Universidade Federal do Rio Grande do Sul

Faculdade de Medicina

Programa de Pós-Graduação em Ciências Médicas: Endocrinologia

AVANÇOS NO TRANSPLANTE HEPÁTICO: DA REDUÇÃO DA INFLAMAÇÃO DO DOADOR COM A UTILIZAÇÃO DE ANÁLOGOS DO GLP-1 À AVALIAÇÃO DO IMPACTO DA DURAÇÃO DA HEPATECTOMIA

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Porto Alegre, agosto de 2023

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Esta tese de Doutorado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Metabolismo e Nutrição da Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de uma breve introdução sobre o assunto, seguida de dois manuscritos originais sobre o tema da tese.

Artigo 1: Does liraglutide alleviates inflammation in brain-dead donors? A randomized clinical trial

Artigo 2: Association of donor hepatectomy time with liver transplantation outcomes: a retrospective study

SUMÁRIO

ÍNDICE DE ABREVIATURAS

1. INTRODUÇÃO E PERSPECTIVAS FUTURAS

BCL2: antiapoptotic B cell lymphoma 2

HSPA5/BIP: heat shock protein family A

DDIT3/CHOP: DNA damage inducible transcript 3

DM2: diabetes melito tipo 2

DPE: disfunção primária do enxerto

DPP-4: dipeptidil peptidase-4

ELISA: enzyme-linked immunosorbent assay

GLP-1: glucagon-like peptide-1

HLA: antígeno leucocitário humano

IFN-γ: interferon-γ

IL-1β: interleucina-1β

IL-6: interleucina-6

IL-8: interleucina-8

IL-10: interleucina-10

ME: morte encefálica

RNA seq: sequenciamento de RNA

SOD2: superoxide dismutase 2

RT-qPCR: quantitative real-time polymerase chain reaction

TNF: fator de necrose tumoral

UCP2: uncoupling protein 2

2. ARTIGOS

BMI: body mass index

BCL2: antiapoptotic B cell lymphoma 2

BD: brain death

HSPA5/BIP: heat shock protein family A

DDIT3/CHOP: DNA damage inducible transcript 3

DAB: diaminobenzidine

DRI: donor risk index

EAD: early allograft dysfunction

ELISA: enzyme-linked immunosorbent assay

GLP-1: glucagon like peptide-1

IFN-γ: interferon-γ

IL-1β: interleukin-1β

IL-6: interleukin-6

IL-10: interleukin-10

ICU: intensive care unit

INR: international normalized ratio

NF-κB: nuclear factor Kappa B

OR: odds ratio

RR: relative risk

RT-qPCR: quantitative real-time polymerase chain reaction

SAPS 3: Simplified Acute Physiology score 3

SD: standard deviation

SOD2: superoxide dismutase 2

STAT3: signal transducers and activators of transcription III

T1: time point 1

T2: time point 2

TNF: tumor necrosis factor

UCP2: uncoupling protein 2

YWHAZ: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

Δ: delta value

95% CI: 95% confidence interval

RESUMO

Ainda hoje a principal fonte de órgãos para transplante é o doador de órgãos em morte encefálica (ME). Durante todo o processo de retirada, estocagem e transplante, os órgãos são submetidos a múltiplos agravos, entre eles: a isquemia fria, as mudanças súbitas de temperatura, o estresse oxidativo e as forças de cisalhamento que atuam sobre o órgão, resultando em lesão tecidual e redução da função e da sobrevida dos enxertos.

Muitos estudos demonstraram que os análogos do *glucagon-like peptide-1* (GLP-1), como a exenatida e a liraglutida, possuem propriedades anti-inflamatórias, pró-proliferativas e antiapoptóticas. Um estudo prévio do nosso grupo demonstrou que a administração de exenatida em ratos submetidos à ME foi capaz de aumentar a viabilidade das ilhotas pancreáticas. Esses efeitos foram atribuídos às modificações na expressão de genes relacionados a estresse oxidativo, a estresse do retículo endoplasmático e à inflamação. Outro estudo mostrou que a administração de exenatida em ratos em ME reduziu marcadores relacionados a dano hepático e à apoptose dos hepatócitos. Com base nesses estudos experimentais, hipotetizamos que a administração de liraglutida a doadores de órgãos em ME poderia melhorar a qualidade dos órgãos para transplante em seres humanos.

Na presente tese, avaliamos por meio de um ensaio clínico randomizado se o tratamento com liraglutida em indivíduos em ME poderia diminuir os danos aos órgãos a serem transplantados quando comparado ao tratamento com placebo. Cinquenta indivíduos doadores em ME foram incluídos no estudo. Nossos resultados mostraram que o uso de liraglutida reduz os níveis de interleucina-6 e de interleucina-10 em doadores em ME, mas não reduz outras citocinas inflamatórias. Além disso, a expressão de genes e de proteínas relacionadas à inflamação, à apoptose e ao estresse oxidativo não foi afetada pelo tratamento com liraglutida.

Também como parte desta tese, estudamos, retrospectivamente, o efeito da duração da hepatectomia do doador nos desfechos dos transplantes hepáticos nos receptores. Foram incluídos 243 pacientes submetidos a transplante hepático de doadores falecidos, cujos dados foram cruzados com os respectivos receptores dos órgãos. Nossos resultados mostraram tempos curtos de hepatectomia, não apresentando associação com o desenvolvimento de disfunção primária do enxerto.

ABSTRACT

The main source of organs for transplantation is the brain-dead organ donor. During the role process of transplantation, from organ recovery and storage to implant, the organs are exposed to multiple injuries, including: cold ischemia, sudden changes in temperature, oxidative stress, and shear forces, resulting in tissue injury, graft dysfunction, and reduced survival.

Many studies have demonstrated that glucagon-like peptide-1 (GLP-1) analogues, such as exenatide and liraglutide, have anti-inflammatory, pro-proliferative and anti-apoptotic properties. A previous study by our group demonstrated that the administration of exenatide to rats in brain death (BD) increased the viability of pancreatic islets. These effects were attributed to changes in the expression of genes related to oxidative stress, endoplasmic reticulum stress, and inflammation. Another study showed that the administration of exenatide to rats in BD reduced markers of liver damage and attenuated hepatocyte apoptosis. Based on these experimental studies, we hypothesized that administration of liraglutide to brain-dead donors would improve the quality of organs for human transplantation.

In this thesis, we evaluated, through a randomized clinical trial, whether treatment with liraglutide in brain-dead individuals would reduce the damage to the organs to be transplanted compared to treatment with placebo. Fifty brain-dead donors were included in the study. Our results showed that the use of liraglutide reduces interleukin-6 and interleukin-10 plasma levels in brain-dead donors, but does not reduce other inflammatory cytokines. Furthermore, the expression of genes and proteins related to inflammation, apoptosis, and oxidative stress was not affected by liraglutide treatment.

Also as part of this thesis, we conducted a retrospective investigation regarding the impact of donor hepatectomy duration on the outcomes of liver transplantation in recipients. Our study included a cohort of 243 patients who underwent liver transplantation from deceased donors, and we cross-referenced their data with that of the respective organ recipients. Our findings revealed no significant association between the duration of donor hepatectomy and the development of primary allograft dysfunction.

1. INTRODUÇÃO

O transplante de órgãos é considerado o tratamento de eleição para várias doenças terminais que afetam rins, pâncreas, fígado, coração e pulmões (1). Atualmente, a principal fonte de órgãos para transplante no Brasil é o doador de órgãos em morte encefálica (ME) (2). Aproximadamente 50% dos órgãos doados não são transplantados e o número de pacientes em lista de espera supera, em muito, o número de doadores de órgãos disponíveis (1, 3).

Durante todo o processo de retirada, estocagem e transplante, os órgãos são submetidos a múltiplos agravos, entre eles: a isquemia fria, as mudanças súbitas de temperatura, o estresse oxidativo e as forças de cisalhamento (4). A esses agravos soma-se o estresse inflamatório produzido pela própria ME, resultando em lesão tecidual e redução da função e da sobrevida dos enxertos (5, 6).

1.1 A morte encefálica

A ME constitui-se de uma síndrome inflamatória, com graves efeitos adversos sobre os desfechos dos transplantes. A influência da ME sobre os órgãos captados foi inicialmente estudada no rim. Em modelo animal, enxertos renais oriundos de ratos falecidos apresentam um curso mais acelerado de rejeição crônica do que os enxertos renais de ratos vivos (7). Da mesma forma, biópsias renais de doadores em ME têm níveis significativamente mais elevados de citocinas 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 antígeno leucocitário humano (HLA)-não relacionados apresentam uma sobrevida maior do que rins de doadores em ME, conforme evidenciado num estudo da década de 1990 com 368 transplantes entre cônjuges (10).

Órgãos provenientes de qualquer doador vivo, relacionado ou não relacionado, demonstram resultados consistentemente superiores quando comparados aos de doadores em ME. Uma lesão cerebral catastrófica leva à ME e essa desencadeia uma série de alterações hemodinâmicas, neuro-humorais e imunológicas que afetam a qualidade dos órgãos a serem transplantados (11-13). 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 (13, 14). Os efeitos deletérios da própria ME na função dos órgãos transplantados tem sido cada vez mais reconhecidos ao longo dos últimos anos. Experimentalmente, ela tem sido associada ao aumento do risco de rejeição aguda e crônica (15).

1.2 A atividade inflamatória da morte encefálica

Modelos experimentais de traumatismo crânio encefálico, antes mesmo do desenvolvimento da ME, tem demonstrado a expressão de diversos mediadores inflamatórios no tecido cerebral, alguns deles com pico mil vezes maior do que os níveis normais (16, 17). Estudos prévios, tanto em modelos experimentais com suínos quanto em humanos, identificaram aumento de mediadores inflamatórios como fator de necrose tumoral (TNF) e interleucina-6 (IL-6) na circulação sanguínea após o início da ME (18-20).

O gatilho inflamatório que afeta adversamente a função dos órgãos transplantados de uma maneira antígeno-independente foi documentado por Kusaka et al., que quantificaram a expressão de TNF, interleucina-1 β (IL-1β) e IL-6 em cobaias submetidas

à ME ou somente à ventilação mecânica. Após cinco dias, ocorreu uma densa infiltração dessas citocinas nos túbulos e glomérulos renais dos ratos em ME (21).

Uma análise de biópsias hepáticas antes da cirurgia de retirada 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, sugerindo que os eventos que permeiam a ME e os cuidados intensivos são importantes fatores de risco no desenvolvimento de disfunção primária do enxerto (DPE) de órgãos transplantados de doadores em ME (22).

Alguns estudos apresentaram aumento de interleucina-8 (IL-8) no lavado broncoalveolar e no tecido pulmonar de pacientes em ME, estando associado a DPE nos receptores transplantados (23, 24). Walwell et al. demostraram em modelo ovino de ME, um aumento da resposta pró-inflamatória, caracterizado por aumento da infiltração de neutrófilos e produção de citocinas na circulação e no lavado broncoalveolar, sendo que efeitos deletérios ocorreram nas funções endoteliais, pulmonares e cardíacas (11).

Dosagens miocárdicas de TNF feitas por RT-qPCR imediatamente antes do transplante foram capazes de predizer a ocorrência de disfunção ventricular direita em 26 receptores após transplante cardíaco (25).

Contreras et al. demonstraram, por sua vez, que a ativação de citocinas pró-inflamatórias tem um impacto importante na função das células β pancreáticas de uma maneira tempo-dependente. Em ratos, a precipitação da ME é seguida de um aumento imediato das concentrações de TNF, IL-1β e IL-6, que induzem à disfunção e à morte da célula beta, principalmente por apoptose (26). Há evidências também da implicação do aumento da interleucina-10 (IL-10) e do interferon-γ (INF-γ) na apoptose de ilhotas nesse cenário (27, 28). Um estudo do nosso grupo de pesquisa do Hospital de Clínicas de Porto Alegre mostrou aumento de TNF e IL-6 no plasma e de TNF no tecido pancreático de

doadores em ME quando comparados a controles submetidos à cirurgia de pancreatectomia (Figura 1) (18) . Mais recentemente, demonstramos que a ME induz inflamação a níveis mais elevados do que a doença crítica e a níveis similares à sepse (29).

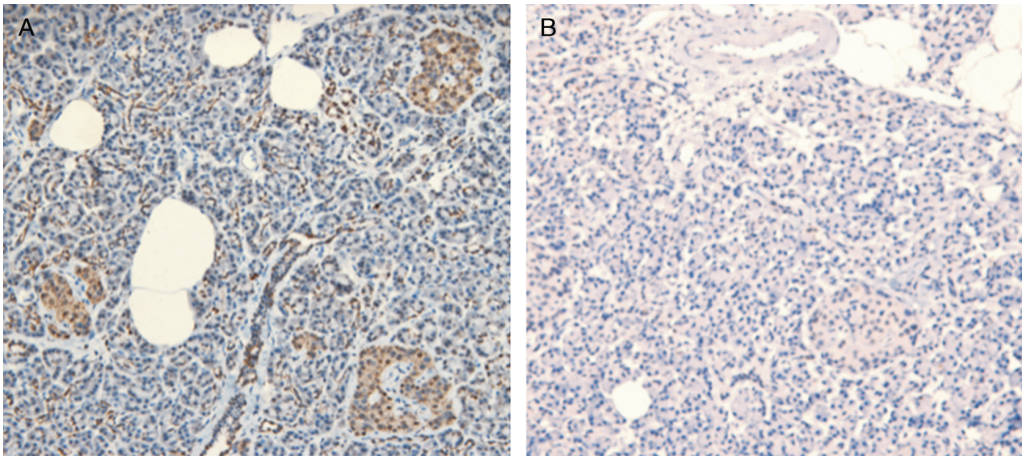

Figura 1. Imunohistoquímica de tecido pancreático humano mostrando ilhotas de Langerhans marcadas com anticorpo policlonal anti-TNF de doador em morte encefálica (A) e de paciente controle (B). Imagem retirada de Rech et al (18).

Há muitas evidências da associação da ME e inflamação, afetando os desfechos dos órgãos transplantados, porém, de uma maneira não completamente compreendida. Estudos observacionais sugerem que o uso de terapias guiadas por metas tenha um feito benéfico em reduzir a taxa de perda de doadores por parada cardíaca e aumentar a taxa de órgãos captados por doador (30, 31), o que será testado por meio do ensaio clínico randomizado denominado DONORS, um grande consórcio multicêntrico brasileiro financiado pelo Ministério da Saúde que visa otimizar a captação de órgãos no Brasil (dados submetidos para publicação). Porém, nenhum estudo até o momento testou em seres humanos e de forma direta o efeito de medicamento com propriedades anti-inflamatórias (exceto corticosteroides) e antiapoptóticas administradas ao doador em ME

em reduzir a inflamação dos órgãos a serem transplantados ou o seu efeito em aumentar a taxa de captação de órgãos por doador.

1.3 Os análogos do GLP-1

O peptídeo-1 semelhante ao glucagon (GLP-1, do inglês, *Glucagon Like Peptide-1*) é um hormônio intestinal que exerce primariamente ações anti-hiperglicemiantes. O GLP-1 está associado a diversos efeitos pleiotrópicos, tais como: redução do esvaziamento gástrico, aumento da saciedade, estimulo à secreção de insulina pela célula-β pancreática, além de desempenhar ações neuroprotetoras, cardioprotetoras, nefroprotetoras e anti-inflamatórias (32-36) (Figura 2). Os incretinomiméticos são agonistas do receptor do GLP-1, conhecidos como incretinomiméticos de curta duração ou análogos do GLP-1 resistentes à inativação enzimática da dipeptidil-peptidase-4 (DPP-4). Existem cinco representantes desta classe dos incretinomiméticos, usados amplamente no tratamento clínico do diabetes melito tipo 2 (DM2): exenatida, liraglutida, lixenatida, dulaglutida e albiglutida, todos de uso por via subcutânea (33, 37).

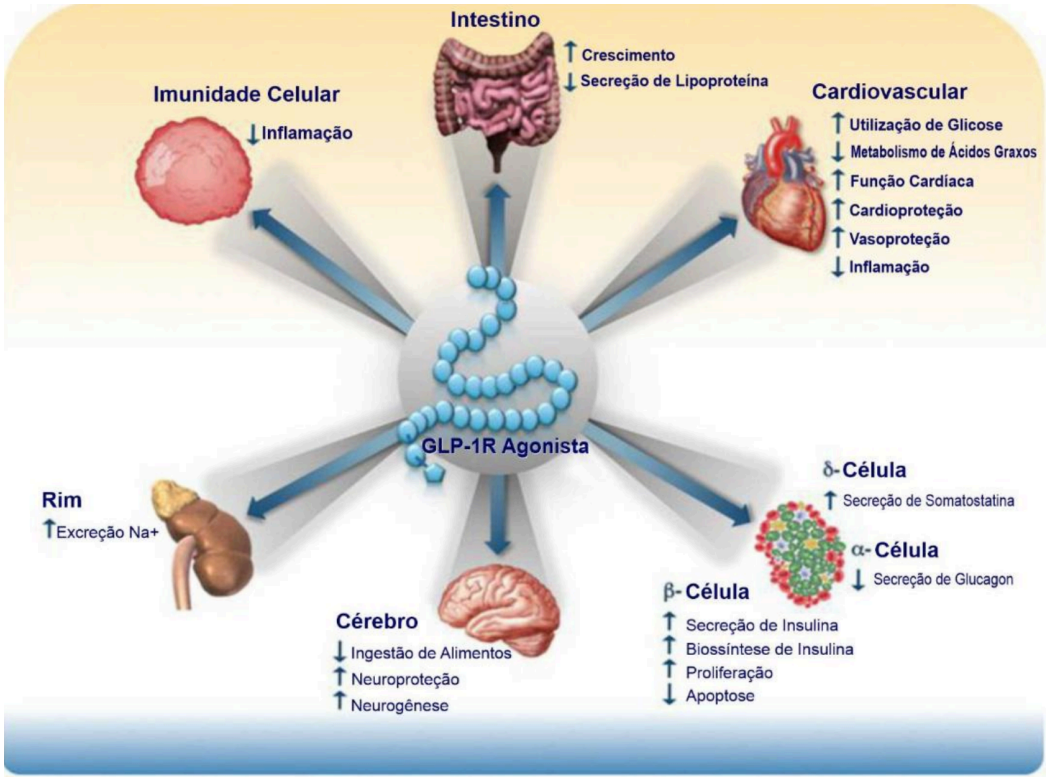

Figura 2. Representação da atuação do *glucagon-like peptide-1* (GLP-1) em diversos tecidos. Imagem adaptada de Campbell (38).

Com o intuito de minimizar os efeitos inflamatórios induzidos pela ME, nosso grupo administrou exenatida em ratos submetidos a um modelo experimental de ME, demonstrando uma diminuição da inflamação e maior viabilidade das ilhotas pancreáticas no grupo em ME que recebeu exenatida em comparação com o grupo submetido à ME que não recebeu exenatida (Figura 3) (39). Além disso, também demonstramos a redução da apoptose de hepatócitos (Figura 4) (40) e a reversão da lesão renal neste mesmo modelo experimental (41).

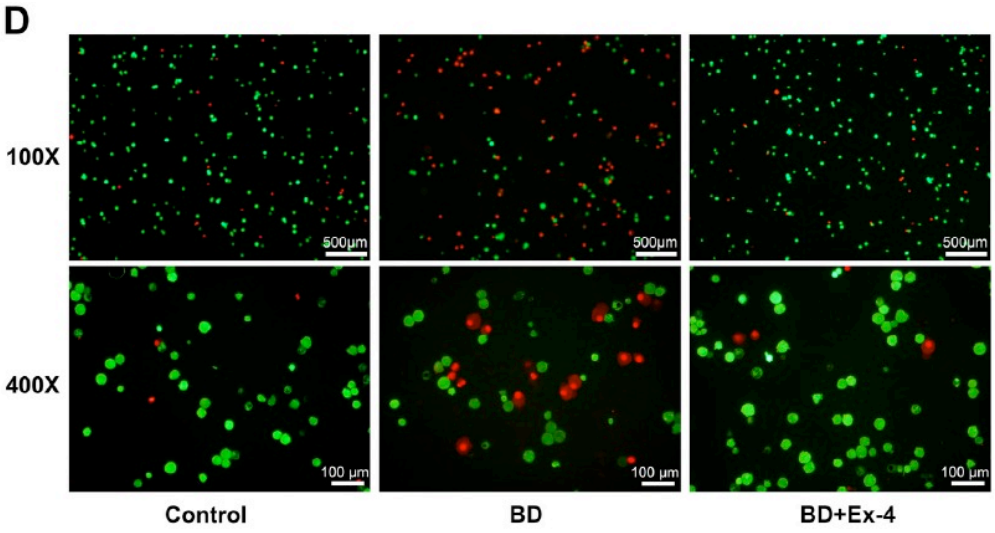

Figura 3. Imagens representativas de fluorescência de ilhotas isoladas de ratos controles (à esquerda), em morte encefálica (ao centro) e em animais tratados com exenatida antes da indução da morte encefálica (à direita). Em verde estão células viáveis e em vermelho, células em apoptose. Pode-se ver que os ratos em morte encefálica tratados com exenatida apresentam grau de apoptose semelhante aos ratos controles. Aumento de 100x e 400x. Imagem retirada de Carlessi et al. (39).

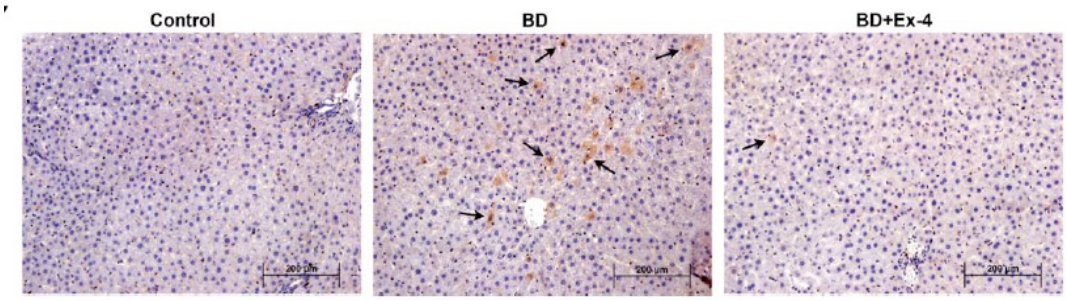

Figura 4. A exenatida protege os hepatócitos contra a apoptose induzida pela morte encefálica, conforme imunohistoquímica do tecido hepático marcado com anticorpos anti-caspase-3 em ratos controles (à esquerda), em morte encefálica (ao centro) e em animais tratados com exenatida antes da indução da morte encefálica (à direita). Imagem retirada de Carlessi et al. (40)

A liraglutida é uma medicação da classe das incretinas aprovada clinicamente para o tratamento de pacientes com DM2 (42). É um análogo do GLP-1 que amplifica a secreção de insulina induzida por glicose e reduz a secreção de glucagon. É segura para uso clínico, com baixo perfil de efeitos adversos e baixo risco de induzir hipoglicemia. Uma revisão sistemática de 2015 não mostrou aumento de casos de pancreatite com o uso

desta classe de medicação, como se sugeria anteriormente (43). Da mesma forma, uma revisão sistemática sobre mortalidade, status cardiovascular e renal em pacientes com DM2 tratados com GLP-1, concluiu que não ouve aumento da incidência de pancreatite ou câncer de pâncreas (44). Como a exenatida foi retirada do mercado pelo fabricante por questões de mercado, decidimos utilizar no presente estudo a liraglutida, que tem efeitos anti-inflamatórios e antiapoptóticos em modelos animais e que podem ser favoráveis neste cenário de ME.

1.4 A hepatectomia do doador

A fim de atenuar os efeitos negativos da isquemia, o transplante se tornou uma corrida contra o tempo. Os esforços têm se concentrado na fase de preservação, reduzindo o tempo de isquemia fria e implementando diferentes técnicas de perfusão de órgãos (45, 46). No entanto, um novo conceito surgiu em relação ao desenvolvimento de disfunção precoce do enxerto: o tempo de hepatectomia do doador, também conhecido como isquemia morna do doador (47, 48). O tempo de hepatectomia é definido pelo tempo entre o pinçamento da aorta e a colocação do fígado em baixas temperaturas. Apesar da curta duração da isquemia morna do doador (minutos) em contraste com a longa duração da isquemia fria (horas), os órgãos são mantidos em temperaturas relativamente altas durante esse período e possuem altas demandas metabólicas (4, 47). Apesar do papel significativo do tempo de hepatectomia do doador, esse aspecto do transplante hepático tem recebido pouca atenção (48, 49). No entanto, recentemente Gilbo et al. demonstraram uma associação entre tempos mais longos de hepatectomia e complicações cirúrgicas precoces (47). Este estudo também mostrou que um aumento de dez minutos na duração da hepatectomia do doador produziu um efeito semelhante a um

aumento de uma hora no tempo de isquemia a frio. Da mesma forma, Adelman et al. demonstraram que o tempo de extração estava independentemente associado à DPE (49).

2. JUSTIFICATIVA

Os órgãos candidatos a transplante são lesados por diversos mecanismos, entre eles estão o processo de estocagem e preservação, a lesão de isquemia-reperfusão, o estresse oxidativo e os eventos inflamatórios induzidos pela ME. Acreditamos que a ME *per se* é um importante gatilho inflamatório responsável por piores desfechos de órgãos transplantados de doadores falecidos. Além disso, demonstramos previamente que a administração de exenatida em ratos com ME melhora marcadores de dano hepático (aspartato aminotransferase e lactato desidrogenase), reduzindo a apoptose dos hepatócitos. Sendo assim, hipotetizamos que a administração de liraglutida a doadores de órgãos em ME poderia melhorar a qualidade dos órgãos para transplante, devido aos seus anti-inflamatórios e antiapoptóticos. Na presente tese, avaliamos se o tratamento com liraglutida comparado a placebo reduz danos hepáticos após o desenvolvimento da ME em seres humanos por meio de um ensaio clínico randomizado.

A fim de analisar outros fatores envolvidos com piores desfechos no transplante hepático como isquemia e temperatura, avaliamos um tempo cirúrgico ainda poruco estudado, o tempo de hepatectomia do doador. Dada a escassez de informação a respeito da duração da hepatectomia em relação ao desenvolvimento de DPE, avaliamos o impacto do tempo de hepatectomia do doador nos desfechos dos receptores de transplante hepático por meio de um estudo observacional retrospectivo.

3. OBJETIVOS

- Comparar os níveis plasmáticos de IL-1β, IL-6, IL-10, INF-γ, TNF e BCL-2 em indivíduos em ME que receberam liraglutida ou placebo, por meio de análises de ELISA (*enzime-linked immunosorbent assay*).
- Comparar os níveis teciduais de *BCL2*, *HSPA5*, *DDIT3*, *SOD2*, *UCP2 e TNF* em biópsias de órgãos de indivíduos em ME que receberam liraglutida ou placebo, por meio de análises de expressão gênica (qRT-PCR) e de SOD2, CHOP e TNF por meio de expressão proteica (imunohistoquímica).
- Avaliar o impacto do tempo de hepatectomia do doador nos desfechos dos receptores de transplante hepático, principalmente relacionado ao desenvolvimento de disfunção primária do enxerto.

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ARTIGO 1

Does liraglutide alleviates inflammation in brain-dead donors? **A randomized clinical trial**

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Does liraglutide alleviates inflammation in brain-dead donors? A randomized clinical trial

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ABBREVIATIONS

BCL2: antiapoptotic B cell lymphoma 2

BD: brain death

HSPA5/BIP: heat shock protein family A

cDNA: complementary DNA

DAB: diaminobenzidine

DDIT3/CHOP: DNA damage inducible transcript 3

ELISA: enzyme-linked immunosorbent assay

GLP-1: glucagon like peptide-1

IFN-γ: interferon-γ

IL-1β: interleukin-1β

IL-6: interleukin-6

IL-10: interleukin-10

NF-κB: nuclear factor Kappa B

OR: odds ratio

RT-qPCR: quantitative real-time polymerase chain reaction

SAPS 3: Simplified Acute Physiology score 3

SD: standard deviation

SOD2: superoxide dismutase 2

STAT3: signal transducers and activators of transcription III

T1: time point 1

T2: time point 2

TNF: tumor necrosis factor

UCP2: uncoupling protein 2

YWHAZ: Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

Δ: delta value

CI: confidence interval

ABSTRACT

Background: Brain death triggers an inflammatory cascade that damages organs before procurement, adversely affecting the quality of grafts. This randomized clinical trial aimed to compare the efficacy of liraglutide compared to placebo in attenuating brain death-induced inflammation, endoplasmic reticulum stress, and oxidative stress.

Methods: We conducted a double-blinded, placebo-controlled, randomized clinical trial with brain-dead donors. Fifty brain-dead donors were randomized to receive subcutaneous liraglutide or placebo. The primary outcome was the reduction in interleukin-6 (IL-6) plasma levels. Secondary outcomes were changes in other plasma pro-inflammatory (IL-1β, IFN-γ, TNF) and anti-inflammatory cytokines (IL-10), expression of antiapoptotic (*BCL2*), endoplasmic reticulum stress markers (*DDIT3/CHOP*, *HSPA5/BIP*), and antioxidant (*SOD2*, *UCP2*) genes, and expression TNF, DDIT3, and SOD2 proteins in liver biopsies.

Results: The liraglutide group showed lower cytokine levels compared to the placebo group during follow-up: Δ IL-6 (−28 [−182–135] vs. 32 [−10.6–70.7] pg/mL; p=0.041) and Δ IL-10 (−0.01 [−2.2–1.5] vs. 1.9 [−0.2–6.1] pg/mL; p=0.042), respectively. The administration of liraglutide did not significantly alter the expression of inflammatory, anti-apoptotic, endoplasmic reticulum stress, or antioxidant genes in the liver tissue. Similar to gene expression, expressions of proteins in liver were not affected by the administration of liraglutide. Treatment with liraglutide did not increase organ recovery rate [OR= 1.24 (95% CI 0.18–8.64), p=0.82].

Conclusions: Liraglutide administration reduced IL-6 and prevented the increase of IL-10 plasma levels in brain-dead donors, without affecting the expression of genes

and proteins related to inflammation, apoptosis, endoplasmic reticulum stress, or oxidative stress.

Keywords: Brain death; liraglutide; inflammation; interleukin-6; transplantation.

INTRODUCTION

Experimental studies have reported an upregulation of cytokines after brain death (BD) [1-3], resulting in harmful effects on organs suitable for transplantation [1,2,4,5]. Our previous research has demonstrated that BD induces inflammation at levels similar to sepsis, which is known to cause significant organ damage [6].

Glucagon-like peptide-1 (GLP-1) is a hormone secreted by the intestinal epithelium L-cells in response to food [7]. Besides its main effect of stimulating glucose-dependent insulin release, it has anti-inflammatory, anti-apoptotic, and cytoprotective properties [7,8], and the administration of the GLP-1 agonist exenatide has been shown to increase pancreatic islet viability [9] and reduce liver damage [10] in rats. Additionally, its use has been shown to protect renal tissue from ischemia-reperfusion damage, an effect mediated by changes in the expression of genes related to oxidative stress, endoplasmic reticulum stress, and inflammation [11,12].

Although liraglutide, another GLP-1 analogue, is an FDA-approved drug for the treating type 2 diabetes and obesity [13-15], its anti-inflammatory and anti-apoptotic properties have not been studied in reducing inflammation, endoplasmic reticulum stress, and oxidative stress of organs from brain-dead donors. Thus, our randomized clinical trial aimed to investigate the potential of liraglutide in attenuating BD-induced inflammation, endoplasmic reticulum stress, and oxidative stress in brain-dead donors compared to placebo.

MATERIAL AND METHODS

Trial design and oversight

A double-blinded, placebo-controlled, single-center, randomized clinical trial was conducted at Santa Isabel Hospital in Blumenau, SC, Brazil. The study protocol was previously registered at ClinicalTrials.gov ID NCT03672812. Ethical approval to conduct the trial was obtained by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre (the reference Ethics Committee in Research, project No. 2018-0170) and by the State Transplant Center of Santa Catarina. Informed consent was obtained from the closest relative, simultaneously with the consent for organ donation. The study adheres to the principles set forth in the Declaration of Helsinki, as well as local standards and Brazilian legislation [16]. The transplant teams were informed about the trial protocol and donor enrollment, but they were not aware of the treatment allocation.

Study population

Patients were deemed eligible for the study after undergoing BD protocol and if they were aged over 18 years. Patients who had received potent anti-inflammatory drugs, such as anti-TNF agents, were excluded from the study. Treatment with corticosteroids was not an exclusion criterion, and type and dose of corticosteroids were recorded. Two physicians not affiliated with the study independently diagnosed BD in accordance with Brazilian legislation [17]. The study adhered the following exclusion criteria: pregnancy, hemodialysis-dependent renal failure, advanced hepatic insufficiency, known allergy to liraglutide, and family refusal to participate. Following the determination of complete eligibility, randomization and initiation of study protocol ensued.

Randomization and trial interventions

Randomization was computer-generated and brain-dead donors were randomized in a 1:1 ratio (using the website *www.randomization.com*) to receive either liraglutide (intervention group) or placebo (placebo group). The dosage of liraglutide administered in our study was equivalent to the highest clinically recommended dose used for treating patients with obesity [18]. The intervention group received 3 mg of liraglutide subcutaneously, corresponding to 0.5 mL immediately after randomization and every six hours until organ recovery. We selected a six-hour timeframe to provide sufficient intervals for the administration of repeated doses, considering that a BD protocol typically does not extend beyond 24 hours. The placebo group received 0.5 mL saline solution immediately after randomization and every six hours subcutaneously until organ recovery. Liraglutide (Victoza®) and placebo were administered subcutaneously. The study pharmacist prepared identical syringes containing either the medication or the placebo, while the research team remained unaware of their respective content. Due to the nature of liraglutide, which does not cause hypoglycemia, no adverse events related to glycemic control were anticipated during treatment.

Data Collection

The management of brain-dead donors was discrete and conducted by the critical care team in accordance with local standards [19-21]. They were unaware of study group allocation. The research personnel collected clinical and laboratory data from the electronic medical records.

Plasma IL-6, IL-1β, IL-10, IFN-γ, TNF, and BCL-2 determinations

A 20-mL whole blood sample was collected at the time of BD diagnosis, immediately before the first dose of either liraglutide or placebo was administered (time point 1, T1), and again before organ procurement (time point 2, T2). Blood samples were centrifuged at 2500g for 10 minutes at 4°C. Plasma was separated and immediately stored at 80°C until analysis. Circulating levels of interleukin-6 (IL-6), interleukin-1β (IL-1β), interleukin-10 (IL-10), interferon-γ (IFN-γ), and tumor necrosis factor (TNF) were assessed by the multiplex enzyme-linked immunosorbent assay (ELISA) method (magnetic bead assay) using the Human Magnetic Custom Luminex® Kit (Invitrogen Life Technologies, Carlsbad, USA) and the Luminex® 200™ magnetic card reader (Luminex, Austin, USA), following the manufacturer's recommendations. Results are expressed as pg/mL. BCL-2 was assessed by ELISA using a commercially available kit and following the manufacturer's recommendations (detection levels: BCL-2 <0.5 ng/mL) (Invitrogen Life Technologies, Carlsbad, USA). The time difference (in hours) between T2 and T1 is the delta value (Δ).

Liver RNA extraction and quantification of BCL2, *HSPA5*, *DDIT3*, *SOD2*, *UCP2 and TNF genes qPCR*

Liver tissue biopsies were obtained during hepatectomy before the liver was flushed with preservation fluid, snap frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was extracted from liver tissue (100 mg) using the Purelink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, USA). The concentration and quality of total RNA samples were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Only RNA samples with adequate purity ratios were used for subsequent analyses [22].

Real-time reverse transcription-PCR (RT-qPCR) was performed in two separate reactions. Firstly, RNA was reverse transcribed into complementary DNA (cDNA). cDNA was then amplified by qPCR. Reverse transcription of 200 ng of RNA into cDNA was carried out using the SuperScript IV Vilo Master Mix (Thermo Fisher Scientific) following the manufacturer's guidelines. qPCR experiments were performed in a Vii7 Fast Real-Time PCR System Thermal Cycler (Thermo Fisher Scientific), following thermal conditions suggested by Thermo Fisher Scientific for the specific qPCR buffer.

For *BCL2*, *HSPA5*, *DDIT3*, *SOD2*, and *UCP2* genes, qPCR experiments were performed by real-time monitoring of the increase in fluorescence of the SYBER Green dye (47). Primer sequences for target and the reference gene *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta) were designed using the Primer Express 3.0 Software (Thermo Fisher Scientific) and are depicted in Supplementary Table 1. qPCR reactions were performed using 5 µL of 2X PowerUp SYBER Green Master Mix, 0.5 µL (1 ng/µL) of forward and reverse primers for target and reference genes, and 1 µL of cDNA template (2.5–10 ng/µL depending on the target), in a total volume of 10 µL. The specificity of the qPCR experiments was verified through melting curve analyses, which showed that all primers generated amplicons that produced a single sharp peak.

For the *TNF* gene, qPCR reactions were performed using 5 µL of 2X TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific), 0,5 µL of 20X TaqMan Gene Expression Assay [for *TNF* (assay ID Hs00174128_m1) or *YWHAZ* (assay ID: Hs01122445_g1), Thermo Fisher Scientific], and 0,5 µL of cDNA template (200 ng/µL), in a total volume of 10 µL.

For all genes, each sample was analyzed in triplicate, and a negative control was included in every experiment. The comparative ΔΔCq method was employed for relative

quantification of genes. The ΔΔCq method estimates changes in gene expression as n-fold changes relative to the calibrator sample (pool of cDNAs) [22,23]. A blinded researcher conducted the experiments.

Immunohistochemistry for DDIT3, SOD2, and TNF proteins in human liver tissue

DDIT3, SOD2, and TNF protein levels were determined by immunohistochemistry in formalin-fixed, paraffin-embedded liver sections. Anti-DDIT3/CHOP mouse monoclonal antibodies (Thermo Fisher Scientific), anti-SOD-2 rabbit monoclonal antibodies (Cell Signaling Technology), and anti-TNF rabbit polyclonal antibodies (Thermo Fisher Scientific) were used to detect DDIT3 (1:50), SOD2 (1:200), and TNF (1:50) protein expression in human liver tissue, respectively. Positive controls for the experiments were brain, spleen, and intestine samples. Immunohistochemical analyses were conducted on 4 μm liver sections using routine immunohistochemical techniques, which included deparaffination and rehydration, antigenic recovery, inactivation of endogenous peroxidase, and blocking of nonspecific reactions. Slides were incubated with primary antibody and then with a biotinylated secondary antibody, streptavidin-horseradish peroxidase conjugate anti-mouse (Santa Cruz Biotechnology, SC-516102) or anti-rabbit (EMD Millipore, code AP132P). The reaction visualization was obtained with Liquid Dab (Dako, K3468), according to the manufacturer's recommendations. For each slide, a blinded researcher captured images of five random fields at 400× magnification. The selection of these fields was determined by the absence of artifacts and the amount of tissue. Images were visualized with a Zeiss microscope (model AXIOSKOP-40; Carl Zeiss, Oberkochen, Germany) and captured using the Cool Snap-Pro CS camera (Media Cybernetics). The staining was done using

diaminobenzidine (DAB) chromogen, and the quantification of DDIT3, SOD2, and TNF proteins were performed using the Image J software with the color deconvolution plugin (National Institutes of Health, NIH). Results are presented in pixels.

Outcomes

The primary outcome was the reduction in IL-6 plasma levels. The secondary outcomes were changes in other pro-inflammatory (IL-1β, INF-γ, TNF) and anti-inflammatory plasma cytokines (IL-10), expression of anti-apoptotic (*BCL2*), endoplasmic reticulum stress markers (*DDIT3*, *HSPA5*), and antioxidant (*SOD2*, *UCP2*) genes, and expression of TNF, DDIT3, and SOD2 proteins in liver biopsies.

Sample size estimation

A sample size of 46 patients was estimated to detect a difference of one log in IL-6 levels between treatment groups considering an 80% power and a 5% α-error rate [4]. To account for possible losses to follow-up, a sample of 50 participants was planned (25 for each group).

Statistical analysis

Categorical variables were expressed as percentages. Continuous data were expressed as mean and standard deviation (SD) if normally distributed, or as median and interquartile range otherwise. Data normality was assessed by visual inspection of the distribution. Groups were compared using Student's *t*-test, the Mann–Whitney *U* test, or the chi-square test. Correlations between variables were estimated using Spearman's test.

Two-way ANOVA was performed to verify the difference between means according to treatment group in the expression of genes considering the median of IL-6 at the first time-point (131 pg/mL). To assess the odds ratio (OR) and 95% confidence interval (95%CI) of the IL-6 and number of donated organs, logistic regression models were built, and the model was adjusted for SAPS 3 score, time from BD to biopsy, and length of hospital stay before BD. To account for potential logistic issues with the study protocol, all analyses were conducted based on the intention-to-treat principle. This approach ensures that participants are analyzed according to their assigned treatment groups, regardless any protocol deviations. Besides, an exploratory analysis per protocol was performed to the main outcome. Two-sided p-values under 0.05 were considered statistically significant. Statistical analyses were conducted in SPSS 21.0 (Chicago, IL, USA). Graphs were created using GraphPad Prism version 9.5 (San Diego, CA, USA).

Authors' Contribution

GC participated in the study design, data collection, data interpretation, and drafted the manuscript. AMM and MRI obtained organ biopsies. DC, NEL, FV, and VMP performed the experiments. CBL participated in the study conception and design, data interpretation, and statistical analysis. THR participated in the study conception and design, data interpretation, statistical analysis, and drafted the manuscript. All authors revised the manuscript.

THR is the guarantor of this study and, as such, has complete access to data and takes full responsibility for the integrity of data and accuracy of analysis.

Compliance with Ethical Standards

All procedures performed in this study were in accordance with the ethical standards of the institutional research committee (Ethics Committee at Hospital de Clínicas de Porto Alegre – project No. 2018-0170) and with the 1964 Helsinki Declaration.

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Informed Consent

Not applicable.

Conflict of Interest

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Consort flow diagram.

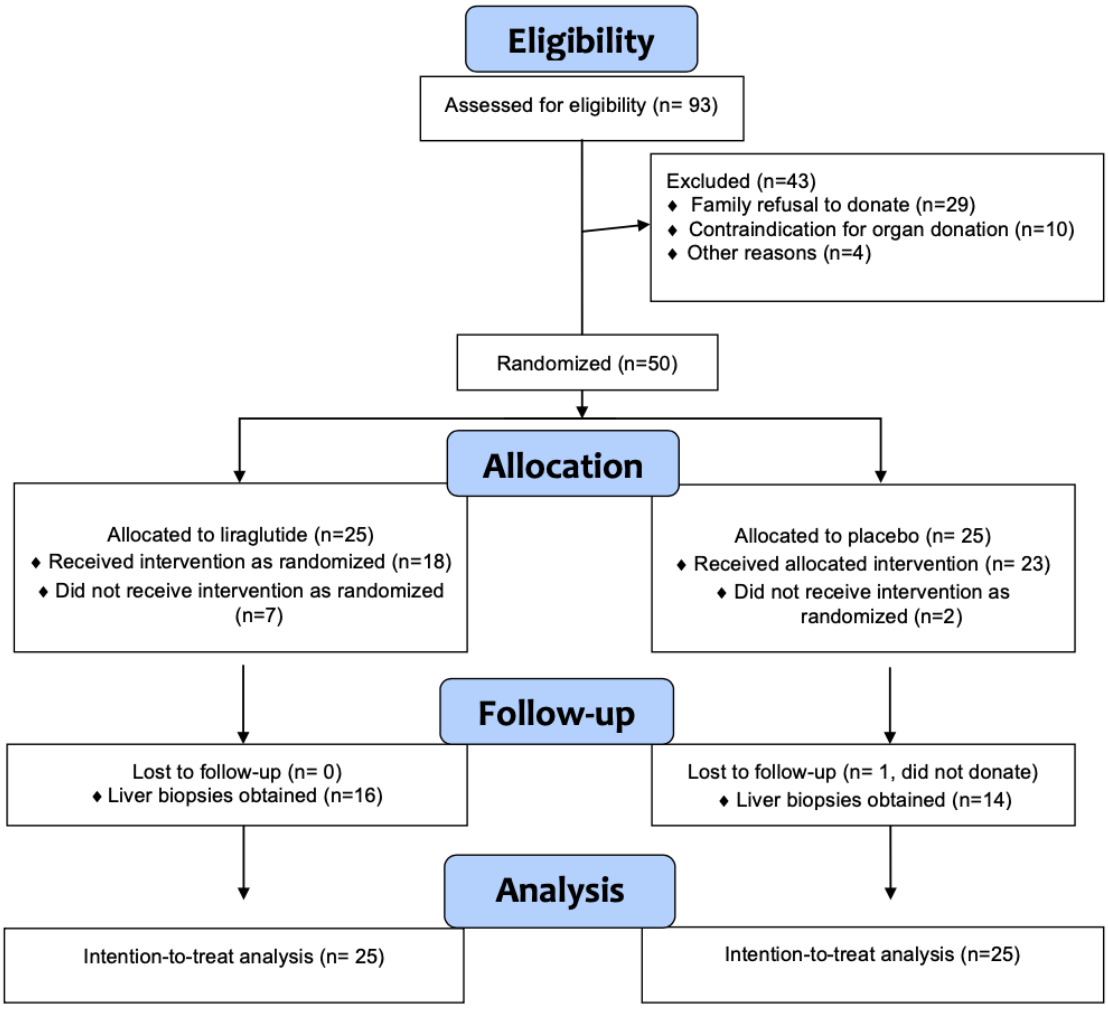

Figure 1. Consort flow diagram.

ARTIGO 2

Association of donor hepatectomy time with liver transplantation outcomes: a retrospective study

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Association of donor hepatectomy time with liver transplantation outcomes: a retrospective study

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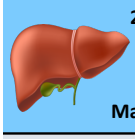
VISUAL ABSTRACT

Is there an association between a shorter duration of donor hepatectomy time and improved post-transplant outcomes?

Population



243 patients underwent liver transplantation

Male 62% | Mean age 41 y

Location

19 centers associated with the state organ distribution center

Design

Retrospective multicenter cohort study

Findings

The median time of donor hepatectomy was 29 (23-40) minutes

Shorter hepatectomy time was associated with BMI <30 Kg/m^2 and with local procurement

The median donor hepatectomy time had no impact on the development of early allograft dysfunction

YES	NO	
25 min	30 min	P=0.126

Conclusion

Shorter duration of donor hepatectomy time was not associated with better outcomes

Custódio et al. *Transplantation*. 07/2023

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ABSTRACT

Background: Prolonged duration of donor hepatectomy may be implicated in early and late complications of liver transplantation. This study aims to evaluate the impact of donor hepatectomy time on outcomes of liver transplant recipients, mainly early allograft dysfunction.

Methods: This is a retrospective multicenter study that included brain-dead donors and adult liver graft recipients. Recipient and donor matching was obtained through a crossover list provided by the regional organ distribution center. Clinical and laboratory data were recorded for both donors and recipients. Cold ischemia, warm ischemia, and donor hepatectomy times were recorded. Primary outcome was early allograft dysfunction. Secondary outcomes were the following: need for retransplantation, length of ICU and hospital stay, and patient and graft survival at 12 months.

Results: From January 2019 to December 2021, a total of 243 patients underwent liver transplantation form brain-dead donors. From them, 57 (25%) developed early allograft dysfunction. The median duration of donor hepatectomy was 29 (23–40) minutes. Patients with early allograft dysfunction had a median hepatectomy time of 25 (22-38) min, while those without had a median time of 30 (24-40) min (p=0.126).

Conclusions: Duration of donor hepatectomy was not associated with early allograft dysfunction, graft survival, or patient survival following liver transplantation.

Keywords: Brain death; hepatectomy; liver transplantation; early allograft dysfunction; graft survival.

INTRODUCTION

The main source of livers for transplantation is the brain-dead donor (1). During liver harvesting and storage processes, the organs are exposed to numerous cellular insults (2). As a result, transplantation becomes a race against time. In order to mitigate the negative effects of ischemia, efforts have focused on the preservation phase, reducing cold ischemia time and implementing different organ perfusion techniques (3, 4).

However, a novel concept has emerged regarding the development of early graft dysfunction: the donor hepatectomy time, also referred to as warm donor ischemia time (5, 6). The hepatectomy time is defined by the time between clamping the aorta and placing the liver in low temperatures. Despite the brief duration of warm donor ischemia (minutes) in contrast to the extended duration of cold ischemia (hours), in warm phase the organs are maintained at relatively high temperatures and at high metabolic demands (5, 7).

Despite the significant role of donor hepatectomy time in graft outcomes, it has received insufficient attention (6, 8). Recently Gilbo et al. have demonstrated an association between longer hepatectomy times and early surgical complications (5). This study showed that a ten-minute increase in the duration of donor hepatectomy time produced a similar effect of one-hour increase in cold ischemia time. Similarly, Adelman et al. demonstrated that the hepatectomy time was independently associated with early allograft dysfunction (8).

To address the shortage of organs and improve liver transplantation outcomes, it is crucial to continuously explore opportunities to enhance donor, graft and recipient care. One such method involves reducing the duration of ischemic phases, which has been demonstrated to be of great importance. Therefore, our study aims to assess the impact of the donor hepatectomy time on outcomes of liver transplant recipients.

METHODS

This is a retrospective multicenter study. The study was approved by the reference Ethics Committee at the Universidade Federal Rio Grande do Sul (PROPESQ UFRGS, project number 5.526.176). The study adheres to the guidelines set forth by the Helsinki Declaration, as well as local standards and Brazilian legislation (9). The Ethics Committee did not require informed consent due to retrospective design and the anonymization of donors and recipients prior to analysis.

Study population

This study included brain-dead donors from 19 regional centers in Santa Catarina State, Brazil and adult liver recipients from brain-dead donors at Santa Isabel Hospital in Blumenau, SC, Brazil between January 2019 to December 2021. In order to be eligible, patients had to be over 18 years and have received a liver transplant from the Liver Transplantation Center at Hospital Santa Isabel. Exclusion criteria were retransplantation, grafts from live-related donors, split liver grafts, and intraoperative death.

Donor matching was obtained through a crossover list provided by the Santa Catarina regional organ distribution center. Clinical and laboratory data were recorded for both donors and recipients, and the Donor Risk Index (DRI) was calculated to assess organ quality (10). DRI scores takes into account eight donor characteristics, namely age, height, ethnicity, cause of death, donor after circulatory death, donor hospital location, split graft, and cold ischemia time. DRI assess the risk of graft loss in comparison to an ideal donor (10, 11). A DRI score ≥1.4 predicts graft failure (11).

Donor hepatectomy time and ischemia times were analyzed. Donor hepatectomy

time, also known as donor warm ischemia time is the duration between the start of aortic cold flush in the donor to the completion of the donor hepatectomy, during which the liver is transferred to ice-cold preservation solution on the back table (7). The cold ischemia time refers to the period between the start of cold flush (both aortic and portal) in the donor and the moment the liver is removed from ice storage and placed in the recipient abdomen for implantation (7). Warm ischemia time in the recipient is defined as the duration between the removal of the liver from the cold solution until organ reperfusion in the recipient (5, 7).

The criteria for early allograft dysfunction were defined as the presence of any of the following postoperative laboratory findings: 1) serum bilirubin >10 mg/dL on day 7 after transplantation; 2) international normalized ratio (INR) >1.6 on day 7 after transplantation; and 3) levels of alanine or aspartate aminotransferases >2,000 IU/L within the initial 7 days after transplantation (12). Graft survival was defined as the time from liver transplantation to either retransplantation or death from any cause (13). Patient survival referred to the time from transplantation until death from any cause. Graft and patient survival were evaluated at 12 months. Patients were follow-up until their last visit to the Liver Transplantation Center at Hospital Santa Isabel.

Primary outcome was early allograft dysfunction. Secondary outcomes were the following: need for retransplantation, length of ICU and hospital stay, and patient and graft survival at 12 months.

Organ Procurement and Transplantation

Livers were procured regionally in 19 centers at the state of Santa Catarina, Brazil. The procedure involved isolating the liver and extracting it through dissection of the

biliary duct, portal vein and hepatic artery, along with en block celiac trunk and aortal patch. The liver was then flushed and cooled through both abdominal aorta and portal vein, and immersed in an ice-cold preservation solution (Institute George Lopez 1 solution). Skilled senior staff members performed all liver transplantations, with most recipients receiving a caval-sparing piggy-back anastomosis, although some required replacement of the inferior vena cava. The portal vein was reconstructed using a standard end-to-end fashion, while the hepatic artery anastomosis was performed end-to-end, with multiple anastomoses done in cases of donor or recipient hepatic artery anatomy abnormalities. Sequential portal and arterial reperfusion were employed. A standard triple immunosuppression regimen consisting of a calcineurin inhibitor, steroids, and an antimetabolite medication was administered to all patients (14).

Statistical analysis

Categorical variables were expressed as percentages. Continuous data were presented as mean ± SD if normally distributed, or median and interquartile ranges if not. Comparison between patients with early allograft dysfunction and patients without were performed using Student's *t* test, Mann-Whitney *U* test or chi-square test, as appropriate. For patient and graft survival analyses, a Kaplan-Meier survival curve with Log-Rank test were constructed while censoring the graft survival for patients who died with a functioning graft to account for competing events. For this analysis, patients were divided below and above the median hepatectomy time. Values were statistically significant if $p<0.05$. Statistical analyses were conducted using the SPSS 21.0 (Chicago, IL, USA).

Authors' Contribution

GC participated in the study design, collection and interpretation of data, statistical analysis, and drafting of the manuscript. AMM and AC performed all liver transplantations. TGP, ABD, GS, and LDOT were involved in data collection. CBL participated in the study conception and design, interpretation of data, and statistical analysis. THR contributed to the study conception and design, interpretation of data, statistical analysis, and drafting the manuscript. All authors reviewed and edited the manuscript.

THR is the guarantor of this work and, as such, had complete access to all data, with full responsibility for the integrity of the data and accuracy of analysis.

Compliance with Ethical Standards

All procedures performed in this study were in accordance with the ethical standards of the institutional research committee (Ethics Committee at Federal University of Rio Grande do Sul, PROPESQ UFRGS, project number 5.526.176)

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Informed Consent

Not applicable.

Conflict of Interest

The authors of this manuscript declare no conflicts of interest.

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CONCLUSÕES GERAIS

Da presente tese podemos tirar as seguintes conclusões:

- Existe atividade inflamatória exacerbada em doadores de órgãos em ME.
- Parte dessa resposta inflamatória e anti-inflamatória é afetada pelo uso de liraglutida, que atua de maneira a atenuar a reposta.
- Não identificamos alterações nas expressões de genes e de proteínas relacionados aos processos inflamatório, de estresse oxidativo e de apoptose, possivelmente por falta de poder do estudo.
- O estudo de expressão gênica foi restrito a um número mínimo de genes, devendo ser expandido para uma análise mais completa.
- O tempo de hepatectomia do doador não foi associado à disfunção primária do enxerto, à sobrevida do enxerto ou à sobrevida do paciente após o transplante hepático, provavelmente por já ser um tempo suficientemente curto.

PERSPECTIVAS FUTURAS

Existem evidências consistentes da atividade inflamatória induzida pela ME, inclusive os próprios resultados desta tese, onde demonstramos níveis muito elevados de citocinas em doadores em ME, que são afetados pelo uso da liraglutida. No nosso estudo, avaliamos um número muito restrito de genes associados à inflamação, apoptose e estresse oxidativo, não encontrando efeito significativo da liraglutida em alterar a expressão desses genes ou mesmo de proteínas no fígado quando comparada ao placebo. No entanto, realizamos também a coleta de biópsias de rim e de pâncreas, que serão analisadas a seguir e permitirá uma avaliação tecido-específica da liraglutida nos seus receptores. Acreditamos que com o aumento amostral e um maior número de genes analisados teremos resultados mais robustos para demostrar os efeitos da liraglutida em reduzir a inflamação.

Acreditamos que a ME *per se* é um gatilho inflamatório responsável por piores desfechos de órgãos transplantados. Alterações do transcriptoma induzidas pela morte encefálica nunca foram estudadas. A análise das alterações do transcriptoma induzidas pela ME poderia auxiliar no entendimento da inflamação induzida pela ME, indicando quais vias de sinalização intracelular podem estar modificadas pela ME. A perspectiva translacional dessas modificações na expressão gênica induzida pela ME poderia abrir novos caminhos no desenvolvimento de terapêuticas anti-inflamatórias na ME.

Transcriptoma é o conjunto completo de transcritos (RNA mensageiro, micro RNA, RNA ribossômico e RNA não codificante) de uma célula ou tecido (1, 2). Alterações no transcriptoma tem sido demonstradas em várias situações fisiológicas, como na resposta imune (3), ou em situações patológica, como no diabetes (4), na sepse (5) e na síndrome da angústia respiratória aguda (6), onde o uso de sequenciamento de

RNA (RNA seq) tem contribuído para as análises de transcriptoma e melhor compreensão dos mecanismos de doença.

O RNA seq é o processo de determinação da ordem dos nucleotídeos em um fragmento de RNA. Esta técnica de biologia molecular é considerada de *next generation*, por permitir análises quantitativas de expressão gênica em larga escala, possibilitando o mapeando do transcriptoma de células e tecidos. Essa capacidade de sequenciamento *high throughput* do RNA seq facilita a dissecção de redes de genes e a elucidação de vias de sinalização em diferentes cenários, como em resposta a diversos estímulos intracelulares ou a RNAs de interferência (1,7). Ao contrário dos estudos de RT-qPCR ou *microarray*, não necessita que os genes ou sequencias de RNA estejam previamente mapeados em placa, podendo detectar qualquer gene presente na célula ou tecido (8).

Nossa hipótese é de que a ME causa graves alterações no transcriptoma das células, o que pode favorecer a expressão de genes e a ativação vias de sinalização envolvidas na inflamação e na apoptose. Assim, pensamos em um estudo ousado, no qual o objetivo principal é avaliar as alterações do transcriptoma induzidas pela ME. Ainda, como objetivos específicos podemos citar:

1- Identificar genes diferentemente expressos em indivíduos em ME em comparação com a expressão gênica de indivíduos controles sem ME por meio de sequenciamento de RNA.

2- Identificar quais vias de sinalização intracelular são modificadas pela ME, especialmente as relacionadas à regulação da inflamação e da apoptose, por meio de análises de Bioinformática.

3- Validar as alterações da expressão gênica de alguns genes selecionados identificados como aumentados ou diminuídos no RNA seq, por meio de *real-time* PCR (RT-PCR).

4- Testar funcionalmente as vias regulatórias, por meio de RNA de interferência, conforme demonstrado abaixo, no fluxograma do estudo.

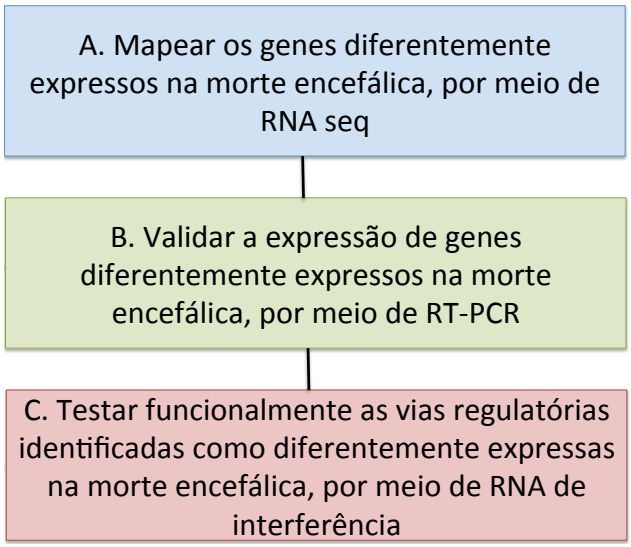

Figura 1. Fluxograma do estudo a ser proposto a seguir.

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