvimento de uma lesão pulmonar aguda [47]. Além disso, um estudo de Acconcia et al.[49] descobriu que em linhagem de células cancerosas o estradiol pode ter um efeito duplo, dependendo da ativação de cada receptor. O ER-α ativou rapidamente várias vias de transdução de sinal, como proteína quinase regulada por sinal extracelular/proteína cinase ativada por mitógeno (ERK/MAPK) e fosfatidilinositol-3-quinase/ proteína quinase B (PI3K/AKT), que promoveram a progressão do ciclo celular e a prevenção da cascata de apoptose. Por outro lado, ER-β induziu fosforilação rápida e persistente de p38/MAPK, que controla a ativação e clivagem da caspase-3 iniciando o ciclo apoptótico. Portanto, seriam necessários mais estudos focando nas ações do estradiol no controle da apoptose em tecido pulmonar, uma vez que o perfil dos receptores de estrogênio no pulmão é principalmente ER-β, o qual tem sido atribuído como o responsável pelo efeito protetor do estradiol no pulmão[15,44]. Por fim, os resultados mostrando a ativação do sistema complemento pela ME em fêmeas até o nível C3, sem efeito no complexo de ataque à membrana (C5b-9) confirma o papel anti-inflamatório do estradiol. A literatura indica que, em comparação com o sexo masculino, o sexo feminino apresenta menor expressão de componentes do sistema complemento terminal[51,52]. Existem estudos sobre o papel direto do sistema complemento por meio das vias clássicas e da lectina na lesão pulmonar induzida pela ME e no sucesso do transplante[53,54]. Em nossos estudos utilizando fêmeas em ME relacionamos o tratamento de estradiol na inflamação pulmonar com a diminuição da liberação de mediadores inflamatórios e com a redução da ativação do sistema complemento, que possivelmente modula os neutrófilos e macrófagos com um efeito protetor duradouro sobre a inflamação do pulmão.

Por fim, no **Capítulo 6**, focamos uma técnica que ganhou importância no transplante de pulmão, a perfusão pulmonar *ex vivo* (PPEV). A qual tem sido usado na avaliação da função pulmonar pré-transplante, auxiliando na decisão do uso pulmões marginais na clínica, mas também permite potenciais tratamentos e reparo de pulmões lesados[55]. Juntamente com o conhecimento da diferença na inflamação pulmonar masculina e feminina após ME[8,9],

desenvolvemos um estudo que permite comparar a inflamação pulmonar em ratos machos e fêmeas em ME, seguida pela PPEV. Nossos dados mostraram que a ME, independente do sexo, reduziu a função pulmonar, diminuindo sua capacidade de oxigenação. Nos parâmetros de ventilação mecânica, descobrimos que a PPEV global se manteve durante as 4h de experimento. Sendo o mais importante que as fêmeas em ME continuaram a apresentar maior inflamação pulmonar, em comparação com os machos após a perfusão. Os pulmões de fêmeas em ME apresentaram maior infiltrado leucocitário, nos quais encontramos elevada presença de neutrófilos. Além disso, constatamos que fêmeas em ME apresentaram maior concentração de IL-1β no perfusato após 4h de PPEV, no meio de cultura pulmonar (24h) e maior expressão gênica. Portanto, é possível sugerir que nos pulmões das fêmeas encontramos um maior número leucócitos, possivelmente mais ativos do que nos machos. Um estudo experimental com doadores em parada cardíaca também analisou a influência do sexo no PPEV. Eles descobriram uma diferença ligada ao sexo na função pulmonar, onde os homens apresentavam capacidade reduzida de transportar oxigênio. O sexo feminino apresentou efeito protetor, resultando em melhor perfusão comparado aos machos. Porém, não foi observado dimorfismo no desenvolvimento do edema pulmonar[56]. É importante ressaltar que doadores em parada cardíaca apresentam perfil semelhante ao observado após modelo de isquemia e reperfusão, no sentido de que os níveis hormonais não se alteram em resposta à morte circulatória, portanto, não apresentam desequilíbrio hormonal e nenhuma redução de estradiol ou corticosterona. Em nossos dados de PPEV, também não observamos nenhuma ligação entre sexo e formação de edema, avaliada por um índice de edema. No entanto, métodos mais precisos de avaliação da permeabilidade microvascular devem ser usados para confirmar esse parâmetro. Com esse dado, podemos reiterar o entendimento de que o desequilíbrio hormonal produzido pelo ME, com ênfase na perda dos hormônios sexuais femininos por fêmeas, produzirá diferentes respostas inflamatórias no pulmão após o ME, as quais a PPEV sozinho não poderia modificar.

Pespectivas Futuras

No conjunto, a tese ampliou nossa compreensão das diferenças sexuais na fisiopatologia da ME e na modulação do estradiol na lesão pulmonar induzida pela ME, possibilitando novos estudos sobre o manejo do doador e melhoria da qualidade do enxerto pulmonar. Tais estudos levarão em consideração a importância dos hormônios sexuais femininos e sua implicação no processo

inflamatório, ao mesmo tempo que serão criadas oportunidades para novos estudos que testam o estradiol como uma opção de terapia viável no tratamento de doadores.

O manejo do doador visa preservar e otimizar a função pulmonar pós-transplante por meio de estratégias que se concentram na otimização dos parâmetros fisiológicos pulmonares e extrapulmonares, ou focam na preservação durante a obtenção e o trânsito do pulmão[57]. Para doadores em ME, o protocolo de manejo pulmonar exige abordagens hemodinâmicas, neuroendócrinas e específicas do pulmão, tratando assim doadores potenciais com vasopressina, glicocorticóides, hormônios da tireoide e insulina[5].

Os dados nos **Capítulos 4** e **5** mostram que o tratamento com estradiol em fêmeas em ME controla a inflamação, reduzindo a expressão de moléculas de adesão e a liberação de citocinas e quimiocinas, resultando em menor infiltrado de leucócitos. Contudo, o estradiol não foi capaz de proteger o pulmão do aumento da permeabilidade microvascular levando à formação de edema. Em nossos estudos, também chamamos a atenção para a redução aguda da corticosterona, acompanhada pela redução do estradiol após a ME. Os glicocorticóides são liberados nos estágios inflamatórios iniciais e são conhecidos por ter um papel sinérgico no controle da inflamação com o estradiol[36]. No caso da insuficiência hipotálamo-hipofisária na ME, observamos uma perda de ambos os hormônios. Portanto, seria benéfico investigar o tratamento combinado com corticosterona e estradiol, pois o tratamento poderia potencialmente ter repercussões positivas, não apenas na inflamação pulmonar, mas também na formação de edema em doadoras do sexo feminino. Um estudo em fêmeas em ME avaliando esse efeito da combinação na lesão pulmonar está de fato em andamento como pesquisa conjunta entre os laboratórios da University of Groningen e da Universidade de São Paulo. Seus resultados preliminares sugerem que o tratamento com metilprednisolona altera a inflamação pulmonar em fêmeas submetidas à ME, ao reduzir a liberação de citocinas e quimiocinas e a expressão de moléculas de adesão, afetando positivamente a infiltração leucocitária alveolar. Uma vez finalizado este estudo, esses resultados irão expandir nosso conhecimento sobre a interação dos hormônios nas repercussões pulmonares causadas pelo ME em mulheres.

Também observamos que poucos estudos investigam a via pela qual o estradiol e seus receptores podem produzir um efeito protetor no pulmão, os quais especificamente têm se concentrado principalmente em modelos de choque trauma-hemorragia. O estradiol apresenta ações por vias genômica e não genômica, com efeitos protetores no pulmão observados principalmente pela via ER-β, assim como encontrados no coração[19,43]. Assim, reconhecendo esta lacuna na literatura, seria importante estudar mais as vias de ação do estradiol na proteção

pulmonar para que tratamentos específicos do impacto da ME no pulmão pudessem ser desenvolvidos. Portanto, experimentos que usam agonistas ou antagonistas específicos dos receptores de estradiol seriam de interesse, para definir como o estradiol modula a lesão pulmonar em um modelo de ME.

Também é importante considerar que os efeitos do tratamento com estradiol em doadores em ME podem alterar positivamente o sucesso do transplante em um período de curto ou longo prazo (rejeição aguda ou disfunção primária do enxerto), uma vez que o estradiol provou ter um efeito protetor sobre a inflamação pulmonar (**Capítulo 4** e **5**). Com esse conhecimento, poderíamos propor uma PPEV que utilize o estradiol como tratamento durante a perfusão, como forma de melhorar a lesão pulmonar e principalmente preservar o pulmão do sexo feminino. Este uso pode trazer alguns efeitos protetores, possivelmente reduzindo a liberação de neutrófilos e macrófagos de mediadores inflamatórios, enzimas proteolíticas e produção de ERO. No entanto, nossos resultados do **Capítulo 6** indicam que o pulmão feminino tinha parâmetros inflamatórios piores do que os pulmões masculinos após ME seguido por PPEV, apontando para a possibilidade de que a PPEV sozinha não pode melhorar a condição dos pulmões do enxerto feminino e enfatizando a necessidade de novas terapias para reduzir inflamação pulmonar antes do uso da máquina PPEV e, consequentemente, melhorar o resultado do transplante. Portanto, é possível especular que o tratamento com estradiol na máquina PPEV poderia produzir resultados diferentes nos pulmões masculinos e femininos.

Já foi proposto o uso de uma membrana adsorvente para remoção de citocinas, que mostrou eficaz na sua redução no perfusato, mas não foi eficaz em reduzir a atividade dos neutrófilos[58]. Nesse sentido, o uso do tratamento com estradiol poderia ser de grande utilidade, uma vez que estaria controlando o processo inflamatório no doador em ME, necessário principalmente em fêmeas por sua resposta acentuada, atenuando assim a inflamação antes da perfusão. Também existem estudos controversos sobre a eficácia dos filtros de leucócitos no PPEV, quanto à sua saturação e tempo de reperfusão[59,60]. Porém, o que devemos chamar atenção é que a maioria dos estudos utilizam pulmões após apenas um período de isquemia, sem de fato realizar a ME, tal protocolo não mimetiza o quadro clínico. Suscitando a questão do que aconteceria com os leucócitos que migram para o parênquima pulmonar e influenciam no desenvolvimento do enxerto pulmonar durante a perfusão. Portanto, supomos que a atividade leucocitária e o infiltrado pulmonar poderiam ser controlados no doador em ME antes da coleta ou inserção do enxerto pulmonar em uma máquina de perfusão *ex vivo*.

Em conclusão, esta tese focou nas diferenças inerentes entre homens e mulheres presentes no caso de ME, destacando o papel do hormônio sexual feminino no desenvolvimento da lesão pulmonar em doadoras do sexo feminino e apontando o sexo do doador como critério de avaliação para manutenção do doador. Além disso, sugerimos o uso terapêutico do estradiol em doadores após a confirmação da ME ou durante a PPEV como perspectivas futuras para melhorar a qualidade do pulmão para o transplante.

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