UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS:

ENDOCRINOLOGIA

ATIVIDADE INFLAMATÓRIA INDUZIDA PELA MORTE ENCEFÁLICA EM COMPARAÇÃO À ATIVIDADE INFLAMATÓRIA INDUZIDA PELA DOENÇA CRÍTICA

DISSERTAÇÃO DE MESTRADO

PATRÍCIA SCHWARZ

Porto Alegre, março de 2017

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LISTA DE ABREVIATURAS

BD *Brain death*

BMI *Body mass index*

ELISA *Enzyme-linked immunosorbent assay*

DM Diabetes melito ou *diabetes mellitus*

FIPE Fundo de Incentivo à Pesquisa e Ensino

GLM *Generalized linear models*

GLP-1 *Glucagon-like peptide 1*

HCPA Hospital de Clínicas de Porto Alegre

HLA *Human leukocyte antigen*

ICU *Intensive care unit*

IFN-γ Interferon-γ ou *Interferon-γ*

IL-1β Interleucina-1β ou *Interleukin-1β*

IL-2 Interleucina-2 ou *Interleukin-2*

IL-6 Interleucina-6 ou *Interleukin-6*

IL-8 Interleucina-8 ou *Interleukin-8*

IL-10 Interleucina-10 ou *Interleukin-10*

ME Morte encefálica

MFI *Mean fluorescence intensity*

mRNA *Messenger ribonucleic acid*

RT-PCR *Reverse transcription polymerase chain reaction*

SD *Standard deviation*

TNF-α Fator de necrose tumoral- α ou *Tumor necrosis factor-α*

UFRGS Universidade Federal do Rio Grande do Sul

UTI Unidade de tratamento intensivo

Esta dissertação de Mestrado será apresentada no formato exigido pelo Programa de Pós-graduação em Ciências Médicas: Endocrinologia. Ela será constituída de uma introdução em português e um artigo em inglês, este formatado conforme as exigências da respectiva revista médica à qual será submetido para avaliação e posterior publicação. O artigo em inglês desta dissertação é um artigo do tipo Artigo Original.

RESUMO

Órgãos provenientes de doadores em morte encefálica (ME) apresentam piores desfechos nos receptores transplantados quando comparados a órgãos de doadores vivos, relacionados ou não relacionados. A lesão desses órgãos dá-se pelos processos relacionados à sua retirada e estocagem e também pela intensa atividade inflamatória presente na ME. Os efeitos dessa inflamação, com aumento da expressão de citocinas, já foram comprovados em rins, fígado, pulmão, coração e pâncreas. A maioria dos estudos avaliou os níveis de citocinas após ter sido estabelecido o diagnóstico de ME ou durante a cirurgia de retirada de órgãos para transplante. Porém, acredita-se que a intensa liberação de catecolaminas que ocorre no momento da instalação da ME possa relacionar-se com elevação precoce desses marcadores. Os pacientes em ME frequentemente apresentam outros insultos que podem desencadear resposta inflamatória sistêmica, como ventilação mecânica, choque hemorrágico, parada cardíaca e sepse. Portanto, o objetivo deste estudo foi comparar o nível de inflamação, por meio de dosagem plasmática de citocinas, entre pacientes em ME e pacientes criticamente doentes, com ou sem sepse, além de avaliar o comportamento das citocinas ao longo do tempo. Demonstramos que a ME está associada a um maior nível de inflamação do que a induzida pela doença crítica e a um nível semelhante à inflamação induzida pela sepse. Mesmo excluindo da análise os pacientes em ME que apresentavam sepse, os achados não se modificaram, corroborando com a hipótese de que a ME, per se, desencadeia uma resposta inflamatória sistêmica.

SUMMARY

Grafts from brain death (BD) donors in have worse outcomes as compared to grafts from living donors, even HLA-unmatched living donors. Besides the injuries related to organ retrieval and transplantation procedure, organs are also exposed to the intense systemic inflammatory response that occurs in BD. The deleterious effects of inflammation, with upregulation of cytokine expression, have already been documented in kidney, liver, lung, heart and pancreas. Most of these studies evaluated cytokine levels after BD confirmation or before organ retrieval. However, it is possible that the massive catecholamine release present at the time of BD installation might lead to a precocious cytokine increase. Moreover, brain-dead patients frequently suffer from other injuries that might also trigger an inflammatory cascade, such as mechanical ventilation, hemorrhagic shock, cardiac arrest and sepsis. Therefore, the purpose of this study was to compare, by means of plasmatic cytokines measurement, the level of inflammation in brain-dead patients and in critically ill patients, septic and non-septic, and to evaluate plasmatic cytokine kinetics in BD. We demonstrated that BD is associated with a higher level of inflammation than that induced by critical illness, which was similar to the induced by sepsis. Even when brain-dead patients with sepsis were excluded from the analysis, the results remain, corroborating the hypothesis that BD itself triggers a systemic inflammatory response.

CAPÍTULO 1

INTRODUÇÃO

O transplante de órgãos é considerado o tratamento de eleição para várias doenças terminais que afetam rins, pâncreas, fígado, coração e pulmão[1]. Atualmente, a principal fonte de órgãos para transplante é o doador de órgãos em morte encefálica (ME)[2].

Durante os processos de retirada, estocagem e transplante, os órgãos são submetidos a múltiplos insultos, tais como isquemia fria, mudanças súbitas de temperatura e estresse oxidativo[3]. Porém, antes mesmo desses agravos ocorrerem, existe uma intensa atividade inflamatória induzida pela ME, que lesa os órgãos candidatos a transplante[4].

Órgãos provenientes de qualquer doador vivo, relacionado ou não relacionado, demonstram resultados consistentemente superiores quando comparados aos de doadores em ME. Uma lesão catastrófica leva à ME e esta desencadeia alterações hemodinâmicas, neuro-humorais e imunológicas que afetam a qualidade dos órgãos[5]. A liberação aguda maciça de catecolaminas, conhecida como tempestade autonômica, é consequência da herniação cerebral e é tanto mais intensa quanto maior a velocidade de instalação da hipertensão intracraniana. Essa liberação explosiva de catecolaminas produz um aumento na expressão de citocinas nos órgãos sólidos, além de mediar a ativação do complemento[6]. A ME é um gatilho inflamatório que afeta adversamente a função dos órgãos transplantados de uma maneira antígeno-independente[7].

Os efeitos deletérios da ME nos diversos órgãos e tecidos

A ME constitui-se de uma síndrome inflamatória, com efeitos adversos conhecidos sobre os desfechos dos transplantes. O efeito da ME sobre os órgãos captados foi inicialmente estudado no rim. Biópsias renais de doadores em ME têm níveis significativamente mais elevados de citocinas e moléculas de adesão do que biópsias de rins de doadores vivos, o que indica um estado inflamatório precoce prévio à retirada dos órgãos[8,9]. Além disso, rins de doadores vivos HLA não relacionados apresentam uma sobrevida maior do que rins de doadores em ME, conforme evidenciado num estudo com 368 transplantes entre cônjuges[10].

Com relação ao fígado, uma análise de biópsias hepáticas antes da cirurgia de retirada do órgão demonstrou um aumento da infiltração de leucócitos CD4 e CD8 e de neutrófilos nos fígados de doadores em ME quando comparados aos de doadores vivos. Estes achados sugerem que os eventos que permeiam a ME e os cuidados críticos são importantes fatores de risco no desenvolvimento de disfunção primária de enxertos transplantados de doadores em ME[11].

Em 2001, Fisher *et al* demonstraram um aumento significativo nos níveis de interleucina-8 (IL-8) no lavado broncoalveolar de pacientes em ME quando comparados ao de controles normais, o que se correlacionou com infiltração neutrofílica no pulmão do doador[12]. Além disso, Kaneda *et al* estudaram biópsias de pulmão de 89 doadores e demonstraram que os receptores que morreram nos primeiros 30 dias pós-transplante apresentavam níveis significativamente mais elevados de interleucina-6 (IL-6) nas amostras coletadas antes da retirada do órgão[13].

Em relação aos enxertos cardíacos, dosagens miocárdicas de fator de necrose tumoral-α (TNF-α) feitas por RT-PCR imediatamente antes do transplante foram capazes de predizer a ocorrência de disfunção ventricular direita em 26 receptores após transplante cardíaco[14,15].

Contreras *et al* demonstraram que a ativação de citocinas pró-inflamatórias tem um impacto importante na função das células beta pancreáticas de uma maneira tempo-dependente. Em ratos, a precipitação da ME é seguida de um aumento imediato das concentrações de TNF-α, interleucina-1β (IL-1β) e IL-6, que induzem a disfunção e a morte da célula beta, principalmente por apoptose[16]. Há evidências também da implicação de aumento da interleucina-10 (IL-10) e de interferon-γ (INF-γ) na apoptose de ilhotas nesse cenário[17,18]. Corroborando esses achados, um estudo do nosso grupo de pesquisa demonstrou aumento de TNF-α e IL-6 no plasma e de TNF-α no tecido pancreático de doadores em ME quando comparados a controles submetidos à cirurgia de pancreatectomia[4]. Como as dosagens de citocinas deste estudo foram realizadas em média 12 horas após o diagnóstico de ME, não podemos descartar que ocorra um pico mais precoce destes marcadores inflamatórios nesses pacientes.

Além disso, outro estudo do nosso grupo testou as propriedades anti-inflamatórias da exenatida, um análogo do GLP-1 (*Glucagon Like Peptide- 1*) utilizado para o tratamento de diabetes tipo 2, em um modelo experimental de ME em ratos. A exenatida foi capaz de atenuar a inflamação induzida pela ME nesses animais, bem como melhorar a viabilidade das células beta pancreáticas e reduzir a apoptose de hepatócitos quando comparada ao grupo não tratado[19], além de reverter a lesão renal induzida pela ME (dados não publicados).

Doença crítica e inflamação

Há evidências da associação da ME com piores desfechos dos órgãos transplantados, porém, de uma maneira não completamente compreendida. Os pacientes em ME podem apresentar vários insultos simultâneos, que incluem desde a lesão cerebral que culminou na ME até choque hemorrágico, necessidade de ventilação mecânica, infecções nosocomiais, sepse e parada cardíaca[20-22]. Esses agravos, muito frequentes nos pacientes em ME, também estão associados a ativação da cascata inflamatória[23-25].

A ME pode, *per se,* ser o gatilho responsável pelos desfechos desfavoráveis observados, mas a contribuição de fatores inerentes à doença crítica como determinantes do estado inflamatório presente no paciente em ME não pode ser afastada. Para avaliar o papel da doença crítica no nível de inflamação de pacientes em ME, realizamos este estudo comparando os níveis plasmáticos de TNF-α, IL-1β, IL-6, IL-8, IL-10 e INF-γ de pacientes em ME com pacientes críticos internados em unidade de terapia intensiva (UTI) por outras patologias. Além disso, a fim de determinar a cinética das citocinas ao longo do curso clínico da ME, realizamos dosagens sequenciais dos mesmos marcadores inflamatórios ao longo do tempo nos pacientes em ME.

Frente ao exposto, esta dissertação tem os seguintes objetivos:

1- Determinar os níveis de inflamação induzidos pela ME em comparação aos níveis induzidos pela doença crítica, sendo os pacientes controles subdivididos em sépticos e não sépticos, por meio de dosagens plasmáticas de TNF-α, IL-1β, IL-6, IL-8, IL-10 e INF-γ;

2- Avaliar o comportamento de TNF-α, IL-1β, IL-6, IL-8, IL-10 e INF-γ ao longo do tempo em pacientes em ME.

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CAPÍTULO 2

Brain death inflammatory activity is similar to sepsis-induced cytokine release

Running title: Brain death and sepsis inflammatory activity

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Key words: brain death, cytokines, sepsis, inflammation

ABSTRACT

Brain death (BD) is associated with a systemic inflammation that leads to worse grafts outcomes. The purpose of this study was to compare plasmatic cytokine levels from brain-dead patients to those from critically ill patients, including septic and non-septic controls, and to evaluate the plasmatic cytokines kinetics in BD. Sixteen brain-dead and 32 control patients (16 with sepsis and 16 without) were included. Plasmatic levels of TNF-α, IL-1β, IL-6, IL-8, IL-10 and INF-γ were measured by magnetic bead assay. The results demonstrate levels of IL-8 and INF-γ were higher in brain-dead and septic patients compared with non-septic patients [IL-8: 80.3 (18.7-169.6) vs. 68.2 (22.4-359.4) vs. 16.4 (9.2-42.7) pg/mL; P=0.006; INF-γ: 2.8 (1.6-6.1) vs. 3.4 (1.2-9.0) vs. 0.5 (0.5-1.8) pg/mL; P=0.012], TNF-α showed a clear tendency to increase in brain-dead patients [2.7 (1.0-4.8) vs. 1.0 (1.0-5.6) vs. 1.0 (1.0-1.0) pg/mL; P=0.051] and IL-6 were higher in brain-dead patients as compared to non-septic control [174.5 (104.9-692.5) vs. 13.2 (7.3-38.6) pg/mL; P=0.002]. Even when brain-dead patients who had also sepsis (n=3) were excluded from the analysis, the differences remained. Levels of IL-1β and IL-10 increased from baseline to the second time point (~6 hours later) [IL-1β: 5.39 (1.93-16.89) vs. 7.11 (1.93-29.13) pg/mL; P=0.012; IL-10: 8.78 (3.62-16.49) vs. 15.73 (5.49-23.98) pg/mL; P=0.009]. In conclusion, BD systemic inflammation is as higher as that induced by sepsis and both are higher than the levels observed in critical illness.

Introduction

Brain-dead donors are the main source of organs for transplantation[1,2]. Brain death (BD) causes an acute and massive catecholamine release, leading to an inflammatory state characterized by cytokine upregulation[3]. Accumulating evidences suggest that BD-induced inflammatory activity adversely affects graft survival[4-8].

Experimentally, renal tubules and glomeruli from rat models of BD demonstrate increased expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) compared to controls[9]. In line with this, renal biopsies from BD donors have shown higher cytokines levels as compared to biopsies from living donors[10,11]. Moreover, kidney grafts from HLA-unmatched living donors have longer survival rates than kidneys from HLA-matched BD donors[12].

Primary lung graft dysfunction is a common complication of lung transplantation and results from ischemia-reperfusion injury, a process that might also be triggered by a systemic inflammatory state of the donor[13]. A significant increase in interleukin-8 (IL-8) levels in bronchoalveolar fluid from BD lung donors has been demonstrated and correlates with early graft dysfunction after lung transplantation[7]. Besides, patients who had died in the first 30 days after lung transplantation had elevated levels of IL-6 in biopsies prior to transplantation[14]. Interestingly, myocardial *TNF-α* mRNA expression during organ retrieval predicted right ventricular dysfunction in heart receptors[15,16]. In line with this, liver biopsies from brain-dead donors have higher CD4 and CD8 infiltration as compared to biopsies from living donors[17]. In a previous study from our group, we observed an increase in IL-6 and TNF-α plasma levels and an upregulation

of *TNF-α* mRNA expression in pancreatic tissue from BD donors as compared to control patients submitted to pancreatectomy due to malignant tumors, but the kinetics of cytokines release during BD was not evaluated[18].

Brain-dead patients suffer from a variety of insults, ranging from the lesion that has led to BD to hemorrhagic shock, mechanical ventilation, cardiac arrest and sepsis. All these injuries are frequently observed in other critically ill patients and might also trigger the inflammatory cascade[19-21]. So far, the contribution of critical illness to BD-associated inflammation has not been studied. Therefore, the aim of this study was to compare plasmatic cytokine levels from brain-dead patients with those from critically ill patients, including septic and non-septic individuals, and to evaluate the cytokine profile following BD in a time-dependent manner.

Patients and Methods

Brain-dead patients and controls

Study protocol was approved by the ethics committee at Hospital de Clínicas de Porto Alegre. Informed consent was obtained from patients or their legal representatives. BD was assessed independently by two physicians and was based on the following criteria: coma with complete unresponsiveness, absence of brain stem reflexes, apnea test, and confirmatory exam with absence of cerebral blood flow, according to Brazilian law[22]. From June 2013 to June 2015, brain-dead patients older than 18 years admitted to intensive care unit (ICU) were prospectively included in the study after the first clinical exam

consistent with BD. Blood samples were collected at the study entrance and every 6 hours thereafter, comprising a total of three samples by patient. Control subjects were defined as critically ill patients without a suspected diagnosis of BD admitted to the same ICU of the cases. For each brain-dead patient, two control patients were included – the first septic patient to the right and the first non-septic patient to the left side of the case. Sepsis was defined as the presence of infection and two or more signs of systemic manifestation of infection[23]. Control patients had blood samples collected once, at the time of study entry. Clinical and laboratory data were recorded from brain dead and control patients.

Plasma cytokine quantifications

Blood samples were immediately centrifuged at 1260 g for 10 min at 4ºC and plasma was stored at -80ºC until analysis. All samples were analyzed at the same time after being thawed at room temperature and centrifuged at 1000 g for 10 min. Plasma levels of TNF-α, IL-1β, IL-6, IL-8, interleukin-10 (IL-10) and interferon-γ (INF-γ) were assessed by magnetic bead assay using the Human Magnetic Custom Luminex® Kit (Invitrogen Life Technologies, Carlsbad, USA) and the Luminex® 200™ magnetic bead plate reader (Luminex, Austin, USA) following the manufacturer's recommendations. Standard curve was generated by serially diluting the reconstituted standard. Samples and standards were incubated with mixed beads overnight at room temperature on an orbital shaker. Beads were washed and then incubated with a detection antibody at room temperature for 1 h and with streptavidin for 30 min. Beads were washed and resuspended and the plate was subsequently analyzed on the Luminex® 200™.

Results are reported as a function of fluorescence intensity. Mean fluorescence intensity (MFI) takes into account the number of fluorescing pixels within the scanned area. Values of MFI under the detection limit were assumed to be equal to the lower detected result (0.91 pg/mL for TNF-α, 1.93 pg/mL for IL-1β, 0.36 pg/mL for IL-6, 2.87 pg/mL for IL-8, 1.17 pg/mL for IL-10 and 0.48 pg/mL for INF-γ). MFI was then transformed to pg/mL based on the standard curve. All samples were analyzed in duplicates.

Statistical Analysis

Categorical variables are presented as percentages. Variables with normal distribution are presented as means ± SD. Variables with skewed distribution are presented as median and interquartile intervals. Groups were compared using one-way ANOVA with Tukey *post-hoc* test, Kruskal-Wallis or Chi-square as appropriate. Plasma levels of cytokines from brain-dead patients were compared at different times points using generalized linear models (GLM). The sample size of this study was calculated considering a power of 80% and an α-error of 5% to find a difference of at least one SD in TNF-α log[18]. Values were considered statistically significant if $P<0.05$. Statistical analyses were performed using SPSS 18.0 (Chicago, IL).

Results

Sixteen brain-dead patients and 32 control patients (16 septic and 16 non-septic) were included in the study and the baseline characteristics of patients are

presented in Table 1. Stroke was the leading cause of BD (62.5%), followed by anoxic encephalopathy (12.5%). Main sources of infection of septic patients were the lung (40.0%) and the abdomen (26.6%). Non-septic patients were admitted to the ICU due to pulmonary embolism (20.0%), hemorrhagic stroke (20.0%), post-cardiac arrest (13.3%), elective cardiac surgery (13.3%) or other causes (33.4%). Age, gender, body mass index and illness severity, assessed by APACHE II score, did not differ significantly between groups. Length of ICU stay and time spent on mechanical ventilation were similar among groups. As expected, brain-dead and septic patients required more vasopressor support than non-septic patients, and hypernatremia and hypothermia were more common in brain-dead patients than in controls.

BD induces inflammation similar to that observed in sepsis

First we examined the plasmatic cytokine profile of brain-dead and control patients obtained at study entrance (1.4 ± 1.3 hours after the first clinical exam consistent with BD) and data are presented in Figure 1. Levels of IL-8 were significantly higher in brain-dead and septic patients as compared to non-septic patients [80.3 (18.7-169.6) vs. 68.2 (22.4-359.4) vs. 16.4 (9.2-42.7) pg/mL; P=0.006]. Similar findings were obtained with INF-γ levels, which were also higher in brain-dead and septic group than in non-septic control group [2.8 (1.6-6.1) vs. 3.4 (1.2-9.0) vs. 0.5 (0.5-1.8) pg/mL; P=0.012]. Regarding IL-6, plasma levels were upregulated in brain-dead patients as compared to non-septic control patients [174.5 (104.9-692.5) vs. 13.2 (7.3-38.6) pg/mL; P=0.002], but not compared to septic patients [174.5 (104.9-692.5) vs. 134.7 (7.2-413.6) pg/mL;

P=0.524]. Conversely, there were no differences in IL-1β levels [5.4 (1.9-16.9) vs. 3.1 (1.9-15.0) vs. 1.9 (1.9-4.0) pg/mL; P=0.271] and IL-10 levels [8.8 (3.6-16.5) vs. 8.5 (4.3-22.9) vs. 3.0 (1.2-16.1) pg/mL; P=0.185] between BD, septic and non-septic patients. TNF-α has a clear tendency to increase in brain-dead patients as compared to other groups [2.7 (1.0-4.8) vs. 1.0 (1.0-5.6) vs. 1.0 (1.0-1.0) pg/mL; P=0.051].

Influence of superimposed sepsis in cytokines levels of BD subjects

Three brain-dead patients (18.7%) had sepsis, all from pulmonary origin. In order to remove this possible confounder, we excluded septic patients from the analysis. IL-6 levels remained significantly higher in brain-dead patients than in non-septic control patients (P=0.003), INF-γ levels continued to be higher in brain-dead and septic patients than in non-septic (P=0.011) and there were no differences in IL-1β (P=0.281) and IL-10 (P=0.213) levels. However, IL-8 levels turned out to be different only between brain-dead and non-septic patients (P= 0.008). Remarkably, TNF-α levels became significantly higher in brain-dead group compared to non-septic group (P=0.025).

Plasma cytokine kinetics overtime in BD subjects

Next we characterized the time course of cytokines during BD and showed that cytokine plasma levels varied overtime (Table 2). Levels of IL-1β and IL-10 increased from baseline (1.4 ± 1.3 hours; first clinical exam consistent with BD; n = 16) to the second time point (7 ± 1.2 hours; second clinical exam consistent

with BD; n = 15), but no statistical differences were observed in the third time point (14.7 ± 2.1 hours; time of organ retrieval; n = 6). INF-γ showed a non-significant increase at post-hoc analysis from time point two to time point three, probably due to the reduced sample size (P=1.0).

As cytokines varied during the period of observation, we therefore compared cytokine plasma levels from control subjects with cytokine plasma levels from BD patients at the second-time point. The results demonstrate that IL-8 and INF-γ plasma levels were significantly higher in brain-dead and septic patients than in non-septic control subjects [IL-8: 68.2 (39.8-222.4) vs. 68.2 (22.4-359.4) vs. 16.4 (9.2-42.7) pg/mL; P=0.003 and INF-γ: 4.0 (1.8-14.3) vs. 3.4 (1.2-9.0) vs. 0.5 (0.5-1.8) pg/mL; P=0.001], similar to the results obtained from baseline analysis. IL-6 also expressed the same pattern from the results obtained at baseline, with higher levels in BD patients at the second time point in comparison with non-septic control subjects [223.7 (142.8-2664.9) vs. 13.2 (7.3-38.6) pg/mL; P=0.001], but both groups were not different from septic patients. Notably, IL-10 and TNF-α, that were not statistically different at baseline, turned out to be higher in brain-dead patients as compared to non-septic control patients at the second time point [IL-10: 15.7 (5.5-24.0) vs. 3.0 (1.2-16.1) pg/mL; P=0.047 and TNF-α: 3.3 (1.0-6.6) vs. 1.0 (1.0-1.0) pg/mL; P=0.01]. IL-1β plasma levels were similar among groups regardless the time point.

Discussion

We presently show that BD is associated with higher systemic inflammation than that induced by critical illness. Interestingly, cytokine levels in

BD were similar to the levels observed in sepsis. This fact is evidenced by the upregulation of IL-6, IL-8, IL-10, INF-γ and TNF-α in brain-dead patients. In addition, we demonstrated an elevation in IL-1β and IL-10 levels during the course of BD diagnosis.

Inflammation secondary to BD is partially responsible for primary graft dysfunction and is induced by the innate and adaptive immune systems[24]. Cytokines participate in both responses and are released in reaction to injury and infection and orchestrate the inflammatory response acting in immune cell differentiation, proliferation and activity. While TNF-α, IL-1β and INF-γ are pro-inflammatory mediators, upregulated early in the inflammatory cascade and with short half-life, IL-6 has both pro and anti-inflammatory effects and a longer half-life and IL-10 has anti-inflammatory properties. Besides, IL-8 is a chemokine with pro-inflammatory function[25]. One proposed mechanism of inflammation in BD is the release of cytokines by monocytes and macrophages stimulated by cell lysis in extensive brain cortical necrosis[26]. In stroke patients, IL-6 correlates with the extension of the ischemic brain lesion[27]. Furthermore, loss of anti-inflammatory response mediated by parasympathetic nervous system due to ischemia of the vagal nucleus in the brain stem is another mechanism implicated in cytokine upregulation after BD[28].

Increased levels of IL-6 have been consistently found in brain-dead patients[6,8,18,29-31]. Our findings corroborate with the presence of a systemic inflammation in BD and clarify the cytokine profile and kinetics during BD. Interestingly, we identified not only increased plasmatic levels of IL-6, but also increased levels of IL-8, INF-γ, IL-10 and TNF-α in brain-dead patients compared to critically ill subjects without sepsis. IL-10 and TNF-α were elevated at the

second time point, which was around the time of BD diagnosis confirmation, in accordance of the period when TNF-α was upregulated in our previous study[18]. By contrast, another study demonstrated higher serum and tissue levels of IL-6 and IL-10 from brain-dead donors as compared to living donors at the time of laparotomy for liver harvest, a later time point than in our study[30].

Studies that performed cytokine time courses showed increased levels of cytokines at the time of BD diagnosis[13,24,29,32]. In humans, a sequential evaluation of plasmatic levels of IL-6 in patients with severe head injury showed levels above the normal range at admission, but patients developing BD had a markedly increase of IL-6 at the time of BD confirmation compared to those who did not[29]. Corroborating the hypothesis that inflammatory mediators peak before organ retrieval, Lopau *et al* showed increased interleukin-2 (IL-2) and TNF-α soluble receptors and IL-6 plasmatic levels at the time of BD diagnosis as compared to controls submitted to neurosurgical procedures, but a decrease in these levels after six hours[32]. In our study, levels of cytokines increased from first to second time point, which was approximately the moment of BD diagnosis confirmation. Different from the animal studies, in most cases we are not able to precisely define the time of BD installation. We also had a third time point measurement, at the time of organ retrieval, but no significant increase in cytokines was observed, probably due to small sample size at this point, as only patients that became effective organ donors were at the ICU at this time.

Other acute critical illness, such as trauma[21], hemorrhagic shock[33] and cardiac arrest[34], may present as a systemic inflammatory response that contributes to the multiple organ dysfunction syndrome. Adrie *et al* studied the inflammatory response of 61 successfully resuscitated out-of-hospital cardiac

arrest patients and compared with septic patients and healthy controls. Plasma levels of IL-6, IL-8 and IL-10 were elevated at admission, with levels as high as in patients with severe sepsis. Besides, the use of vasopressor was associated with increased cytokine levels in non-survivors[35]. Moreover, mechanical ventilation has shown to trigger a pulmonary and systemic inflammation with enhanced levels of TNF-α, IL-1β and IL-6 in plasma and in bronchoalveolar lavage, even in normal lungs [36-40]. In our study, cytokines, with exception of IL-1β, were higher in brain-dead patients than in controls subjects without sepsis, even though, non-septic control patients were not different from brain-dead patients in occurrence of cardiac arrest, disease severity as assessed by APACHE II score, or by the time spent on mechanical ventilation; but, as expected, brain-dead patients were more frequently on vasopressor support. Taken together, these findings suggest that systemic inflammatory response in BD is triggered mainly by BD itself and not by other injuries that develop during critical illness.

Sepsis is a clinical syndrome classically associated with increased inflammation, with both pro-inflammatory and anti-inflammatory cytokines upregulation[41,42]. Increased cytokine release is partially responsible for the clinical manifestations of sepsis and for organ dysfunction[43]. In addition, TNF-α, IL-6 and IL-10 are associated with organ failure and mortality[44-47]. Notably, the levels of inflammation of BD patients in our study were similar to the levels of inflammation of septic patients. Moreover, even when we exclude BD patients with sepsis from the analysis, the resemblance remains.

The importance of inflammation in transplantation outcomes has been demonstrated in a recent study from our group[48]. In an experimental model of

BD, rats treated with exendin-4, a *Glucagon like peptide- 1* (GLP-1) analogue that has anti-inflammatory properties, had reduced of inflammation and higher viability of pancreatic islets as compared to brain-dead control animals[48]. In this study, gene expression of IL-1β was elevated in pancreas brain-dead rats, which decreased with exendin-4 treatment. Though, therapies to reduce inflammation in organ donors, such as pro-inflammatory cytokine blockade, seem to be a promising intervention on donors in order to ameliorate recipient outcomes.

This is the first study that compares plasmatic levels of cytokines from brain-dead patients and critically ill patients, aiming to evaluate the role of sepsis and other intensive care situations on the level of inflammation in BD. The strengths of our study are the exclusion of septic brain-dead patients from the analysis, eliminating a major confounder and the collection of samples at first BD clinical examination and so, before BD confirmation. This approach allowed us to describe cytokine kinetics from a precocious period when we believed the inflammatory response would be pronounced, until organ retrieval or circulatory arrest, when cytokines levels may be decreasing.

This study has some limitations. First, our sample size was based on TNF-α values from a previous study[18] and may be underpowered to detect smaller differences. Second, the significant loss of patient's follow-up at third time point (due to the fact that only few patients became effective organ donors) has limited a better evaluation of cytokines kinetics later after BD diagnosis. In line with this, a time course with more time points, such as one hour and two hours in addition to six and twelve hours, might have demonstrated more consistently the cytokine behavior.

In conclusion, our data suggest that BD-induced systemic inflammation is as high as that induced by sepsis and both BD and sepsis are associated with higher levels of inflammation than that observed in critical illness. These findings corroborate the hypothesis that BD itself triggers inflammatory cascade and prompt clinical evaluation of therapeutic approaches to attenuate this response and improve graft outcomes.

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Table1. Baseline characteristics of brain-dead patients and controls.

	Brain-dead (n=16)	Septic controls (n=16)	Non-septic controls (n=16)	P
Age, (yr)	55 ± 9	48 ± 17	52 ± 21	0.515
Male gender, n (%)	6 (37.5)	8 (50)	5 (31.3)	0.543
APACHE II score	21.8 ± 8	23.9 ± 8	19.8 ± 10	0.481
BMI (kg/m^2) †	24.6 ± 2	26.9 ± 8	29.5 ± 8	0.192
Sepsis, n (%)	3 (18.7)	16 (100)	0	
Time from ICU admission, (days)	2 (1-7.7)	4 (1-10)	4 (2-10)	0.762
Ventilation support, (days)	3.5 (1-7)	2.5 (1-7)	1 (0-10)	0.388
Vasopressor support, n (%)	11 (68.8)	9 (56.3)	4 (25)	0.039§
Episode of cardiac arrest, n (%)	4 (25)	0	3 (18.8)	0.132
Use of steroids, n (%)	6 (37.5)	9 (56.3)	3 (18.8)	0.091
Plasma sodium (mEq/L)	152 ± 6.7	140 ± 6.5	142 ± 4.3	< 0.001¶
Body temperature (Cº)	35 (34.4-36.1)	36.1 (35.1-36.7)	36.2 (36-36.6)	0.031*
Hemoglobin (g/dL)	10 ± 2.2	9 ± 2	8.9 ± 1,8	0.291
White blood count (per mm^3 x1000)	11.8 (7-15)	12.3 (6.5-17.6)	9.8 (7-14.9)	0.916
Creatinine (mg/dL)	1.13 (0.8-1.5)	1.23 (0.6-1.7)	0.76 (0.5-1.7)	0.596

§ Non-septic control patients are different from brain-dead and septic control patients.
¶ Brain-dead and septic control patients and brain-dead and non-septic control patients are different.
* Brain-dead and non-septic control patients are different.
† BMI: body mass index.

Table 2. Plasma cytokines kinetics in brain-dead patients.

	Time 1 (n=16)	Time 2 (n=15)	Time 3 (n=6)	P
TNF-α (pg/mL)	2.70 (0.99-4.76)	3.28 (0.99-6.59)	0.99 (0.99-5.95)	0.278
IL-1β (pg/mL)	5.39 (1.93-16.89)	7.11 (1.93-29.13)	6.48 (1.93-19.21)	0.012[§]
INF-γ (pg/mL)	2.79 (1.6-6.06)	2.79 (1.6-6.06)	4.04 (0.47-8.57)	0.045
IL-6 (pg/mL)	174.48 (104.9-692.51)	223.74 (142.79-2664.93)	395.77 (22.93-5594.31)	0.096
IL-8 (pg/mL)	80.3 (18.72-169.61)	68.24 (39.84-222.37)	90.71 (26.43-241.29)	0.391
IL-10 (pg/mL)	8.78 (3.62-16.49)	15.73 (5.49-23.98)	11.1 (1.17-15.23)	0.009[§]

[§] Time 1 and time 2 are different

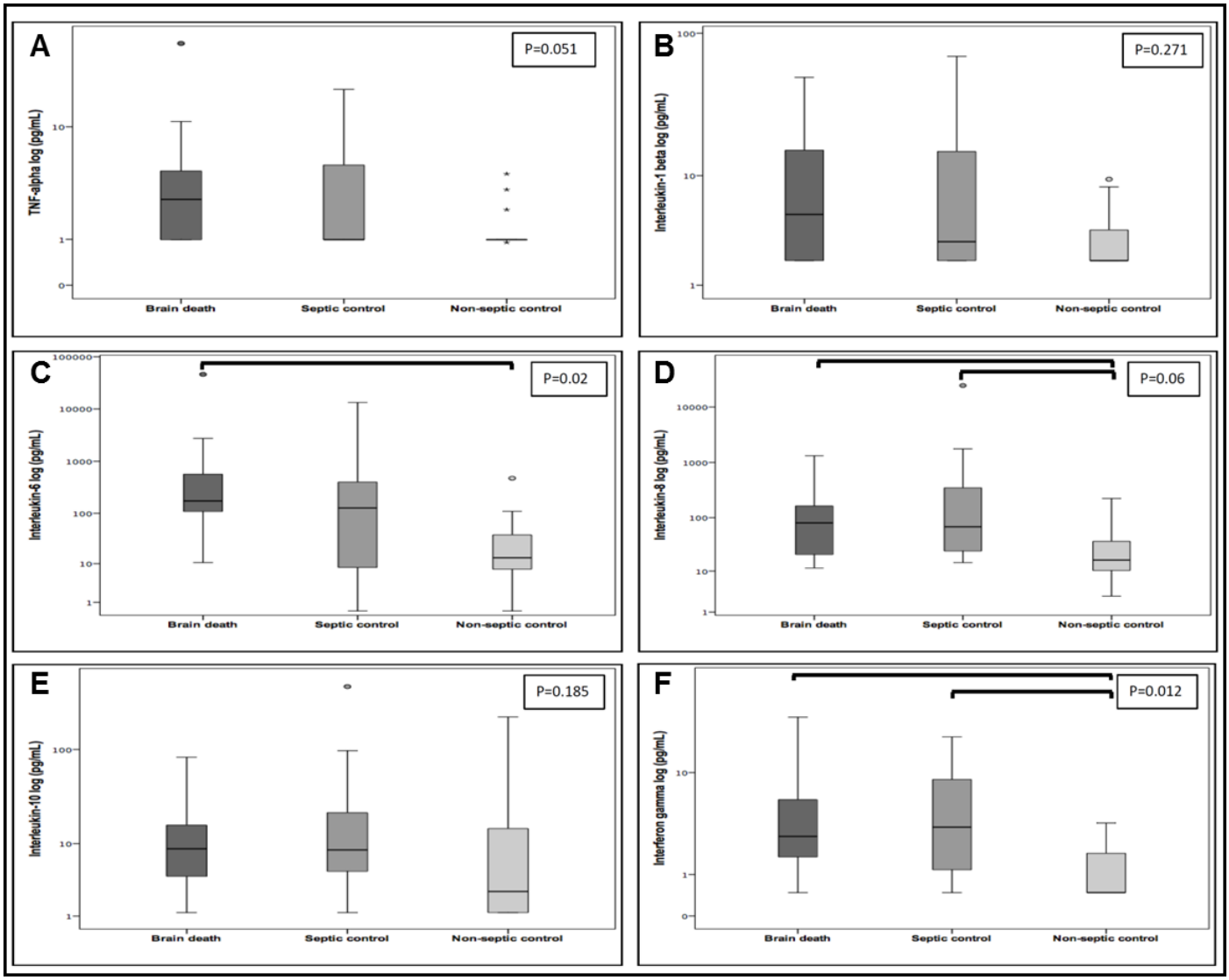

Figure 1. Plasma levels of cytokine determined by magnetic bead assay in brain-dead patients and controls. A. Tumor necrosis factor-α (pg/mL). B. Interleukin-1β (pg/mL). C. Interleukin-6 (pg/mL). D. Interleukin-8 (pg/mL). E. Interleukin-10 (pg/mL). F. Interferon-γ (pg/mL). Kruskal-Wallis with pairwise comparison. Significant statistical differences as indicated by the bars (IL-6: P=0.01 for BD vs. non-septic controls; IL-8: P=0.029 for BD vs. non-septic controls and P=0.01 for septic vs. non-septic controls; INF-γ: P=0.028 for BD vs. non-septic controls and P=0.031 for septic vs. non-septic controls). Graphs are presented in logarithmic scale, representing median and interquartile interval. Dots and asterisks represent outliers.

CAPÍTULO 3

Considerações finais e perspectivas futuras

No mundo todo, o transplante de órgãos sólidos é limitado pelo desbalanço entre a oferta de órgãos e a demanda de pacientes em lista de espera[1]. A principal fonte de órgãos para transplante são os doadores em ME[2]. Entretanto, órgãos provenientes de doadores em ME apresentam piores desfechos quando comparados aos órgãos de doadores vivos, o que se atribui, pelo menos parcialmente, à inflamação sistêmica induzida pela ME[3]. São necessárias, portanto, estratégias para minimizar os efeitos desta resposta inflamatória e otimizar o número de doações. Protocolos para o cuidado de potenciais doadores de órgãos têm sido desenvolvidos e resultaram em menores perdas de doadores por parada cardíaca[4], além de aumentar a quantidade e a qualidade dos órgãos disponíveis[5,6]. Esses protocolos, até o momento, não objetivam o tratamento específico da inflamação, apesar de alguns indicarem o uso de corticosteroides, que têm propriedades anti-inflamatórias.

Em um estudo de Kuecuek *et al*, a expressão sérica e tecidual de IL-6 e IL-10 reduziu com o uso de corticosteroide antes da cirurgia para retirada de órgãos a níveis comparáveis aos dos doadores vivos[7]. Um ensaio clínico randomizado mostrou que o uso de metilprednisolona antes da cirurgia para retirada de órgãos reduziu a taxa de rejeição nos primeiros seis meses após o transplante de fígado[8]. Outras terapias têm sido propostas para reduzir a inflamação e melhorar os desfechos nos órgãos transplantados, por meio do bloqueio de citocinas pró-inflamatórias, como N-acetilcisteína, eritropoietina,

hemadsorção e transferência de genes das citocinas, com resultados incipientes[9].

Uma classe de drogas com potencial benefício para reduzir a resposta inflamatória induzida pela ME são os antagonistas do receptor de TNF-α, que bloqueiam mecanismos apoptóticos mediados pela ativação do fator nuclear- κB e que já mostraram melhorar desfechos no transplante de ilhotas pancreáticas quando utilizados no protocolo de imunossupressão[10,11].

Consideramos que a exenatida possa ser uma droga promissora para ser utilizada neste contexto, tendo em vista os resultados já obtidos em estudos experimentais, como redução da produção de citocinas e ativação de cinases das vias de sinalização intracelular pró-proliferativas e antiapoptóticas em ilhotas pancreáticas humanas isoladas[12]. Um estudo do nosso grupo de pesquisa demonstrou a atenuação da inflamação em modelo animal de ME tratado com exenatida em comparação ao grupo não tratado, com melhora da viabilidade das células beta pancreáticas[13], redução da apoptose de hepatócitos[14] e reversão da lesão renal (dados não publicados). Desta forma, pretendemos testar por meio de um ensaio clínico randomizado em doadores em ME os efeitos da exenatida em atenuar os efeitos inflamatórios da ME em biópsias de órgãos captados para transplante e a sua capacidade de reduzir a ocorrência de disfunção primária desses enxertos.

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