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O papel dos análogos do GLP-1 na função de ilhotas pancreáticas e na morte encefálica

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Porto Alegre, novembro de 2018

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“As nuvens sempre passam. Podem ser nuvens claras ou escuras, mas sempre passam. Talvez tenha que chover tempestade, mas ela também passa. Compreenda que você não é a nuvem, você é o céu.”

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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 dos manuscritos originais sobre o tema da tese.

- **Artigo 1:** "Use of additives, scaffolds and extracellular matrix components for improvement of human pancreatic islet outcomes in vitro: A systematic review"

- **Artigo 2:** "Comparison of two techniques for evaluation of pancreatic islet viability: flow cytometry and FDA/PI staining"

- **Artigo 3:** "Exenatide improves function of rat pancreatic islets exposed to inflammatory stress: an in vitro study"

- **Artigo 4: "**Renal effects of exendin-4 in an animal model of brain death"

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1. INTRODUÇÃO

Ca: cálcio

CIT: *Clinical Islet Transplantation Consortium*

CITR: *Collaborative Islet Transplant Registry*

DM1: diabetes mellitus tipo 1

DM2: diabetes mellitus tipo 2

DPP-4: *dipeptidil peptidase-4*

EXE: exenatida

FDA: acetato de fluoresceína

GIP: *glucose-dependent insulinotropic polypeptide*

GLP-1: *glucagon-like peptide-1*

GLP-1R: receptor do GLP-1

GRP: *gastrin-releasing peptide*

IEQ: islet equivalent

LIRA: liraglutida

ME: morte encefálica

NFAT: fator nuclear de células T ativadas

PC1: *proconvertase 1*

Pdx-1: *pancreas duodenum homeobox-1*

PI: iodeto de propídeo

2. ARTIGOS

7AAD: 7-Aminoactinomycin D

AICAR: 5-aminoimidazole-4-carboxamide1-d-ribofuranoside

AM: calcein-acetoxymethyl

AO: acridine orange

Bcl-2: antiapoptotic B cell lymphoma 2

BclxL: B-cell lymphoma-extra large

BD: brain death

Bim: Bcl-2-like protein 11

BimEL: Bcl-2-like protein 11 extra large

BimL: Bcl-2-like protein 11 large

BimS: Bcl-2-like protein 11 small

Bip: immunoglobulin heavy-chain binding protein

Chop: C/EBP homologous protein

CIT: Clinical Islet Transplantation Consortium

CIT: cold ischemia time

CITR: Collaborative Islet Transplant Registry

CM: collagen matrix

CREB: cAMP response element binding

CypA: cyclophilin A

DAMPs: danger-associated molecular patterns

DAPI: 40,6-diamidino-2-phenylindole

DNA: deoxyribonucleic acid

E2F1: E2F transcription factor 1

ECM: extracellular matrix

ELISA: enzyme-linked immunosorbent assay

EP1013: N-benzyloxycabonyl-Val Asp-fluoromethyl ketone [zVD-FMK]

ER: endoplasmic reticulum

ERK1/2: extracellular signal-regulated kinases 1/2

Ex-4: exendin-4

EXE: exenatide

F573: pan-caspase inhibitor

FACS: Fluorescence-activated cell sorting

FBS: fetal bovine serum

FCS: fetal calf serum

FDA: fluorescein diacetate

FPCM: human fibroblast-populated collagen matrix

GCK: glucokinase

GDNF: glial cell line-derived neurotrophic factor

Glis3: GLIS family zinc finger 3

GLP-1: glucagon like peptide-1

GLP-1R: glucagon like peptide-1 receptor

Glut2: glucose transporter 2

GSIS: glucose-stimulated insulin secretion

GW3965: synthetic nonsteroidal liver X receptor (LXR) agonist

HA: human albumin

HBSS: Hank's balanced salt solution

Hif-1α: hypoxia-inducible factor-1α

HRP: horseradish peroxide

HS: human serum

HSA: human serum albumin

IAC: bis (1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decantionate

IDN-6556: caspase inhibitor

IEQ: islet equivalents

IFN-γ: interferon-γ

IGF: insulin-like growth factor

IK-Bα: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

Il-1β: interleukin-1β

IL-2: interleukin-2

Il-6: interleukin-6

Il-7: interleukin-7

Inos: inducible nitric oxide synthase

INS-1E cells: insulinoma cells

Ins-2: insulin 2

IR: ischemia-reperfusion

IRMA: immunoradiometric assays

IRS-2: insulin receptor substrate 2

LIRA: liraglutide

LSF: lysofylline

MafA: MAF bZIP transcription factor A

MeSH: medical subject headings

MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

MnSOD2: mitochondrial superoxide dismutase 2

MnTMPyP: SOD mimeticmanganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin

MSC: mesenquimal stromal cells

mTOR: mammalian target of rapamycin

Nfkb*:* nuclear factor of kappa light polypeptide gene enhancer in B-cells

Nfkb1α*:* nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α

NG: Newport green

Nlrp3: NLR family, pyrin domain containing 3

NO: nitric oxide

OCR: oxygen consumption rate

PACAP: pituitary adenylate cyclase-activating peptide

PBS: phosphate buffered saline

PBT: poly(butylenes terephthalate)

PDC: perfluorodecalin

PEOT: poly (ethylene oxide terephthalate)

pERK1/2: phosphorylated ERK1/2

PI: propidium iodide

PI3K: phosphatidylinositide 3-kinases

PKA: protein kinase A

PKB: protein kinase B

RIA: radioimmunoassays

ROS: reactive oxygen species

RT-qPCR: quantitative real-time polymerase chain reaction

SD: standard deviation

SD-282: indole-5-carboxamide ATP-competitive inhibitor of p38a MAPK

SE: standard error

SOCS2: suppressor of cytokines signaling 2

Sod2: superoxide dismutase 2

STZ: strepzotocin

T1DM: type 1 diabetes mellitus

T2DM: type 2 diabetes mellitus

TdT: terminal deoxynucleotidyl transferase

TMRE: Tetramethyl rhodamine ethylester

TNF: tumor necrosis factor

TUNEL: tdT dUTP nick-end labeling

TXNIP: Thioredoxin Interacting Protein

Ucp2: uncoupling protein 2

Z-DEVD-FMK:Z-Asp (OMe)-Glu (OMe)-VaL-Asp (OMe)-fluoromethylketone

RESUMO

O diabetes mellitus tipo 1 (DM1) é caracterizado pela destruição autoimune das células-beta pancreáticas, o que causa a deficiência total da produção de insulina e a necessidade de administração de insulina exógena para a sobrevivência. A terapia intensiva com insulina é capaz de reduzir o surgimento e a progressão das complicações crônicas do diabetes. Entretanto, as hipoglicemias severas associadas a este tratamento constituem um grave efeito colateral, especialmente em uma fração dos pacientes com DM1 que apresentam controle metabólico instável e episódios frequentes de hipoglicemias graves sem sintomas adrenérgicos de alerta. Para estes pacientes, o transplante de ilhotas pancreáticas é uma terapia efetiva em restabelecer a secreção de insulina, controle glicêmico e a percepção das hipoglicemias. No entanto, ao longo do processo do isolamento de ilhotas do pâncreas de um doador em morte encefálica (ME) e posterior enxerto no receptor ocorrem perdas importantes no número e qualidade das ilhotas, as quais impactam o desfecho do transplante. Dessa forma, frequentemente, são necessários transplantes de ilhotas de dois ou mais doadores para se atingir a independência à insulina, limitando o número de pacientes que podem se beneficiar dessa terapia. Neste contexto, diversos estudos têm buscado desenvolver estratégias capazes de minimizar a perda das ilhotas durante o isolamento ou cultura das ilhotas a serem transplantadas.

As ilhotas são mantidas em cultura por até três dias antes do transplante, período no qual são realizados testes de viabilidade, função, esterilidade, pureza e contagem do número de ilhotas (*islet equivalents* – IEQs). Diversos estudos avaliaram se o uso de diferentes aditivos no meio de cultura ou cultura das ilhotas sobre componentes da matriz extracelular (ECM) ou outros dispositivos (*scaffolds*) foi capaz de melhorar

desfechos das ilhotas, com resultados bastante variados. Sendo assim, no nosso primeiro artigo, realizamos uma revisão sistemática com o objetivo de sumarizar os resultados desses estudos nos seguintes desfechos: IEQs, viabilidade e função (índice de estimulação de secreção de insulina - SI) das ilhotas. A busca foi feita nos sites PubMed e Embase e 37 artigos preencheram os critérios de elegibilidade e foram incluídos na revisão sistemática. Após a extração dos dados, esses artigos foram divididos nos seguintes grupos: 1) "antiapoptótico/anti-inflamatório/antioxidante"; 2) "hormônios"; 3) "sulfoniluréias"; 4) "soro"; e 5) "*scaffolds* ou componentes da ECM". De uma forma geral, aditivos do grupo "antiapoptóticos/anti-inflamatórios/antioxidantes" parecem diminuir a apoptose das ilhotas e melhorar o SI, calculado após estimulação com concentrações normal e alta de glicose. Além disso, a cultura de ilhotas em *scaffolds* ou sobre componentes de ECM foi capaz de melhorar o SI. Os efeitos dos outros grupos de aditivos sobre os desfechos analisados foram heterogêneos, tornando difícil uma conclusão. Dessa forma, novos estudos são necessários para definir o real impacto desses aditivos na qualidade das ilhotas isoladas e nos desfechos do transplante.

A avaliação da viabilidade das ilhotas é um critério importante para a liberação dessas células para transplante. O teste atualmente utilizado para a avaliação da viabilidade pela maioria dos centros de transplante é a coloração com acetado de fluoresceína (FDA) / iodeto de propídeo (PI). Entretanto, esta técnica apresenta algumas limitações, sendo dependente da experiência dos pesquisadores que vão estimar a porcentagem de células vivas (marcadas pela fluorescência verde – FDA) e mortas (fluorescência vermelha – PI) por ilhota. Neste contexto, um método quantitativo pode ser mais adequado para determinar a viabilidade das ilhotas. Então, no nosso segundo artigo, comparamos a viabilidade das ilhotas estimada com a coloração FDA/PI com

aquela obtida pela citometria de fluxo, usando-se o fluoróforo 7-Aminoactinomycin D (7AAD) e o anticorpo Anexina V-APC. Ilhotas isoladas de 10 ratos Wistar machos foram usadas para avaliar a viabilidade pelas duas técnicas. Na coloração FDA/PI, 50 ilhotas por animal foram analisadas por dois pesquisadores em um microscópio de fluorescência e, então, calculou-se a viabilidade média para as 50 ilhotas/experimento. Para a citometria de fluxo, as ilhotas foram primeiramente dissociadas. Após, 100.000 células dissociadas por animal foram incubadas com 7AAD (marcador de células necróticas ou apoptóticas tardias) e Anexina V-APC (marcador de células em apoptose precoce) e avaliadas no citômetro FACSCanto II. Uma correlação moderada foi encontrada entre as médias de viabilidade obtida pelas duas técnicas ($r= 0,6$, $p= 0,047$). A média da viabilidade medida pela citometria de fluxo foi mais alta do que a média estimada pela coloração FDA/PI (95,5 ± 1,4% *vs.* 89,5 ± 5,0%; $p= 0,002$), também demonstrando uma menor variação entre os experimentos. Embora a citometria de fluxo seja mais cara e mais demorada do que a coloração FDA/PI, é uma técnica quantitativa e não subjetiva. Sendo assim, deve ser a técnica de escolha para uma determinação mais eficaz da viabilidade das ilhotas.

Muitos estudos demonstraram que os análogos do *glucagon-like peptide-1* (GLP-1), como a exenatida (EXE) e liraglutida (LIRA), possuem propriedades anti-inflamatórias, pró-proliferativas e antiapoptóticas em células-beta. A adição de EXE ao meio de cultura das ilhotas parece ser capaz de melhor a viabilidade e o SI de ilhotas humanas. No nosso terceiro artigo buscamos confirmar se a adição de EXE ou LIRA ao meio de cultura das ilhotas isoladas de ratos melhora a viabilidade e função dessas células, protegendo-as de danos inflamatórios decorrentes do isolamento e cultura. Para isto, ilhotas isoladas de ratos Wistar machos foram cultivadas nas seguintes condições, por 72h: 1) Controle; 2) *Pool* de citocinas pró-inflamatórias (TNF, IFN-γ e IL-1β;

adicionadas nas últimas 48h de cultura); 3) EXE; 4) EXE + citocinas; 5) LIRA; e 6) LIRA + citocinas. Após 72h de cultura, foram avaliados o SI e a viabilidade das ilhotas, a qual foi quantificada por citometria de fluxo, utilizando-se 7AAD/Anexina V-APC. O tratamento com EXE melhorou o SI das ilhotas submetidas à inflamação (grupo 4) comparado ao grupo tratado somente com citocinas (grupo 2): 1,21 ± 0,40 *vs.* 0,60 ± 0,22 (p= 0,037). Já a LIRA não influenciou o SI das ilhotas na presença ou ausência de citocinas (p= 0,469). A viabilidade das ilhotas não foi modificada pela presença de EXE ou LIRA (p >0,05). Em conclusão, a EXE parece possuir um papel protetor sobre a função das ilhotas de ratos frente a um estresse inflamatório direto. Estamos realizando experimentos adicionais de expressão gênica para avaliar por quais mecanismos essa melhora do SI acontece.

Um estudo prévio do nosso grupo demonstrou que a administração de EXE a ratos com morte encefálica (ME) foi capaz de aumentar a viabilidade e o SI das ilhotas isoladas. Esses efeitos foram devidos a modificações em genes relacionados ao estresse oxidativo, estresse do retículo endoplasmático e inflamação. Sendo assim, hipotetizamos que a administração de EXE a doadores de órgãos em ME poderia melhorar a qualidade das ilhotas humanas para transplante. Entretanto, antes que isso possa ser testado, precisa-se demonstrar que esse hormônio também melhora (ou pelo menos não piora) parâmetros associados a outros órgãos a serem doados. Recentemente, relatamos que a administração de EXE a ratos com ME também melhora marcadores de dano hepático (aspartato aminotransferase e lactato desidrogenase), também reduzindo a apoptose dos hepatócitos. Na presente tese, avaliamos se o tratamento com EXE também pode diminuir danos renais após o desenvolvimento da ME em ratos. Ratos Wistar machos foram divididos em três grupos: 1) controle (sem lesão do sistema nervoso central); 2) ME (morte encefálica induzida experimentalmente); e 3) ME+EXE

(ME induzida experimentalmente, seguido pela administração intraperitoneal imediata de EXE). A partir das amostras de rins coletadas após 6h de ME, realizou-se a extração de RNA total e proteínas. Também se avaliou os níveis plasmáticos de um marcador de dano renal (creatinina). Ratos tratados com EXE tiveram níveis menores de creatinina comparado aos controles ($p < 0.05$). A ME induziu estresse oxidativo nos rins através do aumento da expressão de *Ucp2*, *Sod2* e *Inos*, enquanto a administração de EXE foi capaz de reduzir a expressão desses genes. A ME também aumentou a expressão de *Tnf* e do inflamasoma *Nlrp3* comparado aos controles ($p < 0.05$), mas a EXE não teve nenhum efeito na expressão desses genes ($p > 0.05$). Além disso, o tratamento com EXE aumentou a expressão de *Bcl2*, um gene antiapoptótico. As expressões de *Il-1β*, *IL-6* e *Nfkb1a* foram similares entre os grupos. Conclui-se que a administração de EXE a ratos com ME pode reduzir danos renais induzidos pela ME através da diminuição da expressão de genes relacionados ao estresse oxidativo e aumento de *Bcl2*.

ABSTRACT

Type 1 diabetes mellitus (T1DM) is characterized by the autoimmune destruction of pancreatic beta-cells, which causes a total deficiency in insulin production and the requirement of exogenous insulin administration for survival. Intensive insulin therapy is able to reduce the onset and progression of diabetic chronic complications. However, the severe hypoglycemia episodes associated with this treatment are a serious side effect, especially in a fraction of T1DM patients who have unstable metabolic control and frequent episodes of severe hypoglycemia without adrenergic warning symptoms. For these patients, pancreatic islet transplantation is an effective therapy for restoring insulin secretion, glycemic control and hypoglycemia awareness. Nevertheless, during islet isolation from a pancreas of a donor with brain death (BD), and subsequent graft in the recipient, there are significant losses in the number and quality of the islets, which affect transplantation outcomes. Thus, islet transplantations from two or more donors are required to achieve insulin independence, limiting the number of patients who may benefit from this therapy. In this context, many studies have sought to develop strategies to minimize islet losses during isolation and culture.

Islets are kept in culture for up to three days prior to transplantation, period in which are performed tests for islet viability, function, sterility, purity, and quantification of islet equivalent (IEQ) numbers. Several studies have evaluated if the use of different additives in the culture medium or islet culture on extracellular matrix (ECM) components or other devices (scaffolds) was able to improve islet outcomes, with quite different results. Thus, in our first article, we performed a systematic review aiming to summarize the results of these studies regarding the following outcomes: IEQs, viability and function (stimulation index - SI). PubMed and Embase repositories were searched,

and 37 articles met the eligibility criteria and were included in the systematic review. After data extraction, these articles were divided into the following groups: 1) "antiapoptotic/anti-inflammatory/antioxidant"; 2) "hormones"; 3) "sulfonylureas"; 4) "serum"; and 5) "scaffolds or ECM components". In general, additives of the "antiapoptotic/anti-inflammatory/antioxidant" group seem to decrease islet apoptosis and improve SI, which is calculated after stimulation with normal and high glucose concentrations. In addition, culturing islets on scaffolds or ECM components was able to improve SI. The effects of the other groups of additives on the analyzed outcomes were heterogeneous, making a conclusion difficult. Thus, new studies are needed to define the real impact of these additives on the quality of isolated islets and transplantation outcomes.

Islet viability assessment is an important criterion for the release of these cells for transplantation. The currently islet viability test used by the majority of the Transplant Centers is the fluorescein diacetate (FDA) / propidium iodide (PI) staining. However, this technique has some limitations, being dependent on the experience of the researchers that will estimate the percentage of live (dyed by green fluorescence - FDA) and dead (red fluorescence - PI) cells per islet. In this context, a quantitative method may be more appropriate to determine islet viability. Thus, in our second article, we compared the islet viability estimated using FDA/PI staining with that obtained by flow cytometry, using the 7-Aminoactinomycin D (7AAD) fluorophore and the Annexin V-APC antibody. Islets isolated from 10 male Wistar rats were used to assess viability by the two techniques. After FDA/PI staining, 50 islets per animal were analyzed by two researchers in a fluorescence microscope and, then, the mean viability for 50 islets/experiment was calculated. For flow cytometry, islets were first dissociated. Afterwards, 100,000 dissociated cells per animal were incubated with 7AAD (dyes

necrotic or late apoptotic cells) and Annexin V-APC (identifies early apoptotic cells) and evaluated on the FACSCanto II cytometer. A moderate correlation was found between mean islet viability obtained by the two techniques (r= 0.6, P= 0.047). The mean viability measured by flow cytometry was higher than the mean viability estimated by FDA/PI staining (95.5 ± 1.4% *vs*. 89.5 ± 5.0%, P= 0.002), also showing lower variation among experiments. Although flow cytometry is more expensive and time-consuming than FDA/PI staining, it is a quantitative rather than a subjective technique. Therefore, it should be the technique of choice for a more effective determination of islet viability.

Many studies have shown that glucagon-like peptide-1 (GLP-1) analogs, such as exenatide (EXE) and liraglutide (LIRA), have anti-inflammatory, proproliferative and antiapoptotic properties in beta-cells. The addition of EXE to the islet culture medium seems to improve both viability and SI of human islets. In our third article, we evaluated whether the addition of EXE or LIRA to the culture medium of islets isolated from rats improves viability and function of these cells, protecting them from the isolation and culture-related inflammatory damage. For this, islets isolated from male Wistar rats were cultured under the following conditions, for 72h: 1) Control; 2) Pool of proinflammatory cytokines (TNF, IFN-γ and IL-1β, added in the last 48h of culture); 3) EXE; 4) EXE + cytokines; 5) LIRA; and 6) LIRA + cytokines. After 72h of culture, we evaluated SI and islet viability, which was measured by flow cytometry using 7AAD/Annexin V-APC. EXE treatment improved the SI of islets submitted to inflammation (group 4) compared to the group treated only with cytokines (group 2): 1.21 ± 0.40 *vs*. 0.60 ± 0.22 (P= 0.037). However, LIRA did not influence SI of islets in the presence or absence of cytokines (P= 0.469). Islet viability was not modified by the presence of EXE or LIRA (P >0.05). In conclusion, EXE seems to play a protective role

on islet function against a direct inflammatory stress. We are conducting additional gene expression experiments to evaluate by which mechanisms this improvement of SI occurs.

A previous study from our group demonstrated that the administration of EXE to rats with brain death (BD) was able to increase viability and SI of the isolated islets. These effects were due to modifications in genes related to oxidative stress, endoplasmic reticulum stress and inflammation. We therefore hypothesized that EXE administration to organ donors with BD could improve the quality of human islets for transplantation. However, before this can be tested, it needs to be demonstrated that this hormone also improves (or at least does not worsen) parameters associated with other organs to be donated. We have recently reported that EXE administration to rats with BD also improves liver damage markers (aspartate aminotransferase and lactate dehydrogenase), also reducing hepatocyte apoptosis. In the present thesis, we evaluated whether EXE treatment may also decrease renal damage after the development of BD in rats. Male Wistar rats were divided into three groups: 1) control (without central nervous system injury); 2) BD (experimentally induced ME); and 3) BD + EXE (experimentally induced BD, followed by immediate intraperitoneal administration of EXE). Total RNA and proteins were extracted from kidney samples collected after 6h of ME. Plasma levels of a renal damage marker (creatinine) were also evaluated. Rats treated with EXE had lower creatinine levels compared to controls ($P < 0.05$). BD induced oxidative stress in kidneys by increasing the expression of *Ucp2*, *Sod2* and *Inos*, while EXE administration was able to reduce the expression of these genes. BD also increased *Tnf* and *Nlrp3* inflammasome expression compared to controls ($P < 0.05$), but EXE had no effect in the expression of these genes ($P > 0.05$). In addition, EXE treatment increased expression of *Bcl2*, an antiapoptotic gene. Expressions of *IL-1β*, *IL-*

6 and *Nfkb1a* were similar between groups. In conclusion, the administration of EXE to BD rats might reduce BD-induced renal damage by decreasing expressions of oxidative stress-related genes and increasing *Bcl2*.

1. INTRODUÇÃO

1.1 Diabetes mellitus tipo 1

O diabetes mellitus tipo 1 (DM1) corresponde a ≈ 10% dos casos de diabetes e é caracterizado pela destruição autoimune das células-beta das ilhotas de Langerhans, resultando na ausência de secreção de insulina (1). A destruição das células-beta pode levar meses ou anos, mas, em geral, o DM1 é clinicamente detectado após a destruição de mais de 80% dessas células nas ilhotas pancreáticas (2). Uma complexa interação entre fatores genéticos e ambientais contribui para a ativação imunológica que desencadeia essa destruição (2-4).

A incidência de DM1 está aumentando nos últimos anos na maioria dos países, especialmente em crianças menores que 15 anos. Por mais que existam grandes diferenças na incidência dessa doença de acordo com a região geográfica, o crescimento anual do DM1 em todo o mundo é de aproximadamente 3%. Atualmente existem 1.106.500 crianças e adolescentes com essa doença e cerca de 130 mil novos casos por ano (5). O país com maior número absoluto de crianças com DM1 é o Estados Unidos, com 169.900 crianças afetadas, seguido pela Índia com 128.500 crianças. O Brasil encontra-se em terceiro lugar nesse ranking com 88.300 crianças diagnosticadas com DM1 até o ano de 2017 (5). Dessa forma, o DM1 vem se tornando, cada vez mais, um sério problema de saúde pública devido ao aumento de sua prevalência na população e à sua associação com o desenvolvimento de complicações crônicas de elevada morbidade e mortalidade em indivíduos jovens em idade produtiva (5).

A incapacidade progressiva de produzir insulina faz com que os pacientes com DM1 necessitem de tratamento com insulina exógena para sua sobrevivência (1). A terapia intensiva com insulina reduz o surgimento e progressão das complicações crônicas do DM; entretanto, está associada com o risco aumentado de hipoglicemias

severas (6, 7), especialmente em uma fração dos pacientes com DM1 com difícil controle glicêmico ou "DM1 lábil". O "DM1 lábil" é definido por amplas variações da glicemia capilar ao longo do dia, tipicamente maiores do que 200 mg/dl e que interfiram na qualidade de vida do paciente ou hipoglicemias graves sem sintomas adrenérgicos de alerta (8-10). Para esses pacientes, a substituição das células-beta através do transplante de pâncreas inteiro ou das ilhotas pancreáticas é a única maneira de se restabelecer a secreção de insulina endógena (11-13).

1.2 Transplante de ilhotas pancreáticas

O transplante de pâncreas como órgão inteiro resulta no controle glicêmico adequado e na prevenção das complicações crônicas do diabetes, sendo indicado para pacientes com DM1 com doença renal crônica terminal durante ou após o transplante renal (14, 15). Apesar do índice de sucesso do transplante de pâncreas ser de cerca de 95% após 1 ano (16), esse tipo de transplante não é a melhor opção para pacientes com DM1 sem indicação de transplante renal devido à morbimortalidade associada ao procedimento (14, 17). Neste contexto, o transplante de ilhotas pancreáticas é uma opção terapêutica mais interessante para pacientes com "DM1 lábil", visto que possui morbidade e mortalidade desprezíveis associadas ao procedimento. Isto porque as ilhotas são infundidas na veia porta dos receptores, sem necessidade de intervenção cirúrgica, sendo este procedimento realizado por radiologista intervencionista treinado (11, 13, 18).

O primeiro transplante de ilhotas em humanos foi realizado em 1974, na Universidade de Minnesota (19). Esta modalidade de transplante continuou sendo realizada de forma experimental e restrita até 1988, quando Camilo Ricordi *et al*. (20) introduziram um método "automatizado" de isolamento de ilhotas, o qual modificou, de forma dramática, o panorama de isolamento e do transplante de ilhotas. Nos anos

seguintes, o transplante de ilhotas passou a ser realizado em diversos centros ao redor do mundo; entretanto, as taxas de sucesso (independência à insulina) reportadas não atingiram níveis satisfatórios (11, 21).

Em 2000, Shapiro *et al*. (22) reportaram que sete pacientes alcançaram independência à insulina após segmento de 1 ano. O protocolo realizado nesse estudo ficou conhecido como protocolo de Edmonton e a melhora do transplante foi atribuída aos seguintes fatores: regime de imunossupressão sem corticoide, múltiplas infusões de ilhotas de diferentes doadores para aumentar a massa de ilhotas transplantadas, além da limitação de receptores com índice de massa corporal elevado (22). Em 2005, o mesmo grupo publicou os resultados de um seguimento de 5 anos de 65 pacientes transplantados com ilhotas usando esse protocolo (23). A maior parte desses pacientes necessitou reiniciar com insulina após 5 anos, mas ainda assim os pacientes mantiveram secreção de insulina detectável, com controle glicêmico estável e menor risco de hipoglicemias (23). Dessa forma, atualmente, o sucesso do transplante de ilhotas é considerado satisfatório se houver prevenção de hipoglicemias graves e estabilização dos níveis de glicose (24).

Dados do *Collaborative Islet Transplant Registry* (CITR), o qual registra os resultados do transplante de ilhotas de vários centros mundiais, mostram claramente que o transplante de ilhotas tem apresentado, progressivamente, melhores resultados ao longo dos últimos anos (12). Cerca de 50% de todos os pacientes que receberam transplantes de ilhotas entre 2007-2010 (n = 208) permaneceram livres de insulina três anos após o transplante (12). Mais recentemente, Hering *et al.* (25) publicou resultados de um estudo de Fase 3 do *Clinical Islet Transplantation* (CIT) *Consortium* que envolveu 8 centros de Transplante de Ilhotas da América do Norte e 48 pacientes com "DM1 lábil". Nesses pacientes, o transplante de ilhotas melhorou o controle glicêmico e

impediu o desenvolvimento de episódios de hipoglicemias severas até o final do seguimento. Moassesfar *et al*. (26) avaliaram a taxa de pacientes livres de insulina em comparação aos resultados do transplante de pâncreas inteiro e os resultados foram semelhantes (em 1 ano – Ilhotas 90% *vs*. Pâncreas 93%; 3 anos – Ilhotas 70% *vs*. Pâncreas 64%). Esses autores também demonstraram que os custos dos dois tipos de transplantes são similares. Nos dias atuais, devido aos bons resultados apresentados, diversos países, como Canadá, Reino Unido, Suíça, Bélgica, Países Nórdicos (Dinamarca, Noruega, Suécia) e Austrália, consideram o transplante de ilhotas como um tratamento efetivo para o DM1 de difícil controle glicêmico e os sistemas de saúde públicos e privados patrocinam a sua realização (13).

Uma das limitações do transplante de ilhotas é que a independência à insulina e um controle glicêmico adequado pós-transplante exigem que um grande número de ilhotas seja transplantado [>10.000 equivalentes de ilhotas (IEQ)/kg do recipiente] (27, 28). Dessa forma, mesmo atualmente, transplantes sequenciais de dois ou mais pâncreas podem ser necessários para se atingir a independência à insulina, limitando o número de pacientes que podem se beneficiar dessa terapia, devido à escassez de órgãos (12, 13, 29). Isto pode ser explicado pela grande perda de ilhotas (≈ 20-30%) que ocorre desde a retirada do pâncreas até o transplante propriamente dito (30). Já no processo de retirada e transporte do pâncreas, as ilhotas são submetidas a múltiplos fatores deletérios celulares, entre eles a isquemia fria, as mudanças súbitas de temperatura, o estresse oxidativo, as forças de cisalhamento que agem sobre o órgão, além do processo de digestão necessário ao isolamento das células (31, 32). A esses agravos soma-se o intenso estresse inflamatório produzido pela morte encefálica (ME), resultando em lesão tecidual e redução da função e da sobrevida dos enxertos (33).

A maior fonte de órgãos para transplante e a única fonte substancial de pâncreas é o doador falecido em ME (34). A ME é uma síndrome inflamatória com efeitos adversos graves bem definidos sobre os desfechos dos transplantes (35-39). Órgãos provenientes de qualquer doador vivo, relacionado ou não relacionado, demonstram resultados consistentemente superiores quando comparados aos de doadores falecidos (37, 40). Acredita-se que alterações hemodinâmicas, neuro-humorais e imunológicas decorrentes da ME sejam os principais fatores que alteram a qualidade de diversos órgãos, tais como rins, coração, fígado, pulmões e mesmo as ilhotas pancreáticas (41-46). Dessa forma, diversas estratégias para melhorar a qualidade dos órgãos a serem doados têm sido estudadas e são de grande interesse (43, 45-48).

1.3. Isolamento e cultura das ilhotas e critérios de controle de qualidade do isolamento

O protocolo de isolamento de ilhotas pancreáticas envolve muitas etapas, iniciando pela retirada, transporte, limpeza e canulação do pâncreas (**Figura 1**). Após essas etapas, a fase de digestão é realizada, onde o tecido pancreático será digerido com o auxílio de uma enzima colagenase que quebra as cadeias de colágeno, fazendo com que as células presentes no pâncreas sejam liberadas. Em seguida, a etapa de purificação das ilhotas é realizada para separar as ilhotas pancreáticas do restante das células do pâncreas. Ao final do processo, as células são cultivadas por até 3 dias para avaliação da qualidade e quantidade de ilhotas isoladas e que serão posteriormente transplantadas (11, 20, 49).

Atualmente, os critérios de controle de qualidade das ilhotas isoladas, os quais são usados para liberação das mesmas para transplantes, são os testes de: viabilidade, pureza, contagem do número de ilhotas (IEQs) e testes de esterilidade (para detecção de

bactérias aeróbicas e anaeróbicas, coloração gram, micoplasmas e endotoxinas) (11, 28) (**Tabela 1**). A viabilidade das ilhotas (% de células vivas / total células) é avaliada pela integridade da membrana usando corantes fluorescentes [acetato de fluoresceína (FDA - cora as células vivas das ilhotas em verde) e iodeto de propídeo (PI - cora as células mortas em vermelho)], em um microscópio de fluorescência. A pureza (% ilhotas / % de tecido exócrino) e o número de IEQs (ilhotas >150 μM) são avaliados em um microscópio do tipo lupa, após coloração das ilhotas com ditizona, um corante que marca os grânulos de insulina das células-beta. Também se pode avaliar o índice de secreção de insulina após estimulação por glicose (SI). O SI [concentração de insulina após estimulação com glicose alta (28 mM) / concentração de insulina após estimulação com glicose baixa (2,8 mM)] dever ser maior que 1, indicando que as ilhotas são funcionais (11, 28).

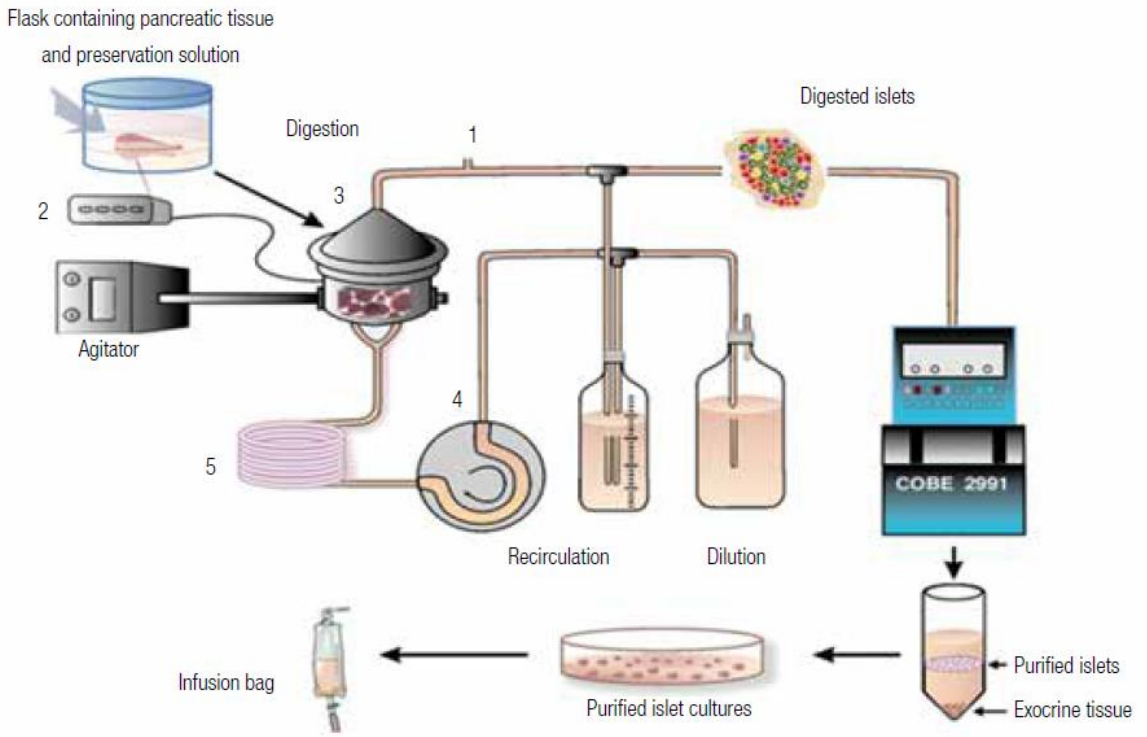

Figura 1. Etapas de digestão e purificação do protocolo de isolamento de ilhotas pancreáticas humanas (11).

Tabela 1 - Critérios de qualidade para liberação das ilhotas para o transplante.

Nome do Teste	Método do Teste	Especificações
Viabilidade	Integridade da membrana usando corantes fluorescentes (FDA/PI)	≥70%
Coloração Gram	Método Gram (cristal violeta)	Ausência de germes
Pureza	% de ilhotas para % de não-ilhotas através de coloração com DTZ	≥50%
Endotoxina	*Quantitative Limulus Amebocyte Lysate test* (LAL)	<5 EU/kg do peso do recipiente*
Contagem de IEQs	Contagem de IEQs usando DTZ	Contagem de IEQs: ≥5000 IEQs/kg
Volume do tecido	Volume do tecido de ilhotas no dia do transplante	<10 ml tecido / infusão
SI*	Secreção de insulina estimulada por glicose	>1

DTZ = ditizona; FDA = diacetato de fluoresceína; IEQs = "islet equivalents" (equivalentes de ilhotas = ilhotas com mais de 150 μM); PI = iodeto de propídeo; SI = índice de estimulação. * O SI não é um critério de liberação, mas é comumente utilizado pela maioria dos centros. Adaptado de: (28).

O sucesso do transplante depende do número de ilhotas isoladas e de fatores associados à qualidade das ilhotas (viabilidade, pureza e SI). Portanto, para melhores resultados do transplante de ilhotas pancreáticas necessita-se o aperfeiçoamento de vários aspectos do isolamento e cultura das ilhotas, bem como da avaliação da qualidade das ilhotas isoladas (11, 50, 51).

1.4 Análogos do GLP-1

Recentemente, um importante alvo de pesquisas na área do DM tem sido as incretinas, predominantemente, o *glucagon-like peptide-1* (GLP-1). O GLP-1 é originado do proglucagon, através da proconvertase 1 (PC1) e suas 2 formas livres possuem uma alanina na posição 2 que são rapidamente degradadas pela dipeptidil peptidase-4 (DPP-4) (52). O GLP-1 é produzido nas células L enteroendócrinas do intestino delgado e do cólon e sua secreção é dependente da presença de nutrientes no lúmen do intestino (**Figura 2**) (53). Os níveis plasmáticos de GLP-1 aumentam rapidamente poucos minutos após a ingestão de alimentos; portanto, parece provável que tanto fatores neurais quanto endócrinos promovam a secreção de GLP-1 a partir das células L distais, bem antes de os nutrientes digeridos atravessarem o intestino delgado para entrar em contato direto com células L enteroendócrinas (52, 54, 55). Embora o *gastrin-releasing peptide* (GRP) e o *glucose-dependent insulinotropic polypeptide* (GIP) estimulem a secreção de GLP-1 em algumas espécies, a identidade dos fatores endócrinos ou neurais que promovem a liberação rápida de GLP-1 em humanos ainda não está clara (52). Sua liberação na corrente sanguínea estimula a secreção de insulina dependente de glicose e inibe a secreção de glucagon e, como resultado, a produção de glicose hepática e a concentração sanguínea da mesma caem. Além da atuação direta no pâncreas e no fígado, o GLP-1 possui um papel coadjuvante em diversos outros tecidos, como músculo, coração, estômago, dentre outros (**Figura 3**) (54).

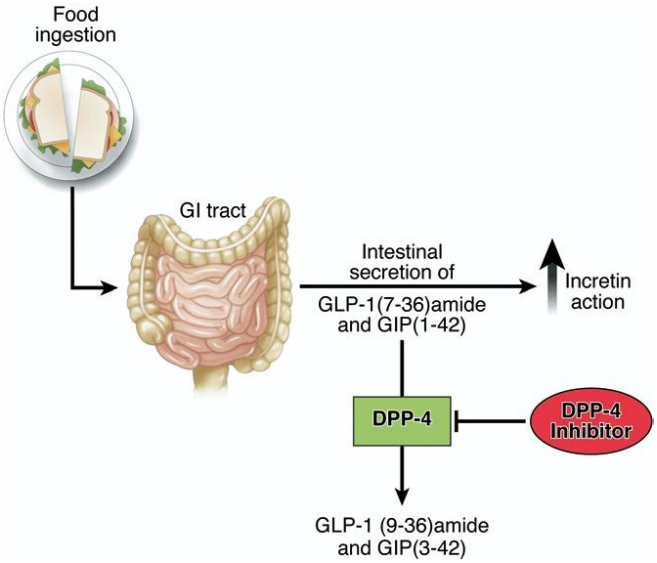

Figura 2. Secreção de *glucagon-like peptide-1* (GLP-1) pelo intestino delgado após a ingesta de alimentos. GI: trato gastrointestinal; DPP-4: dipeptidil peptidase-4 (53).

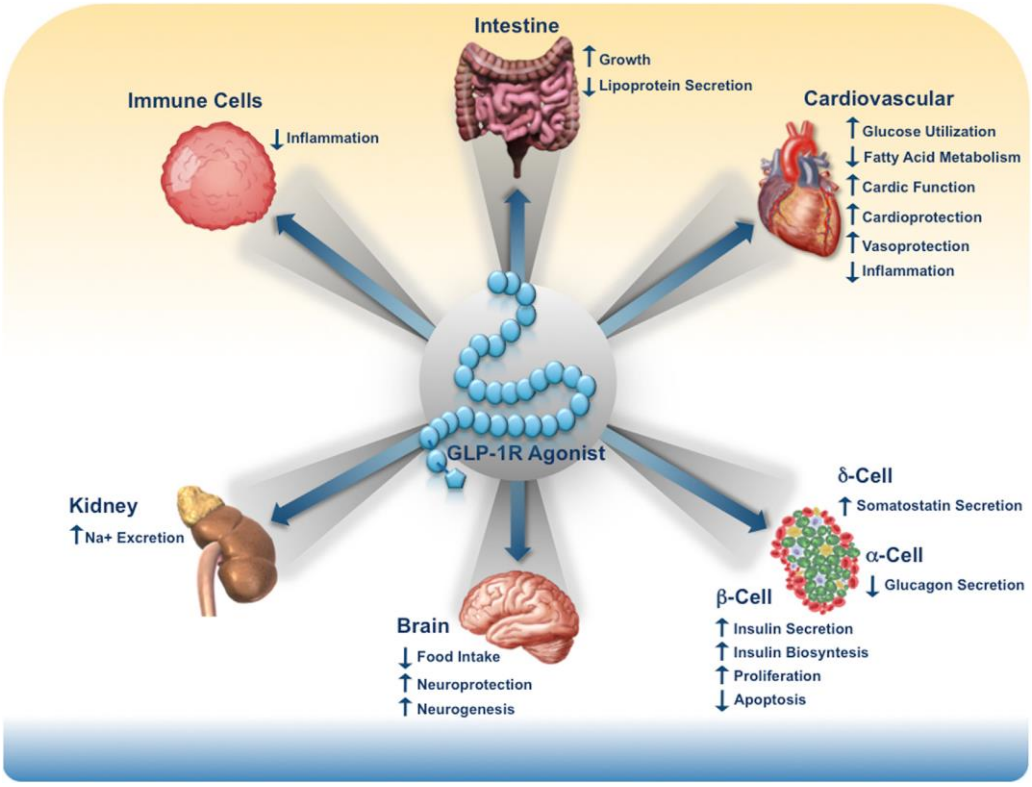

Figura 3. Representação da importante atuação do *glucagon-like peptide-1* (GLP-1) em diversos tecidos (54).

Os mecanismos moleculares da ação das incretinas no pâncreas acontecem basicamente nas ilhotas através do receptor do GLP-1 (GLP-1R), que é predominantemente presente nas células-beta (52-54). A ligação de GLP-1 ao GLP-1R nas células-beta leva à ativação e produção de AMPc da mesma forma que as altas concentrações de glicose na corrente sanguínea (56). O GLP-1 estimula a secreção de insulina através da inibição direta dos canais de K_{ATP} que levam à despolarização da membrana das células-beta, aumentando os níveis e mobilização de reservas de Ca^{2+} intracelular e a síntese de ATP mitocondrial, dessa forma, provocando efeitos diretos na exocitose de grânulos de insulina pela célula-beta (**Figura 4**) (53).

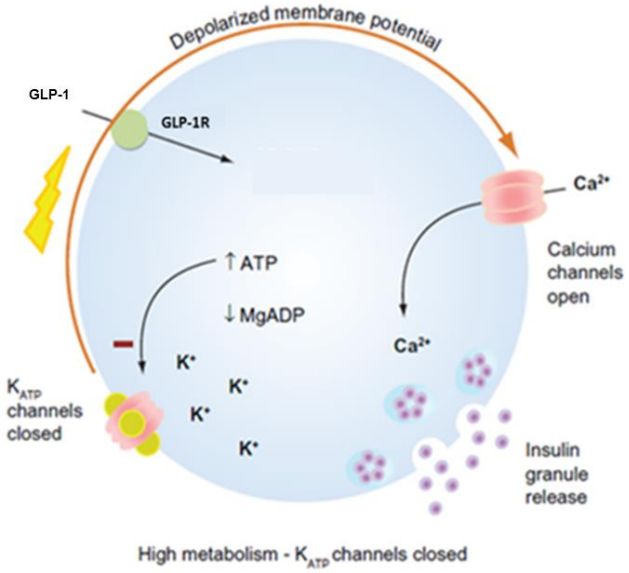

Figura 4. Secreção de insulina estimulada por GLP-1 na célula-beta. Adaptado de (56).

Além disso, o GLP-1 atua sinergicamente com a glicose para promover a transcrição, biossíntese e estabilidade do RNAm do gene da insulina, evitando o esgotamento das reservas de insulina e repondo os estoques na célula-beta (57-59). O fator nuclear de células T ativadas (NFAT) também pode ser um importante mediador da transcrição do gene da insulina induzida por GLP-1, assim como o *Pancreas duodenum homeobox 1* (Pdx-1), um fator de transcrição essencial para o

desenvolvimento pancreático e função das células-beta (53, 60). Além desses mecanismos, o GLP-1 ativa vias antiapoptóticas e bloqueia vias de estresse do retículo endoplasmático (**Figura 5**).

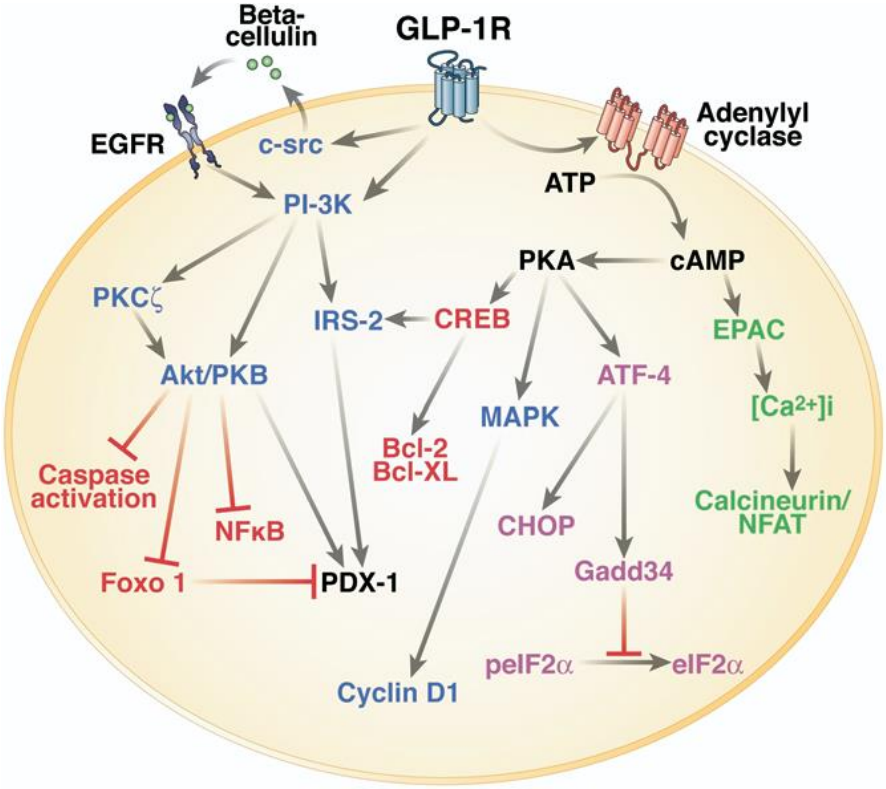

Figura 5. Mecanismos de ação dos GLP-1 e seus análogos na célula-beta pancreática (53).

As propriedades antidiabetogênicas do GLP-1 levaram à intensa pesquisa e ao desenvolvimento de análogos do GLP-1, utilizados atualmente como tratamento para pacientes com diabetes mellitus tipo 2 (DM2). Os análogos de GLP-1 mais comumente utilizados são a exenatida (EXE, Byetta. AstraZeneca, London, England) e a liraglutida (LIRA, Victoza. Novo Nordisk, Bagsvaerd, Denmark). A EXE é originada de um composto natural encontrado na glândula salivar do lagarto *Heloderma suspectum* (Monstro de Gila) e é um agonista do GLP-1R mais resistente a ação da DPP-4, o que

faz com que ela tenha uma meia-vida mais prolongada (1h-1,5h) do que o GLP-1 natural (< 3 minutos). A LIRA, um agonista de longa ação do GLP-1R, também tem mostrado efeitos similares ao GLP-1 e tem uma meia-vida (10h-14h) mais longa do que a EXE (**Figura 6**) (61).

Além do seu uso terapêutico, os análogos do GLP-1 demonstram propriedades citoprotetoras para ilhotas pancreáticas isoladas e outros tecidos oriundos de doadores de órgãos em ME (45, 46, 62, 63). Estudos *in vitro* demonstraram que os análogos do GLP-1 estimulam o aumento da regeneração e proliferação celular e redução da apoptose e da inflamação tanto em ilhotas murinas como em ilhotas humanas isoladas (63-67). Além disso, um estudo realizado pelo nosso grupo demostrou que quando a EXE foi administrada em ratos submetidos à ME, a viabilidade e função das ilhotas melhoraram em comparação ao grupo que sofreu apenas ME. A expressão de genes relacionados à inflamação e estresse oxidativo foi menor nas ilhotas do grupo em ME tratadas com EXE em comparação ao grupo com ME não-tratado com a droga (46). Em outros tecidos, como fígado (45), por exemplo, essa droga também parece atuar em vias de apoptose e estresse oxidativo, protegendo as células dos efeitos da ME. Assim, seu potencial como agente citoprotetor das células-beta pancreáticas no transplante de ilhotas para pacientes com DM1 foi sugerido e testado clinicamente (68-70). Dessa forma, a adição de análogos do GLP-1 ao meio de cultura das ilhotas ou a administração dos mesmos em doadores em ME, parecem ser estratégias válida para melhorar a qualidade de ilhotas e de órgãos transplantados.

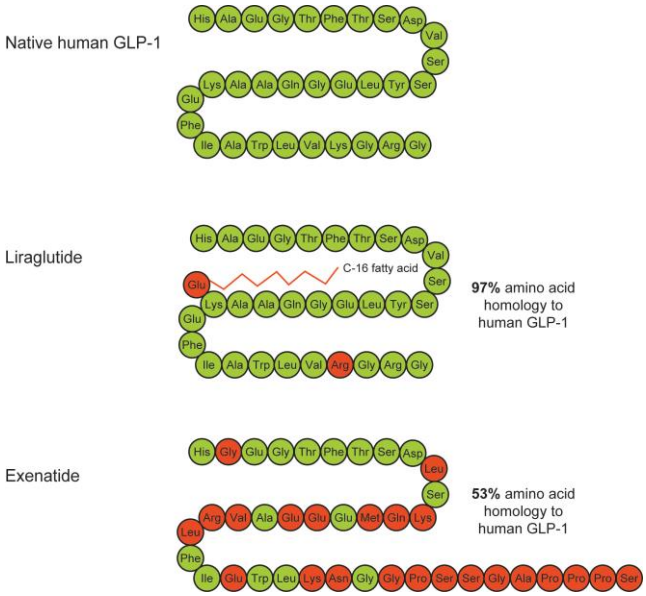

Figura 6. Diferenças entre a exenatida e a liraglutida, ambos análogos do GLP-1 (61).

2. JUSTIFICATICA

O transplante de ilhotas pancreáticas é uma terapia efetiva em restabelecer a secreção de insulina, controle glicêmico e a percepção das hipoglicemias em pacientes com DM1 de difícil controle glicêmico. Entretanto, ao longo dos processos de isolamento das ilhotas de um pâncreas de um doador em ME, cultura das ilhotas e posterior enxerto no receptor ocorrem perdas importantes no número e qualidade das ilhotas, as quais impactam o desfecho do transplante. Dessa forma, frequentemente, são necessários transplantes de ilhotas de dois ou mais doadores para se atingir a independência à insulina, limitando o número de pacientes que podem se beneficiar dessa terapia. Neste contexto, diversos estudos têm buscado desenvolver estratégias capazes de minimizar a perda das ilhotas durante o isolamento ou cultura. Essas estratégias incluem a adição de diferentes aditivos ao meio de cultura das ilhotas, bem como o tratamento do doador de órgão em ME, visando a obtenção de ilhotas em maior quantidade e com viabilidade e função adequadas.

Dessa forma, a identificação de quais aditivos do meio de cultura das ilhotas, com especial ênfase nos análogos de GLP-1, podem melhorar a viabilidade e função dessas células foi o foco dessa tese. Além disso, em um estudo prévio demonstramos que a administração de um análogo de GLP-1 (EXE) a ratos com ME foi capaz de aumentar a viabilidade e o SI das ilhotas isoladas. Sendo assim, hipotetizamos que a administração dessa droga a doadores de órgãos em ME poderia melhorar a qualidade das ilhotas humanas para transplante. Entretanto, antes que isso possa ser testado em humanos, precisa-se demonstrar que esse hormônio também melhora (ou pelo menos não piora) parâmetros associados a outros órgãos a serem doados. Recentemente, relatamos que a administração de EXE a ratos com ME também melhora marcadores de

dano hepático. Na presente tese, avaliamos se o tratamento com EXE também pode diminuir danos renais após o desenvolvimento da ME em ratos.

3. OBJETIVOS

- Realizar uma revisão sistemática de todos os estudos encontrados na literatura que avaliaram o papel de diferentes aditivos, *scaffolds* ou componentes de matriz extracelular na cultura de ilhotas pancreáticas humanas sobre desfechos como: viabilidade, apoptose, SI e IEQ.

- Comparar a citometria de fluxo com a coloração FDA/PI para avaliação da viabilidade de ilhotas pancreáticas isoladas de ratos.

- Avaliar o efeito dos análogos do GLP-1 (EXE e LIRA) sobre a viabilidade e o SI de ilhotas pancreáticas isoladas de ratos frente a um estresse inflamatório direto.

- Avaliar os efeitos da administração da EXE em ratos submetidos à ME sobre marcadores de função renal, apoptose de células renais e expressão gênica e proteica de genes relacionados ao estresse oxidativo, inflamação e proliferação.

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ARTIGO 1

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RESEARCH PAPER

Use of additives, scaffolds and extracellular matrix components for improvement of human pancreatic islet outcomes in vitro: A systematic review

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ABSTRACT

Pancreatic islet transplantation is an established treatment to restore insulin independence in type 1 diabetic patients. Its success rates have increased lately based on improvements in immunosuppressive therapies and on islet isolation and culture. It is known that the quality and quantity of viable transplanted islets are crucial for the achievement of insulin independence and some studies have shown that a significant number of islets are lost during culture time. Thus, in an effort to improve islet yield during culture period, researchers have tested a variety of additives in culture media as well as alternative culture devices, such as scaffolds. However, due to the use of different categories of additives or devices, it is difficult to draw a conclusion on the benefits of these strategies. Therefore, the aim of this systematic review was to summarize the results of studies that described the use of medium additives, scaffolds or extracellular matrix (ECM) components during human pancreatic islets culture. PubMed and Embase repositories were searched. Of 5083 articles retrieved, a total of 37 articles fulfilled the eligibility criteria and were included in the review. After data extraction, articles were grouped as follows: 1) "antiapoptotic/anti-inflammatory/antioxidant," 2) "hormone," 3) "sulphonylureas," 4) "serum supplements," and 5) "scaffolds or ECM components." The effects of the reviewed additives, ECM or scaffolds on islet viability, apoptosis and function (glucose-stimulated insulin secretion - GSIS) were heterogeneous, making any major conclusion hard to sustain. Overall, some "antiapoptotic/anti-inflammatory/antioxidant" additives decreased apoptosis and improved GSIS. Moreover, islet culture with ECM components or scaffolds increased GSIS. More studies are needed to define the real impact of these strategies in improving islet transplantation outcomes.

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Introduction

Pancreatic islet transplantation is an established treatment for patients with type 1 diabetes mellitus (T1DM) that suffer from hypoglycemia unawareness with frequent episodes of hypoglycemia and marked glycemic lability.[1-5] A publication from The Collaborative Islet Transplant Registry (CITR) in 2012 showed a clear improvement in islet transplantation outcomes in the recent era. Insulin independence, 3 y after transplantation, improved from 27% in 1999–2002 (n = 214) to 37% in 2003–2006 (n = 255) and to 44% in the most recent period, 2007–2010 (n = 208).[4] Recently, Brennan *et al.*[6] reported the results from a 12 -year follow-up of 7 subjects initially assigned for the Edmonton protocol in 2000. One patient experienced graft failure only 10.9 y after islet transplantation.

The other 6 patients continued to have sustained C-peptide and improved glycemic control without episodes of severe hypoglycemia after islet transplantation even in this long follow-up, although all of them have lost insulin independence in different timepoints. Hering *et al.*[7] recently published results from the Phase 3 study of Clinical Islet Transplantation (CIT) Consortium. They demonstrated that purified human pancreatic islets transplantation provided good glycemic control, restoration of hypoglycemia awareness, and protection from severe hypoglycemia.

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Also related to CIT, Ricordi *et al.*[8] demonstrated the feasibility of implementing a harmonized process at multiple facilities for the manufacture of a complex cellular product of human islet for transplantation.

An important criteria for the achievement of long-term insulin independence is the total number of viable islets transplanted per Kg of the recipient́s weight.[9] It is generally assumed that a combined implant mass of at least 10,000 islet equivalents (IEQ) per kg is required to routinely achieve insulin-independence.[4] Nowadays, most of the islet isolation facilities keep the isolated islets in culture for 24–48 hours prior transplantation, allowing them to recover from the stress generated during the isolation process and also allowing the preparation of the recipient, including the administration of immunosuppressive induction therapy.[10] During this culture period, up to 10–20% of the total islet mass is lost, which may, in turn, compromise the success of the transplant.[11]

Studies have shown that the loss of islets during the culture period is due, in part, to the apoptosis that is triggered along the whole process of procurement (due to brain death catecholamine storm and cold ischemia time) and also during the islet isolation process.[12-15] In this context, protective strategies to preserve islets from damage during culture time have been studied as a way to improve islet transplant outcomes. These strategies include the use of different additives in the islet culture media or novel culture methods, such as scaffolds or extracellular matrix (ECM) components.[16-20] However, the high variability of additives and culture methods tested makes it difficult to draw a conclusion on the subject. So, the aim of this systematic review was to summarize the research findings on the use of medium additives, scaffolds or ECM components to improve viability and function during human pancreatic islet culture.

Results

Literature search

Fig. 1 is a flow diagram showing the strategy used to identify and select studies for inclusion in this systematic review. All studies that analyzed effects of additives added to the human pancreatic islet culture medium on IEQ, viability/apoptosis and/or function (assessed by glucose-stimulated insulin secretion - GSIS) were selected for inclusion. In addition, studies that cultured islets on different scaffolds or ECM components were also selected for inclusion. A total of 5083 possible relevant citations were retrieved by searching the electronic databases, and 5032 of them were excluded following the reading of titles and abstracts. Fifty articles remained to be fully evaluated. However, after careful analysis of the complete texts, another 14 articles were excluded due to use of islet encapsulation or co-culture.

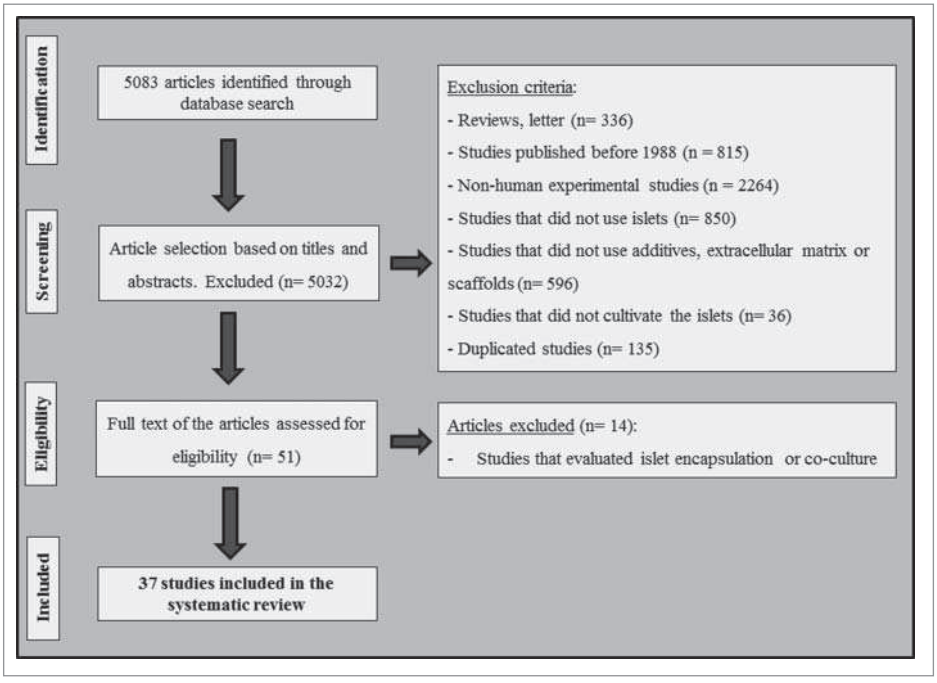

Figure 1. Flowchart illustrating the search strategy used to identify studies for inclusion in the systematic review.

Thirty-seven articles[16-19,21-53] fulfilled the eligibility criteria and were included in the systematic review (Fig. 1). After data extraction, the studies were grouped to better describe and summarize the results, as follows: 1) "antiapoptotic/anti-inflammatory/antioxidant" additives, 2) "hormone" additives, 3) "sulphonylurea" agents, 4) "serum supplements," and 5) "scaffolds or ECM components."

Main characteristics of the eligible studies

Table 1 shows the main characteristics of the 37 studies included in this systematic review. In brief, the number of donors ranged from 2 to 18 and the donor's mean age ranged from 24 y to 70 y among studies. Of note, some studies did not reported donor's characteristics. Purity (%) of the islet preparations were reported by 21 studies, but only in the pre-culture period, and no description was available after the interventions.

All studies included in this systematic review used the Ricordi's semi-automated technique for islet isolation.[54] Twenty-three studies (62.2%) used CRML 1066 as the islet culture media after isolation,[16,17,19,24,25,27,32-35,37-39,41-43,47-53] 4 studies used Miami media,[22,23,29,30] 3 used M199 media,[21,31,44] 6 of them did not report the media used,[18,26,28,36,40,45] and one study used Ham's/F10 media.[46] All studies had an experimental group where modifications

Table 1. Main characteristics of the pancreas donors and purity of the isolated islets described in the included studies.

Groups of additives	N° of donors	Mean age	Gender (%male)	CIT (h)	Pre-culture purity (%)
1st Author (year)[Ref]					
"Antiapoptotic/anti-inflammatory/antioxidant"					
Emamaullee (2008)[32]	—	—	—	—	—
Mancarella (2008)[18]	8	49	50	—	—
McCall (2011)[33]	—	—	—	—	—
Mita (2008)[23]	9	—	—	—	Pure: >90; Impure: 40–60
Moriscot (2007)[24]	—	—	—	—	—
Mwangi (2011)[34]	4	45.8	25		89.2
Nakano (2004)[17]	12	41.8	50	—	70–95
Omori (2010)[25]	—	—	—	—	>70
Pepper (2017)[35]	2	—	—	—	43.8
Scholz (2009)[26]	5	57.6	20	9.52	50–95
Yang (2005)[27]	3	46	80	11	50–95
Zhang (2004)[19]	6	43	—	16	57
"Hormones"					
Farilla (2003)[21]	3	—	—	—	>90
Liu (2009)[28]	—	—	—	—	—
Miki (2014)[22]	—	—	—	—	—
Sakuma (2009)[29]	—	—	—	—	—
Toso (2010)[16]	14	53	21.4	10	—
Yamamoto (2010)[30]	14	48	64.3	11.5	—
"Sulphonylureas"					
Del Guerra (2005)[31]	18	51	55.6	—	—
Maedler (2005)[47]	7	38–70	—	—	>75
"Serum"					
Avgoustiniatos (2012)[48]	—	—	—	—	>70
Bucher (2003)[53]	—	—	—	—	85
Kerr-Conte (2010)[49]	—	—	—	—	50–80
Lee (2008)[50]	—	—	—	—	>50
Matsumoto (2003)[51]	—	47.4	20	35.8	62
Nacher (2013)[52]	15	—	—	—	—
"Scaffolds or ECM components"					
Benti-Barnes (2008)[36]	8	—	—	—	>70
Buitinga (2013)[37]	4	—	—	—	—
Daoud (2010)[38]	—	—	—	<8	>80
Daoud (2011)[39]	—	—	—	<8	>80
Kitzmann (2014)[40]	—	—	—	—	low purity
Maillard (2011)[41]	8	24–61	—	5.5–9	—
Matsushima (2016)[42]	—	—	—	—	—
Marchioli (2015)[43]	—	—	—	—	—
Murray (2009)[44]	—	—	—	—	—
Papas (2005)[45]	3	—	—	—	>90
Zhang (2012)[46]	—	—	—	—	~90

CIT: Cold ischemia time (in hours); ECM: extracellular matrix.

were added to the culture and a control group without modifications added to the culture. The culture time varied from 16 hours to 10 d. The seeding density ranged from 30 IEQ/cm^2 to 5000 IEQ/cm^2, and the volume density varied from 300 IEQ/mL to 1500 IEQ/mL.

Table 2 shows the interventions made to the islet culture, including type of modification, concentrations of additives, ECM components or substances used in scaffolds, and the number of replications per each study. Twelve studies analyzed "antiapoptotic/anti-inflammatory/antioxidant" additives, 6 studies analyzed "hormones," 2 study investigated "sulphonylurea" agents, 6 studies analyzed "serum supplements," and 11 studies investigated the use of "scaffolds or ECM components" in islet culture. Concentrations of these substances

Table 2. Additives, scaffolds and ECM components used in included studies and their concentrations.

Groups of additives	Component	Concentration	n experimental
1st Author (year)[Ref]			
"Antiapoptotic/anti-inflammatory/ antioxidant"			
Emamaullee (2008)[32]	EP1013	1mg/mL	3
Mancarella (2008)[18]	IAC	10μM/L	8
Mc Call (2011)[33]	IDN-6556	100μM	3
Mita (2008)[23]	Sirolimus	30ng/mL	3
Moriscot (2007)[24]	MnTMPyP	25μM/L	3
Mwangi (2011)[34]	GDNF	100ng/mL	4
Nakano (2004)[17]	Z-DEVD-FMK	25 and100μM/L	—
Omori (2010)[25]	SD-282	0.1μM and 0.3μM	3
Pepper (2017)[35]	F573	100μM	2
Scholz (2009)[26]	GW3965	1μM/L	10
Yang (2005)[27]	LSF	20, 50 e 100μM/L	3
Zhang (2004)[19]	Polyphenol (green tea extract)	0, 30, 60, 125, 250 and 500μg/mL	—
"Hormones"			
Farilla (2003)[21]	GLP-1	10nM	3
Liu (2009)[28]	β-E2 and α-E2 (17α-estradiol)	10^{-8}M	5
Miki (2014)[22]	Exendin-4	10nM	3
Sakuma (2009)[29]	PACAP	10^{-12}M/L	—
Toso (2010)[16]	Liraglutide	1μM/L	—
Yamamoto (2010)[30]	Prolactin	500μg/L	—
"Sulphonylureas"			
Del Guerra (2005)[31]	Glimepiride, glibenclamide and chlorprapamide	10μM, 10μM and 600μM; respectively	10
Maedler (2005)[47]	Repaglimide, nateglimide and glibenclamide	0.01 or 1μM, 10 or 1000μM, 0.1 or 1 or 10 or 100nM; respectively	3
"Serum"			
Avgoustiniatos (2012)[48]	FBS and HSA	10% and 0.5%; respectively	10
Bucher (2003)[53]	FCS, HSA and human AB serum	10%, 0.625% and 2.5%; respectively	5
Kerr-Conte (2010)[49]	HSA and human AB serum	0.625% and 2.5%; respectively	9–15
Lee (2008)[50]	HSA and human AB serum	0.5% and 10%; respectively	4
Matsumoto (2003)[51]	FBS and HA	10% and 1.4%; respectively	—
Nacher (2013)[52]	HSA and HS	—	—
"Scaffolds or extracellular matrix"			
Bentsi-Barnes (2008)[36]	Gas-permeable membranes	—	1–7
Buitinga (2013)[37]	PEOT/PBT	—	3
Daoud (2010)[38]	Collagen I/IV, fibronectin and laminin	6.25μg/cm^2	3
Daoud (2011)[39]	Collagen I gel with or without ECM components, micro-fabricated scaffold	4mg/mL and 100 g/mL; respectively	3
Kitzmann (2014)[40]	Silicone rubber membrane	—	5–6
Maillard (2011)[41]	Fibrinogen, thrombin and PDC	20mg/mL, 10mU/mL and 10%; respectively	7–8
Matsushima (2016)[42]	Fibroblasts	—	5–6
Marchioli (2015)[43]	Alginate/gelatingel	—	—
Murray (2009)[44]	Pancreatic duct-derived epithelial cells	—	6
Papas (2008)[45]	Silicone rubber membrane	—	3
Zhang (2012)[46]	CM and FPCM	—	3

EP1013: N-benzyloxycabonyl-Val Asp-fluoromethyl ketone [zVD-FMK]; IAC: bis (1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decantionate; IDN-6556: caspase inhibitor; MnTMPyP: SOD mimeticmanganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP); GDNF: glial cell line-derived neurotrophic factor; Z-DEVD-FMK: Z-Asp (OMe)-Glu (OMe)-Val-Asp (OMe)-fluoromethylketone; SD-282: indole-5-carboxamide ATP-competitive inhibitor of p38α MAPK; F573: pan-caspase inhibitor; GW3965: synthetic nonsteroidal liver X receptor (LXR) agonist; LSF: lysofylline; GLP-1: glucagon-like peptide-1; PACAP: pituitary adenylate cyclase-activating peptide; FBS: fetal bovine serum; FCS: fetal calf serum; HSA: human serum albumin; HA: human albumin; HS: human serum; PEOT: poly(ethylene oxide terephthalate); PBT: poly(butylenes terephthalate); ECM: extracellular matrix; PDC: perfluorodecalin; CM: collagen matrix; FPCM: human fibroblast-populated collagen matrix.

Table 3. Methods used to evaluate the outcomes of interest.

	Methods		
1st Author (year)[Ref]	Viability assesment	Apoptosis assessment	Insulin secretion
Emamaullee (2008)[32]	Sytogreen/ethidium bromide	—	Static incubation/RIA
Mancarella (2008)[18]	—	—	Static incubation/IRMA
Mc Call (2011)[33]	Sytogreen/ethidium bromide	TUNEL/DAPI	—
Mita (2008)[23]	FACS using NG, TMRE and 7AAD	—	Perifusion/ELISA
Moriscot (2007)[24]	FACS using Live/Dead kit	—	Static incubation/RIA
Mwangi (2011)[34]	—	TUNEL/DAPI	Static incubation/ELISA
Nakano (2004)[17]	—	DNA fragment using ELISA	Static incubation/ELISA
Omori (2010)[25]	—	TUNEL	Perifusion/ELISA
Pepper (2017)[35]	Sytogreen/ethidium bromide	TUNEL/DAPI	Static incubation/ELISA
Scholz (2009)[26]	ApoGlow Kit/XTT Assay/CellTiter-Glo	Apo-ONE caspase assay/ELISA	Perifusion/ELISA
Yang (2005)[27]	—	Apo Percentage apoptosis assay kit	Static incubation/ELISA
Zhang (2004)[19]	FDA/PI	—	Static incubation/ELISA
Farilla (2003)[21]	—	DAPI	Static incubation/RIA
Liu (2009)[28]	—	Hoechst	—
Miki (2014)[22]	FACS using NG, TMRE and 7AAD	—	Perifusion/ELISA
Sakuma (2009)[29]	FACS using NG, TMRE and 7AAD	—	Perifusion/ELISA
Toso C (2010)[16]	SYTO_13 Kit/ethidium bromide	TUNEL	Static incubation/ELISA
Yamamoto T (2010)[30]	FACS using NG, TMRE and 7AAD	—	—
Del Guerra (2005)[31]	—	TUNEL/ELISA	Static incubation/IRMA
Maedler (2005)[47]	—	TUNEL	RIA
Avgoustiniatos (2012)[48]	OCR	—	—
Bucher (2003)[53]	—	Cell Death Detection ELISA	Static incubation / -
Kerr-Conte (2010)[49]	Tripan-blue	Cell Death Detection Kit	Static incubation/RIA
Lee (2008)[50]	—	—	Static incubation/Immulite immunometric assay
Matsumoto (2013)[51]	AO/PI	—	Static incubation/ELISA
Nacher (2013)[52]	—	TUNEL/DAPI	Static incubation/ELISA
Bentsi-Barnes (2008)[36]	—	—	Perifusion/ELISA
Buitinga (2013)[37]	—	—	Static incubation/ELISA
Daoud (2010)[38]	—	—	Static incubation/ELISA
Daoud (2011)[39]	—	—	Static incubation/ELISA
Kitzmann (2014)[40]	OCR/FDA/PI	—	—
Maillard (2011)[41]	FDA/ethidium bromide	Caspase 3/ELISA	Static incubation/ELISA
Matsushima (2016)[42]	AM/PI	—	Static incubation/ELISA
Marchioli (2015)[43]	—	—	Static incubation/ELISA
Murray (2009)[44]	—	—	Static incubation/ELISA
Papas (2008)[45]	OCR	—	—
Zhang (2012)[46]	Viability/cytotoxicity assay kit	—	Static incubation/ELISA

FACS: Fluorescence-activated cell sorting; NG: Newport green; TMRE: Tetramethyl rhodamine ethylester; 7AAD: 7-Aminoactinomycin D; FDA: Fluorescein diacetate; PtdIns: Propidium iodide; OCR: oxygen consumption rate; AO: acridine orange; AM: calcein-acetoxymethyl; TUNEL: Terminal deoxynucleotidyl transferase dUTP nickend labeling; DAPI: 4′,6-diamidino-2-phenylindole; ELISA: enzyme-linked immunosorbent assay; RIA: Radioimmunoassay; IRMA: Immunoradiometric assay.

varied among studies, and the number of experimental replications per study ranged from 2 to 15.

The methods used to assess viability, apoptosis and function (GSIS) varied widely among studies, as depicted in Table 3. Twenty out of 37 studies evaluated islet viability,[16,19,22-24,26,29,30,32-35,40-42,45,46,48,49,51] but only 3 of them applied the standard technique used to evaluate islet viability for transplantation [fluorescein diacetate (FDA) and propidium iodide (PI)].[19,34,40] Apoptosis evaluation was reported in 16 studies, with Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL),[16,25,31,33-35,47,52] and ELISA[17,26,31,41,53] being the most used techniques. The majority of studies used ELISA for GSIS evaluation.[16,17,19,22,23,25-27,29,34-39,41-44,46,51,52] Some studies analyzed GSIS using radioimmunoassays (RIA)[21,24,32,47,49] or immunoradiometric assays (IRMA).[18,31] Islet equivalents (IEQ) were calculated using diphenylthiocarbozone dye (Dithizone).[16,17,25]

Results of studies that evaluated "antiapoptotic/anti-inflammatory/antioxidant" additives

Results of the studies that evaluated the effect of "anti-apoptotic/anti-inflammatory/antioxidant" additives added to the culture media on islet viability, apoptosis, GSIS or IEQs are summarized in Table 4. A total of 12 studies analyzed the effects of this group of additives on the islet outcomes of interest. Among them, 7 studies analyzed islet viability. Three of them showed an increased in viability using the pan-caspase inhibitor F573,[35] the caspase inhibitors EP1013[32] and IDN-

Table 4. Summary of the effects of the additives, scaffolds and ECM components on islet outcomes of interest.

Groups of additives	N of studies	Results
		Viability
"Antiapoptotic/anti-inflammatory/antioxidant"	7	↑ 3 studies found increased viability in treated groups (EP1013;[32] IDN-6556;[33] F573[35]) ↔ 4 studies found no differences between groups.[19,23,24,26]
"Hormones"	3	↑ 2 studies found increased viability in treated groups (exendin-4;[22] PACAP[29]) ↔ 1 study found no differences between groups[16]
"Serum"	3	↑ 2 studies found increased viability in treated groups (FBS *vs* HSA;[48] AB serum *vs* HSA[49]) ↔ 1 study found no differences between groups[51]
"Scaffolds or ECM components"	6	↑ 4 studies found increased viability in treated groups (SRM;[40] collagen I, IV, fibronectin and laminin;[38] fibroblasts;[42] CM and FPCM[46]) ↔ 2 studies found no differences between groups[41,45]
		Apoptosis
"Antiapoptotic/anti-inflammatory/antioxidant"	7	↔ 2 studies found no differences between groups[25,26] ↓ 5 studies found decreased apoptosis in treated groups (Z-DEVD-FMK;[17] LSF;[27] IDN-6556;[33] F573;[35] GDNF[34])
"Hormones"	3	↓ 2 studies found decreased apoptosis in treated groups (GLP-1;[21] β-E2 or α-E2 estradiol[28]) ↔ 1 study found no difference between groups[16]
"Sulphonylureas"	2	↔ 1 study found no difference between groups[31] ↑ 1 study found increased apoptosis in treated groups (repaglimide, nateglimide and glibenclamide[47])
"Serum"	3	↓ 3 studies found decreased apoptosis in treated group (AB serum *vs* HSA,[49] and *vs* FCS;[53] HS *vs* HSA[52])
"Scaffolds or ECM components"	1	↓ 1 study found decreased apoptosis in treated groups (fibrinogen, thrombin and PDC[41])
		GSIS
"Antiapoptotic/anti-inflammatory/antioxidant"	11	↑ 7 studies found increased insulin secretion in treated groups (Z-DEVD-FMK;[17] IAC;[18] Sirolimus;[23] GW3965;[26] LSF;[27] F573;[35] GDNF[34]) ↔ 3 studies found no differences between groups[19,25,32] ↓ 1 study found decreased insulin secretion in treated group (MnTMPyP[24])
"Hormones"	4	↑ 2 studies found increased insulin secretion in treated groups (GLP-1;[21] exendina-4[22]) ↔ 2 studies found no differences between groups[16,29]
"Sulphonylureas"	2	↓ 1 study found decreased insulin secretion in treated group (glimepiride, glibenclamide and chlorprapamide[31]) ↑ 1 study found increased insulin secretion in treated group (Glibenclamide[47])
"Serum"	5	↑ 3 studies found increased insulin secretion in treated groups (AB serum *vs* HSA,[49,53] and *vs* FCS;[53] HS *vs* HSA[52]) ↔ 2 studies found no differences between groups[50,51]
"Scaffolds or ECM components"	9	↑ 6 studies found increased insulin secretion in treated groups (gas-permeable membrane;[36] collagen I gel with or without ECM components, micro-fabricated scaffold;[39] fibrinogen, thrombin and PDC;[41] fibroblasts;[42] pancreatic duct-derived epithelial cells;[44] CM and FPCM[46]) ↔ 2 studies found no differences between groups[37,43] ↓ 1 study found decreased insulin secretion in treated group (collagen I, IV, fibronectin and laminin[38])
		IEQ
"Antiapoptotic/anti-inflammatory/antioxidant"	2	↑ 1 study found increased IEQ in treated group (Z-DEVD-FMK[17]) ↔ 1 study found no differences between groups[25]
"Hormone"	1	↑ 1 study found increased IEQ in treated group (liraglutide[16])

↔: No difference in outcome between treated and non-treated islets; ↑: increase of the outcome in treated islets; ↓: decrease of the outcome in the treated islets. ECM: extracellular matrix components; GSIS: glucose stimulation insulin secretion; IEQ: islets equivalents. EP1013: N-benzyloxycabonyl-Val Asp-fluoromethyl ketone [zVD-FMK]; IDN-6556: caspase inhibitor; F573: pan-caspase inhibitor; PACAP: pituitary adenylate cyclase-activating peptide; FBS: fetal bovine serum; HSA: human serum albumin; SRM: silicone rubber membrane; CM: collagen matrix; FPCM: human fibro-blast-populated collagen matrix; Z-DEVD-FMK: Z-Asp (OMe)-Glu (OMe)-Val-Asp (OMe)-fluoromethylketone; LSF: lysofylline; GDNF: glial cell line-derived neurotrophic factor; FCS: fetal calf serum; GLP-1: glucagon-like peptide-1; HS: human serum; PDC: perfluorodecalin; IAC: bis (1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl) decantionate; GW3965: synthetic nonsteroidal liver X receptor (LXR) agonist; MnTMPyP: SOD mimetic manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin.

6556[33] additives. Four studies did not observe any difference in viability between additive-treated and non-treated islets. The additives tested in these 4 studies were: polyphenol (green tea extract);[19] sirolimus;[23] SOD mimetic manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP),[24] and synthetic non-steroidal liver X receptor (LXR) agonist (GW3965).[26]

Regarding apoptosis, P38 inhibitor SD-282[25] and GW3965[26] additives had no significant effect on this outcome, while caspase-3 inhibitor Z-DEVD-FMK,[17] lysofylline (LSF),[27] IDN-6556,[33] F573,[35] and glial cell line-derived neurotrophic factor (GDNF)[34] additives were associated with decreased apoptosis rates compared with control condition.

Eleven of the 12 studies in the "antiapoptotic/anti-inflammatory/antioxidant" group evaluated GSIS. Islets treated with Z-DEVD-FMK,[17] radical scavenger IAC,[18] sirolimus,[23] GW3965,[26] LSF,[27] GDNF[34] and F573[35] additives had increased GSIS compared with non-treated islets. In contrast, one study showed that the MnTMPyP[24] additive added to the culture media decreased GSIS in comparison to the control group. Three other studies found no significant effects of polyphenol (green tea extract),[19] SD-282[25] and EP1013[32] additives on GSIS.

Nakano *et al.*[17] reported that Z-DEVD-FMK also was associated with an increase in IEQ in relation to the non-treated group, which was in agreement with a protective effect of this additive against apoptosis. The other study that evaluated IEQ showed no effect of SD-282 on this outcome.[25]

Based on above-mentioned studies, it is possible to suggest that some additives with "antiapoptotic/anti-inflammatory/antioxidant" effects added to the islet culture media have the potential to improve GSIS and decrease apoptosis (Table 4).

Results of studies that evaluated "hormones" additives

Six studies reported the effects of hormones added to the culture medium on islet outcomes (Table 4). Viability was evaluated in 3 studies. Exendin-4[22] and pituitary adenylate cyclase-activating peptide (PACAP)[29] additives were associated with increased viability compared with control conditions. In contrast, liraglutide[16] did not modify islet viability. Moreover, glucagon-like peptide-1 (GLP-1)[21] and estradiol[28] were able to decrease apoptosis rates compared with non-treated islets. Liraglutide added to the culture medium did not alter apoptosis rates.[16]

Four studies analyzed the effects of culture medium additives on GSIS (Table 4). Islet treated with GLP-1[21] or Exendin-4[22] had an improvement in GSIS compared with non-treated islets. Liraglutide[16] and PACAP[29] had no significant effect on this outcome. However, liraglutide in the culture medium of islets was associated with higher IEQ than the control condition.[16]

Results of studies that evaluated "sulphonylureas" agents

Only 2 studies by Del Guerra *et al.*[31] and Maedler *et al.*[47] added "sulphonylurea" agents to the culture media. Del Guerra *et al.*[31] showed that glimepiride, glibenclamide, and chlorpropamide had no effect on apoptosis rate but decreased GSIS. Maedler *et al.*[47] reported that repaglimide, nateglimide and glibenclamide caused an increase in islet apoptosis, but only glibenclamide was able to increase GSIS. Viability was not assessed in these studies.

Results of studies that evaluated "serum supplements"

Six studies analyzed "serum supplements" added to the islet culture media: 3 evaluated viability, 3 evaluated apoptosis and 5 evaluated GSIS. Regarding viability, 2 studies found an increase in viability when using fetal bovine serum (FBS)[48] or AB serum[49] while one study found no difference between islet cultured with FBS or human albumin.[51] The 3 studies that analyzed apoptosis showed that AB serum,[49,53] and human serum[52] decreased apoptosis. These 3 supplements were also able to improve GSIS.[49,52,53] Two other studies reported no difference on GSIS when comparing human albumin *vs.* AB serum[50] or FBS *vs.* human albumin.[51]

Results of studies that evaluated "scaffolds or extracellular matrix components"

Eleven studies were included in the group "scaffolds or ECM components." Of them, 6 evaluated viability, one analyzed apoptosis, and 9 evaluated GSIS. In relation to viability, 4 studies demonstrated an improvement in this outcome when culturing islets with collagen I/IV, fibronectin and laminin,[38] collagen human fibroblast-populated collagen matrix (FPCM),[46] on a silicon rubber membrane[40] or on a fibroblast matrix.[42] Two other studies found no differences in viability between groups.[41,45] Only one study in this group evaluated apoptosis, showing a reduction in this outcome when using a perfluorodecalin (PDC)-enriched fibrin matrix.[41]

Regarding GSIS, 6 studies demonstrated an improvement in insulin secretion when islets were cultured on gas-permeable membranes,[36] collagen I with or without ECM components or micro-fabricated scaffold with ECM,[39] PDC-enriched fibrin matrix,[41] fibroblasts,[42] pancreatic duct-derived epithelial cells,[44] and collagen or FPCM.[46] Two studies were not able to find any differences between experimental groups,[37,43] and one study observed a decrease in GSIS when islets

were cultured on collagen I/IV, fibronectin and laminin matrixes.[38]

Discussion

Since the initial era of islet isolation and transplantation, many advances have been achieved in respect to the islet isolation process and its standardization, and also a better knowledge of the handling and implantation (transplantation) of the islets was acquired. Despite these improvements, islet loss during isolation, culture period and right after implantation still represent a barrier for a widespread utilization of this therapy. There is evidence to link early graft loss following islet transplantation to isolation-induced β-cell apoptosis.[15] It has been established, *in vitro*, that apoptosis participates in the death of freshly isolated islets cultured under standard conditions and it might be related, in part, to anoikis and lack of growth factors.[55-58]

The ability to maintain isolated islets in culture have been essential for the improvements of islet transplantation outcomes.[59] Factors that may augment or even preserve β-cell mass are of particular interest in the field of islet transplantation because, not unfrequently, the number of viable islets isolated from one pancreas is not sufficient to perform the transplant.[3,60]

As shown in this systematic review, many additives, ECM components and scaffolds have been investigated as potential agents to increase or preserve islet mass before and after transplantation.[61-68] For a better analysis in this systematic review, we classified the additives, ECM components and scaffolds used during culture of human islets in groups according to its main mechanism of action. Regarding "antiapoptotic/anti-inflammatory/antioxidant" additives, 3 studies[32,33,35] were able to show an improvement in viability, 5 studies[17,27,33-35] demonstrated a reduction in apoptosis rate and 7 studies showed an increase in GSIS.[17,18,23,26,27,34,35]

The research group from University of Alberta (Edmonton, AB, Canada) has shown, in 3 different studies,[32,33,35] the improvement of viability when adding anti-apoptotic additives in culture media of human islets. Especially during culture time, it seems that the use of caspase inhibitor additives has the ability to distress human islets leading to substantial reduction in cell death; thereby, improving viability and reducing islet mass required for transplantation. Nakano *et al.*[17] showed that caspase-3 has a crucial role in apoptosis of human islets immediately after isolation and that its inhibitor ameliorates the function of isolated islets. Moreover, the caspase-3 inhibitor Z-DEVD-FMK prevented apoptosis in a dose-dependent manner and also improved islet yield.[17] According to these findings, Yang *et al.*[27] showed that *in vitro* short-term treatment with LSF enhanced human islet metabolism and β-cell insulin secretion, also reducing apoptosis as compared with the control group. These effects were associated with promotion of mitochondrial metabolism since mitochondrial function regulates β-cell insulin secretion and controls the end point of apoptosis. Unexpectedly, this occurred through inhibition of TNF, which induces apoptosis in β-cells through suppression of caspase-8 pathway but not through caspase-3, contradicting the results of Nakano *et al.* In addition to an antiapoptotic effect, studies that used other "antiapoptotic/anti-inflammatory/antioxidant" additives in the human islets culture also have shown an improvement in GSIS.[17,18,23,26,27,34,35] However, the study that used sirolimus in islet culture showed no improvement in viability or GSIS.[23] Sirolimus is an immunosuppressive drug that inhibits IL-2 pro-inflammatory cytokine and, consequently, inhibits the activation and proliferation of T lymphocytes through mTOR.[23] This result should be expected by the authors since sirolimus is anti-proliferative and decreases insulin secretion.[69]

In relation to "hormones," some of them seem to enhance islet "health" Sakuma *et al.*[29] observed a significant increase of viability (4.2%) in islets cultured with PACAP. Miki *et al.*[22] verified that exendin-4 increased islet viability up to 1.85 fold in relation to the control group. However, it is uncertain to what extent these increases in viability are clinically relevant. It is known that viability above 80% is a release criterion to perform islet transplantation and that the majority of the isolations reach viability post-culture above 80% with the standard culture media. So, we still do not have the answer if, above 80%, small increments in viability may impact outcomes.

Besides viability, some "hormone" additives enhanced GSIS and reduced apoptosis. We ought to highlight results from studies using GLP-1 analogs, a 30-amino-acid peptide hormone secreted from the L-cells of the intestinal epithelium in

response to meals. Its analogous (exendin-4 and liraglutide) were approved as a therapy for type 2 diabetes, since they enhance glucose-stimulated postprandial insulin release, and inhibit inadequate glucagon secretion and gastrointestinal motility.[70,71] Lately, anti-inflammatory, antiapoptotic and cytoprotective properties of the GLP-1 analogues have been revealed, opening new therapeutic perspectives for this class of drugs.[72-75]

Farilla *et al.*[21] demonstrated that GLP-1 analogues delayed the morphological changes that occurs in human islets in culture, as indicated by a longer-lasting preservation of their 3D structure. GLP-1 analogs also promoted an increase in expression of the antiapoptotic protein Bcl-2 and a downregulation of the active form of caspase-3.[21] Moreover, these authors verified that GLP1-treated islets contained more insulin and were capable of a greater glucose-dependent insulin secretion.[21] Miki *et al.*[22] showed that exendin-4 supplementation in the culture media significantly reduced pro-inflammatory cytokine/chemokine production from human islet preparations and improved β-cell survival through increased Erk2 phosphorylation, which may be helpful for possible β-cell proliferation after islet transplantation.

Regarding "sulphonylurea" additives, 2 studies were found.[31,47] These oral hypoglycemic agents reduce blood glucose levels by stimulating insulin release from β-cells.[76] Their actions occur through ATP-sensitive potassium (K-ATP) channel, fundamental to the control of β-cell function.[76,77] Based on their mechanism of action, it is expected that the use of sulfonylureas in islet culture media would improve, at least, insulin secretion, as seen in Maedler *et al.*[47] study but not by Del Guerra *et al.*[31]

On the topic of "serum supplements," the use of AB serum,[49,53] and human serum[52] decreased apoptosis and increased GSIS when compared with HSA, the most widely used serum supplement. AB serum has its rational based on serum derived from AB blood donors, making it less immunoreactive. AB serum[49] and FBS[48] improved viability compared with HSA, also suggesting that the quality of clinical islet preparations might be improved when culture is performed in media supplemented with serum instead of albumin.

Scaffolds and ECM components had positive effects on islets in many studies, especially related to viability,[38,40,42,46] and function.[34,37,39,40,42,44] In general, this group of cell culture modifications transmits a variety of chemical and mechanical signals to the islets, mediating key aspects of cellular physiology, such as adhesion, migration, proliferation, differentiation, and death.[78] Probably for these reasons the "scaffolds and ECM components" group showed a good performance on the evaluated outcomes.

An emerging strategy to improve islet viability and function and, thus, graft survival, involves the co-culture of pancreatic islets with mesenchymal stem cells (MSCs). This topic was not included in this study because our group has recently published a systematic review and meta-analysis on it.[79] Souza *et al.*[79] evaluated 20 studies of co-culture of human islets with MSCs, showing that the co-culture with MSCs improved both islet viability and GSIS compared with islets cultured alone. Thus, this co-culture system has the potential for protecting islets from injury after isolation and during culture period.

The results of this systematic review should be interpreted in the context of some limitations. First, the fact that studies included in the systematic review were experimental studies. Second, most additives and scaffolds/ECM components were tested only once, not been replicated in other studies, making difficult to draw firm conclusions. Third, despite the increased number of human isolation facilities inaugurated around the world in the last decade, allowing studies in human islets, most of the studies with additives, ECM components and scaffolds were performed in murine islets, which was not the scope of our systematic review. These facts limited the number of included studies. Fourth, small variation in the composition of the standard culture media used in different clinical centers might have influenced the results of the analyzed studies; however, this information was not described in the articles.

This systematic review results allowed us to draw a better picture on the effect of additives and scaffolds/ECM components in culture of human islets. Overall some "antiapoptotic/anti-inflammatory/antioxidant" additives appear to offer an increment in islets outcomes after culture period by improving GSIS and reducing apoptosis. Moreover, culture of islets on scaffolds or ECM components is able to improve GSIS. More studies, especially with human islets, are needed to define the real impact of these therapeutic strategies in improving islet transplantation, as well as the combination of more than one approach.

Material and methods

Selection criteria and search strategy

PubMed and Embase repositories were searched to identify all articles that analyzed the effect of additives added to the culture medium of human pancreatic islets on IEQ, viability, apoptosis and/or GSIS outcomes. In addition, studies that cultured islets on different scaffolds or with ECM components were also selected for inclusion. The following medical subject headings (MeSH) were used for this search: ("Cell Culture Techniques" OR "Primary Cell Culture" OR "Batch Cell Culture Techniques") OR ("Culture Media" OR "Culture Media, Conditioned" OR "Culture Media, Serum-Free") OR ("Tissue Scaffolds") OR ("Extracellular Matrix" OR "Extracellular Matrix Proteins") AND ("Islets of Langerhans Transplantation" OR "Islets of Langerhans"). The search was restricted to human islet studies and it was completed on January, 2017. All articles identified were also searched manually to detect other relevant citations. This systematic literature search was designed and described in agreement with current guidelines.[80]

Study selection and data extraction

Eligibility evaluation was made by 2 pairs of independent investigators (A.C.B. and N.E.L.; A.P.B. and J. R.), through title and abstracts reviews. When abstracts did not provide sufficient data, the full text of the paper was retrieved for analysis. Disagreements were resolved by discussion between the investigators and, when required, a third reviewer (DC) was consulted. Articles were excluded from the systematic review if: 1) were published before 1988 (data of publication of Ricordi's semi-automated technique);[54] 2) did not use human pancreatic islets; or 3) were review articles, letter or abstracts without description of results (Fig. 1). If data were duplicated and had been published more than once, the most complete study was chosen.

Information of interest from each study was independently extracted by 2 investigators (N.E.L. and L.A.B.) using a standardized extraction form and consensus was sought in all extracted items. When consensus could not be achieved, differences in data extraction were decided by reading the original publication. The data extracted from each study were as follows: (1) general characteristics of the studies, including name of first author and publication year; (2) brain-dead donor characteristics, such as age, gender, cold ischemia time (CIT) of the pancreas; (3) pre-culture islet isolation characteristics as purity and total IEQ; (4) additives or scaffolds/ECM components used and their concentrations (5) post-culture outcomes of interest: number of IEQ, purity, viability, apoptosis and function (assessed by GSIS).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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ARTIGO 2

Comparison of two techniques for evaluation of pancreatic islet viability: flow cytometry and FDA/PI staining

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Comparison of two techniques for evaluation of pancreatic islet viability: flow cytometry and FDA/PI staining

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Abstract

In patients with unstable type 1 diabetes mellitus, pancreatic islet transplantation is a therapeutic option to restore insulin secretion and improve glycemic control. However, the success of islet transplantation is dependent, in part, on the number of isolated islets as well as their quality, which is assessed by functional and viability tests. The currently employed test to evaluate islet viability, used by the Collaborative Islet Transplant Registry to release clinical islet product for transplantation, is the FDA/PI staining. However, the efficacy of this method is dependent on the researcher experience. In this context, a quantitative method may be useful. **Objectives:** Therefore, the aim of this study was to compare islet viability assessed by flow cytometry and FDA/PI staining. **Design & methods:** Viability was evaluated in islets isolated from 10 male Wistar rats. Upon FDA and PI staining, 50 islets from each animal were analyzed under fluorescence microscope by two well-trained researchers, and the percentages of living and dead cells per islet were estimated. For flow cytometry, islets were dispersed, and then 100,000 single cells were incubated with 7AAD fluorophore (dyes necrotic and late apoptotic cells) and Annexin V-APC antibody (marker of early apoptotic cells). **Results:** A moderate correlation was found between the two techniques (r= 0.6; P= 0.047). The mean islet viability measured by flow cytometry was higher than the viability estimated using FDA/PI staining (95.5 ± 1.4% *vs*. 89.5 ± 5.0%; P= 0.002). **Conclusions:** Although flow cytometry is more expensive and time-consuming than FDA/PI staining, it is a quantitative technique, not dependent of the researcher experience. Thus, flow cytometry appears to be the technique of choice when aiming a more precise determination of islet viability.

Keywords: islet transplantation; islet isolation; viability; flow cytometry.

Introduction

Pancreatic islet transplantation is an established treatment for patients with type 1 diabetes mellitus that suffer from hypoglycemia unawareness and marked glycemic lability (1-5). An important criteria for the achievement of long term insulin-independence with islet transplantation is the total number of viable islets transplanted per Kg of recipient's weight (6). It is generally assumed that a combined implant mass of at least 10,000 islet equivalents (IEQ) per Kg is required to routinely achieve insulin-independence (1, 7).

Nowadays, most of the islet isolation Centers keep the isolated islets in culture for 24-48 hours prior transplantation, allowing them to recover from the stress generated during the isolation process and also allowing the preparation of the recipient, which includes the administration of immunosuppressive induction therapy (7-9). During this culture period, approximately 20% of the total islet mass is lost, which may, in turn, compromise the success of the transplant (10). Studies have shown that the loss of islets during the culture period is due, in part, to the apoptosis that is triggered along the whole process of procurement (due to brain death catecholamine storm and cold ischemia time) and also during the islet isolation process (11-15).

Therefore, evaluation of islet viability is an important quality test for the release of the isolated islets for transplantation. Currently, the standard method used by the world transplant Centers participating in the Collaborative Islet Transplant Registry (CITR) to assess islet viability is DNA-binding dye exclusion technique using fluorescein diacetate (FDA) and propidium iodide (PI) (7, 16, 17). In this method, living cells actively convert the non-fluorescent FDA into the green fluorescent compound fluorescein, while dead cells show red fluorescence in their nuclei due to penetration of PI through the permeabilized membrane (18). A limitation of FDA/PI staining method

is that PI only enter cells that lost selective membrane permeability; thus, it does not dye early apoptotic cells (17). Moreover, this method is not able to discriminate between cell subsets and, in particular, it does not allow us for selectively defining beta-cell viability (17, 19).

Hence, alternative methods have been tested to determine islet viability before clinical transplantation, including flow cytometry (17, 19-23). Through using different fluorescent dyes, flow cytometry may offer a more precise quantification of islet viability, while also allows the differentiation of cell death (necrosis, early or late apoptosis). Another advantage of this technique compared to FDA/PI staining is that flow cytometry is a quantitative method, not dependent on a researcher to determine the percentage of living cells (24, 25). In this context, in the present study, we compared islet viability percentages using flow cytometry and FDA/PI staining.

Methods

Animals and experimental design

Male Wistar rats fed with standard laboratory diet *ad libitum*, weighing 300 to 350 g, were used in the study. All animals were kept in the animal facility of Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) and were cared following the guidelines for use and care of laboratory animal (26). The study was approved by the local ethics committee for the use of animals in research. A total of 10 consecutive islet preparations were analyzed for viability using the methods described below.

Islet isolation and dispersion

Islet isolation was performed following the protocol suggested by Carter *et al*. (27). After euthanasia, the bile duct was cannulated to allow pancreatic perfusion with 10 mL of cold Hank's balanced salt solution (HBSS, Sigma-Aldrich, Saint Louis, MO, USA)

containing 0.5 mg/mL of collagenase P (Roche Diagnostics, Mannheim, BW, Germany). The perfused pancreas was removed and digested for 15 min at 37 °C. Digestion was stopped by the addition of RPMI 1640 medium (11 mmol/L glucose) (Thermo Fisher Scientific, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 25 mmol/L HEPES (Thermo Fisher Scientific). Islet purification was performed through discontinuous density gradient of Histopaque (Sigma-Aldrich), at 1.119, 1.100, and 1.077 g/mL density layers. An aliquot of purified islets was counted under a microscope to obtain the total number of isolated islets. Then, they were divided into two aliquots for further evaluation of viability by cytometry and FDA/PI staining.

Islet viability assessment

For FDA/PI staining, 43 µl of the pellet of islets isolated from each animal were stained with 0.46 µM FDA and 14.34 µM PI (Sigma-Aldrich) in 457 µl DPBS buffer (Sigma-Aldrich), and immediately analyzed by two well-trained researchers, under a Nikon Eclipse TS100 microscope (Nikon, Minato, Tokyo, Japan), to estimate the percentage of living and dead cells by each islet. Each researcher analyzed 50 islets in the suspension. Then, the final islet viability was the mean percentage of living cells in the 50 islets analyzed by each researcher.

For islet viability assessment using flow cytometry, first we dispersed 100 islets into single cells. For this, isolated islets were immediately washed with HBSS (without calcium chloride and magnesium sulphate; Sigma-Aldrich) and dispersed with 0.125% Trypsin–EDTA (Thermo Fisher Scientific) for 5 min at 37 °C. Dispersion was stopped by addition of supplemented RPMI 1640 medium. Next, the obtained single cells were resuspended in phosphate buffered saline (PBS, LGC, Cotia, SP, Brazil) 1x and Annexin V-APC binding buffer (BD, Franklin Lakes, NJ, USA). Then, 5 µL of Annexin

V-APC antibody (label early apoptotic cells; BD) was added in the preparation of cells, which were incubated for 15 min at room temperature, protected from light. After this incubation, 5µL of 7AAD fluorophore (BD) was added. Next, 100,000 cells were acquired on the FacsCanto II (BD). Results were analyzed using Infinicyt flow cytometry software (Cytognos, Santa Marta de Tormes, Salamanca, Spain). Cells stained with both 7AAD and Annexin V-APC were those that had undergone rupture of the membrane, being in the process of late apoptosis or necrosis. Cells marked only with Annexin V-APC were those that had intact membrane, but were already in process of early apoptosis. Those cells that did not stain with any dye were living cells.

Statistical analysis

Variables are shown as mean ± standard deviation (SD) or %. Mean viability values obtained using flow cytometry and FDA/PI staining for the 10 islet isolation experiments were compared using student's *t*-tests. The correlation in the viability values obtained using the two techniques of interest was calculated using *Pearson* correlation test. P values <0.05 (two-tailed) were considered statistically significant, and all analyses were performed using SPSS 18 (SPSS Inc., Chicago, IL, USA).

Results

Figure 1A illustrates the FDA/PI staining of two islets, where the green color (FDA staining) represents the living cells inside an islet, and the red color (PI staining) represents dead cell/islet. In this figure, islet 1 was considered as having 95% of living cells and 5% of dead cells, while islet 2 had 100% of living cells. **Figure 1B** shows the gating strategy used in flow cytometry for the assessment of islet viability after 7AAD and Annexin V-APC treatment. The lower left quadrant shows living cells that did not

stain with Annexin V-APC and 7AAD. The lower right quadrant shows cells in early apoptosis process (those cells stained only with Annexin V-APC), while the upper right quadrant depicts cells suffering late apoptosis or necrosis (cells stained with both 7AAD and Annexin V-APC).

Figure 2 and **Table 1** show viability results (%) obtained using flow cytometry and FDA/PI staining for each of the islet preparations isolated from the 10 Wistar rats. For FDA/PI staining, 50 islets/isolation were evaluated by each of the two researchers that estimated viability, and the results are shown as mean percentages of living cells/islet isolation experiment (**Table 1** and **Figure 1**). For flow cytometry, the results are shown as mean percentages of viable cells, as well as the corresponding percentages of early apoptotic or late apoptotic/necrotic cells (**Table 1**), by 100,000 single cells analyzed/isolation. The total percentage of dead cells obtained by flow cytometry was the sum of the percentage of early apoptotic cells with the percentage of late apoptotic/necrotic cells.

Considering all experiments together, the mean islet viability obtained by FDA/PI staining was lower than the viability measured by flow cytometry (89.5% ± 5.03% *vs.* 95.5% ± 1.43%, P = 0.002) (**Figure 3**). A moderate correlation was observed between mean percentages of viability obtained using the two techniques (r = 0.638, P = 0.047). Of note, islet viability assessed by FDA/PI staining presented higher variability (SD = 5.03) than when assessed by flow cytometry (SD = 1.43), and this is mainly due to the variation on the values obtained by each research in the FDA/PI staining: the SD between researchers ranged from 4.71 to 20.74%.

Discussion

Regulatory agencies obligate the manufactured product of islet isolation to be tested for sterility, purity, number of IEQs, function (glucose-stimulated insulin secretion), and viability prior to releasement to transplantation (7, 9). Hence, islet viability higher than 70% is needed for clinical islet product release (7). In Centers belonging to the CITR, islet viability is commonly assessed by FDA/PI staining (7, 18), which is a simple and cheap technique, but has some limitations. Therefore, in an effort to stablish a more sensitive and specific method for evaluating islet viability, we compared viability values using flow cytometry with 7AAD and Annexin V-APC to islet viability estimated using FDA/PI staining. Our results demonstrated a significant difference in mean islet viability evaluated by the two techniques, with flow cytometry showing less variation among experiments.

Although FDA/PI staining is the current standard method used for islet viability estimation in the clinical transplantation setting; as already mentioned, this assay has important limitations (23). First, the manual method for determining viability using FDA/PI is highly subjective, since usually two researchers estimate the percentages of living and dead cells under a fluorescence microscopy, with the final result being the mean of their values (19). Accordingly, in the present study, we reported that the differences between FDA/PI derived-viability percentages obtained by each of well-trained researchers that estimated this outcome ranged from 4.71 to 20.74%, increasing the SD among experiments. Even in those Centers that use software to quantify FDA/PI fluorescence in the islet preparation, differences in exposure time and camera/software settings can also impact interpretations of viability (22). The greater sensitivity of image acquisition with a CCD camera might lead to inadequate evaluations merely due to manipulation of exposure time or other image capture parameters (22).

Second, because FDA nonspecifically penetrates all living cells present in the islet preparation, including islets and contaminating acinar cells, this complicates an accurate viability estimation of the islet population (19). Third, the non-fluorescent FDA is converted to the green fluorescent dye fluorescein by an esterase present in the cytoplasm of the living cells. However, some dead cells may still exhibit residual esterase activity, complicating the identification of dead cells that show staining with both dyes (22). In addition, PI only enter those cells that lost selective membrane permeability, not being able to identify early apoptotic cells, which still have an intact membrane permeability (17, 19). Fourth, several studies have shown that FDA/PI viability assay does not correlate well with post-transplantation islet function (19, 22, 28, 29). For example, Papas *et al*. (29) demonstrated that the FDA/PI assay was not adequate to predict diabetes reversal in nude mice transplanted with human islets compared to measurement fractional viability using oxygen consumption rate (OCR)/DNA rate. Islets with high ratio of OCR/DNA has higher chances of reversing diabetes in mice than islets with low OCR/DNA, despite the fact that islets in either category had excellent viability (≅ 90%) based on FDA/PI assay. Lastly, other issues related to the stability of the dyes in storage, stock solutions, and staining incubation time may also influence the intensity of the cell staining, thus, influencing the final scoring of viability (22). These limitations suggest that FDA/PI assay may not be the optimal approach to assess islet viability, and alternative methods need to be tested.

In comparison to FDA/PI assay, flow cytometry is an automated technique that provides more accurate measurements and allows the use of specific antibodies that can detect rare populations in a certain cell group (24, 25). In the case of islet preparations, it can allow the identification of cells that are suffering early apoptosis that, when assessed by FDA/PI staining, are counted as living cells. The correct identification of

living cells is a topic of great importance since the number of transplanted functional islets is highly correlated with the success of the transplant (17, 23, 30). Even though flow cytometry is more precise than FDA/PI staining in estimating islet viability, it is a more expensive technique considering the dyes/antibodies that are necessary as well as the requirement of a cytometry equipment. Besides that, it is a more time-consuming technique (≅ 2 h compared to ≅ 30-45 min for FDA/PI), but this duration could be done timely during the final evaluation of the islet product before releasement for islet transplantation. Based on the current viability parameter used for clinical islet product release (≥ 70% of living islets), both tested techniques would have reached islet product approval for transplantation. However, accumulated evidence in the last few years has shown that even small increments in islet viability measured with techniques other than FDA/PI are associated with better islet transplantation outcomes (22, 28, 31-33).

In conclusion, although flow cytometry is a more expensive and time-consuming technique than FDA/PI staining, it is a more accurate and informative technique since the use of 7AAD and Annexin V-APC allow the differentiation of living islets, early apoptotic islets and islets that already underwent apoptosis/necrosis. The differentiation among these cells may be useful to better understanding the quality of the islet recovery after culture. Therefore, we believe that assessing islet viability using flow cytometry should be the technique of choice to release islet for transplantation.

Conflicts of interest:

The authors declare no conflict of interest.

Ethical approval:

This study was approved by the Ethics Committee for the Use of Animals (CEUA) from Hospital de Clínicas de Porto Alegre (project number: 13-0166), Rio Grande do Sul, Brazil.

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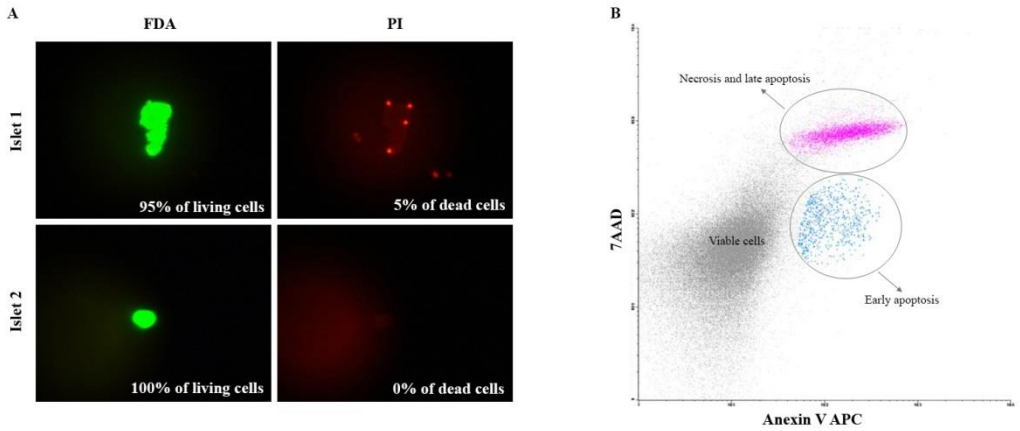
Figure legends

Figure 1. Representative figures of FDA/PI and flow cytometry use for assessing islet viability. A) Rat pancreatic islet stained with FDA (green) and PI (red), showing the estimated percentage of living (green) and dead (red) cells by a well-trained research. **B)** Gate strategies used to assess islet viability by flow cytometry using 7AAD and Annexin V-APC. Islet were dissociate as single cells before treatment with 7AAD and Annexin V-APC. Approximately 100.000 cells were evaluated in this technique. Early apoptotic cells are those marked only with Annexin V-APC while necrotic/late apoptotic cells are marked with both 7AAD and Annexin V-APC.

Figure 2. Islet viability results (% of living cells) assessed by FDA/PI staining (dark grey) or flow cytometry (lighter grey) for each of the ten experiment performed after islet isolation from Wistar rats. For FDA/PI staining, islet viability percentage in each experiment is the mean value obtained by the two-trained researchers that estimated this parameter.

Figure 3. Mean viability percentages obtained using FDA/PI staining and flow cytometry. Results are the mean values for 10 experiments in each technique. (* P = 0.002).



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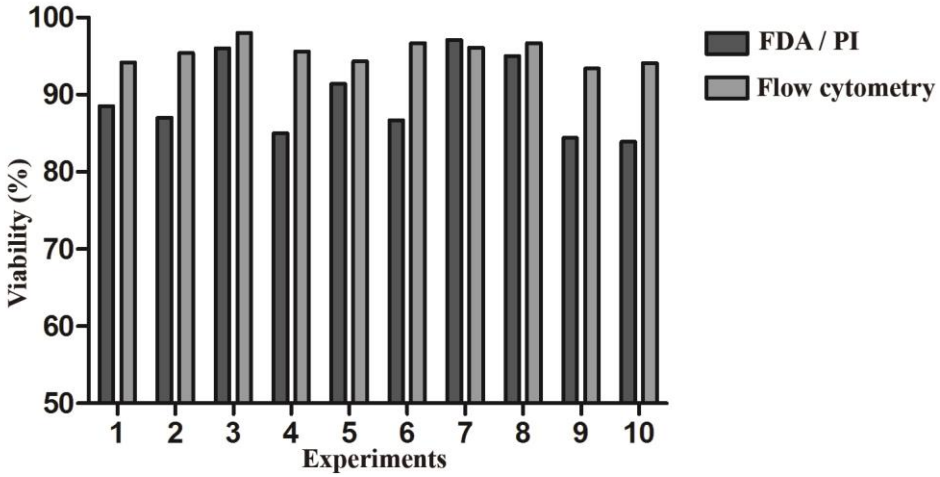

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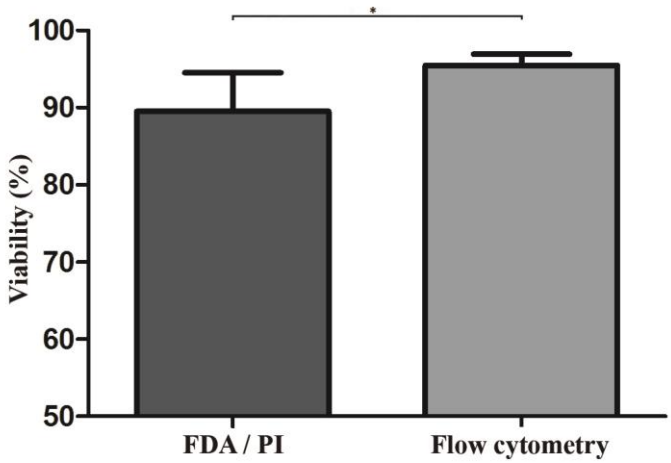

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ARTIGO 3

Exenatide improves function of rat pancreatic islets exposed to inflammatory stress: an in vitro study

Nota:

O presente artigo não é a versão final a ser enviada para publicação. Ele apresenta apenas os resultados do estudo do efeito da adição de dois análogos de GLP-1 no meio de cultura das ilhotas pancreáticas de ratos submetidas a estresse inflamatório sobre os desfechos viabilidade e função (secreção de insulina). Ainda estamos realizando experimentos adicionais para avaliar se esses análogos mudam a expressão de genes relacionados a rotas de apoptose/inflamação [*Bip, Chop, IK-Bα, Nlrp3, Bcl-2, BclxL* e *Bim* e suas isoformas (*BimEl, BimL e BimS*)], estresse oxidativo e hipóxia (*Ucp2, Sod2, Inos, Hif-1α*) e sinalização da insulina (*Ins-2, Glis3, MafA e Glut2*). Além da expressão gênica, também serão realizadas expressões proteicas de Bim e suas isoformas (BimEL, BimL e BimS), Bcl2 e ERK1/2. Esses dados não foram incluídos na presente tese, pois os experimentos para extração de RNA e proteína das ilhotas tratadas e não-tratadas com os análogos estão sendo feitos em animais diferentes daqueles já usados para avaliação de viabilidade e função. Visto que tivemos problemas técnicos durante a realização desses experimentos (dificuldade em conseguir o número suficiente de animais e troca de lote de colagenase) necessitaremos de mais alguns meses para finalização do estudo.

Exenatide improves function of rat pancreatic islets exposed to inflammatory stress: an in vitro study

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Abstract

Background: The isolation of viable and functional pancreatic islets from donors with brain death is crucial for successful islet transplantation in patients with unstable type 1 diabetes mellitus. Therefore, many studies have tested a variety of additives of culture medium to preserve islet quality. Glucagon-like peptide-1 (GLP-1) analogues have been described as having anti-inflammatory, antiapoptotic and cytoprotective properties on islets. Thus, here, we analyzed if the addition of two GLP-1 analogues, exendin-4 (EXE) and liraglutide (LIRA), to the culture medium of islets could preserve their viability and function (GSIS) under an inflammatory environment. **Methods**: Islets isolated from male Wistar rats were cultured under the following conditions: 1) Control; 2) "Pool of proinflammatory cytokines (TNF, IFN-γ and IL-1β); 3) EXE; 4) EXE + cytokines; 5) LIRA; and 6) LIRA + cytokines. After 72 h of culture, we quantified islet viability by flow cytometry, using 7AAD/Annexin V-APC markers, and stimulation index (SI) through glucose-stimulated insulin secretion. **Results**: As expected, cytokine treatment decreased islet viability; however, EXE or LIRA treatment did not improve islet viability ($P > 0.05$). EXE treatment was able to improve the SI of islets submitted to inflammation compared to islets treated with cytokines only (group 4: 1.21 ± 0.40 *vs*. group 2: 0.60 ± 0.22 ($P= 0.037$). LIRA did not influence the SI of islets in the presence or absence of cytokines ($P= 0.469$). **Conclusion**: EXE seems to play a protective role on islet function of rats against a direct inflammatory stress.

Key-words: pancreatic islets; GLP-1 analogues; insulin secretion; viability.

Introduction

Pancreatic islet transplantation is an effective therapeutic option for patients with type 1 diabetes mellitus (T1DM) that suffer from hypoglycemia unawareness with frequent episodes of hypoglycemia and marked glycemic lability (1-5). Although improvement in glycemic control and hypoglycemia awareness are normally achieved in T1DM patients transplanted with a sup-optimal islet mass, insulin independence usually requires islet transplantation from two or more donors, limiting the number of patients who may benefit from this therapy (1, 2). This occurs, in part, due to the marked loss of viable islets during isolation and culture period, which is a consequence of a number of insults, such as brain-death's (BD) donor-related factors, oxidative, hypoxic, and pro-inflammatory stresses, nutrient deprivation, and harmful enzymes released by acinar tissue (6-8). In this context, developing new strategies to prevent islet quality and quantity loss during islet isolation and culture is a topic of great interest.

Currently, most of the islet isolation centers keep the isolated islets in culture for 24–72 hours prior transplantation, allowing them to recover from the stress generated during the isolation and also allowing the preparation of the recipient for islet transplantation (6, 9). This culture period also provides a window for opportunity of applying strategies that will help islet recovery, such as the addition of different additives in the islet culture medium (9-14). Among the additive tested in these studies are the incretin hormones, predominantly the glucagon-like peptide 1 (GLP-1) and its analogues; however, their roles on islet quality improvement are still not clear (9, 10, 15, 16).

GLP-1 is produced in the enteroendocrine L cells of the small intestine and colon, and its secretion is dependent on the presence of nutrients in the intestinal lumen (17). Its release into the bloodstream stimulates glucose-dependent insulin secretion and

inhibits glucagon secretion, consequently reducing glucose production by the liver and blood glucose concentrations (17). Accordingly, GLP-1 analogues are approved therapy for type 2 diabetes mellitus (T2DM) (18). Exenatide (EXE) and liraglutide (LIRA) are the two main GLP-1 analogues currently used. EXE is a GLP-1 receptor (GLP1-R) agonist produced from a natural compound found in the salivary gland of the lizard Gila Monster (*Heloderma suspectum*). It is more resistant to the action of dipeptidyl peptidase-4 (DPP-4), having a longer half-life (1 - 1.5 h) than the natural GLP-1 (< 3 minutes). LIRA, a long-acting agonist of the GLP-1R has a half-life 14 h longer than EXE (19).

In addition to their insulinotropic effects, GLP-1 analogues have also been shown to directly prevent beta-cells apoptosis triggered by lipotoxicity, pro-inflammatory cytokines, glucocorticoids, streptozotocin and BD (9, 20-24). In human islets, addition of GLP-1 or EXE to the culture medium seems to increase both viability and insulin stimulation index (SI) compared to non-treated islets (15, 16, 25-28). However, LIRA treatment did not improve viability and function of human islets (10). Therefore, the aim of this study was to evaluate if the addition of GLP-1 analogues (EXE and LIRA) to the culture medium could improve viability and SI of rat pancreatic islets submitted to an inflammatory stress.

Methods

Animals and experimental design

Male Wistar rats fed with standard laboratory diet *ad libitum*, weighing 300 to 350 g, were used in the study. All animals were kept in the animal facility of the Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) and were cared following the guidelines for use and care of laboratory animal (29). The study was approved by the local ethics

committee for the use of animals in research. Twenty-one animals were used in the experiments, being 12 used to SI evaluation (3 by experiment, totalizing 4 experiments) and 9 used to viability tests (3 by experiment, totalizing 3 experiments). The following experimental groups were analyzed: 1) control islets (culture medium only); 2) islets treated with a "pool" of proinflammatory cytokines (TNF, IFN-γ and IL-1β); 3) Islets treated with EXE; 4) Islets treated with EXE + cytokines; 5) Islets treated with LIRA; and 6) Islets treated with LIRA + cytokines.

Islet isolation and culture conditions

Islet isolation was performed following the protocol reported by Carter *et al*. (30). After euthanasia, the bile duct was cannulated to allow pancreatic perfusion with 10 mL of cold Hank's balanced salt solution (HBSS, Sigma-Aldrich, Saint Louis, MO, USA) containing 0.5 mg/mL of collagenase P (Roche Diagnostics, Mannheim, BW, Germany). The perfused pancreas was removed and digested for 15 min at 37°C. Digestion was stopped by the addition of RPMI 1640 medium (Thermo Fisher Scientific, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 25 mmol/L HEPES (Thermo Fisher Scientific). Islet purification was performed using a discontinuous density gradient of Histopaque (Sigma-Aldrich) at 1.119, 1.100, and 1.077 g/mL density layers. An aliquot of purified islets was counted under a microscope to obtain the total number of isolated islets. Then, they were plated, in duplicates, accordingly to the 6 experimental conditions described above. Immediately after plating, 10 nM EXE (Byetta, AstraZeneca, London, England) was added to groups 3, 4 and 100 nM LIRA (Victoza, Novo Nordisk, Bagsvaerd, Denmark) to groups 5 and 6. The concentrations of these GLP-1 analogues were selected based on previous studies (28, 31-34). The culture medium of all groups was changed after the first 24 h and EXE and LIRA were replaced

again accordingly to groups. The “pool” of proinflammatory cytokines was added in the last 48 h of culture to groups 2, 4, and 6, and it was composed by 1000U/mL TNF, 100U/mL IFN-γ, and 50U/mL IL-1β (Thermo Fisher Scientific) (20, 35). Islets were maintained in culture for 72 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Both islet viability and function (SI) were analyzed immediately after the 72 h of culture.

Assessment of islet viability

For islet viability assessment using flow cytometry (36, 37), first we dispersed 100 islets/condition into single cells (n = 3 experiments). For this, isolated islets were immediately washed with HBSS (without calcium chloride and magnesium sulphate; Sigma-Aldrich) and dispersed with 0.125% Trypsin-EDTA (Thermo Fisher Scientific) for 5 min at 37 °C. Dispersion was stopped by addition of supplemented RPMI 1640 medium. Next, the obtained single cells were re-suspended in phosphate buffered saline 1x (PBS, LGC, Cotia, SP, Brazil) and Annexin V-APC binding buffer (BD, Franklin Lakes, NJ, USA). Then, 5 µL of Annexin V-APC antibody (BD) was added in the preparation of cells, which were incubated for 15 min at room temperature, protected from light. After this incubation, 5µL of 7AAD fluorophore (BD) was added. Next, 100,000 cells were acquired on the FacsCanto II (BD). Results were analyzed using Infinicyt flow cytometry software (Cytognos, Santa Marta de Tormes, Salamanca, Spain). Cells stained with both 7AAD and Annexin V-APC were those that had undergone rupture of the membrane, being in the process of late apoptosis or necrosis. Cells marked only with Annexin V-APC were those that had intact membrane, but were already in process of early apoptosis. Those cells that did not stain with any dye were the living cells.

Assessment of insulin stimulation index (SI)

In vitro insulin secretion was evaluated using statistic incubation of isolated islets (n = 4) with two glucose concentrations (2.8 mM and 28 mM). Briefly, 100 islets by each experimental group were collected from plates and transferred to 2 mL tubes. After a few minutes, 100 µL of the pelleted islets was transferred to 24-well plates and pre-incubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer (KRB) (20) containing 2.8 mM glucose (Sigma-Aldrich). Then, the medium was replaced with 1 mL of fresh KRB + 2.8 mM glucose (basal glucose stimulation), followed by 1 h of incubation under the same condition. Next, the supernatant was replaced with KRB + 28 mM glucose (high glucose stimulation), and plates were incubated for an additional 1 h at 37°C. Both after basal- and high-glucose stimulations, supernatants were collected and stored at -80°C until measuring of secreted insulin using a commercial rat ELISA kit (Millipore, Billerica, MA, USA), following manufacturer's instructions. Secreted insulin was normalized by total DNA, which was extracted by boiling islets in 100 µL PBS buffer for 20 min, and quantified using the Quanti-iT-PicoGreen kit (Thermo Fisher Scientific). The SI was defined as the ratio of normalized insulin levels after high glucose stimulation to that of normalized insulin levels after basal glucose incubation (20).

Statistical analysis

Variables are shown as mean ± standard deviation (SD). Differences among groups were tested using student's *t*-tests. P values <0.05 (two-tailed) were considered statistically significant. All analyses were performed using SPSS 18 (SPSS Inc., Chicago, IL, USA).

Results

Effects of EXE and LIRA treatment on islet viability

Figure 1 shows the gate strategies used in flow cytometry with 7AAD and Annexin V for assessment of islet viability accordingly to the different experimental groups. The lower left quadrant shows living cells that did not stain with Annexin V and 7AAD. The lower right quadrant shows cells in early apoptosis process (those cells stained only with Annexin V), while the upper right quadrant depicts cells suffering late apoptosis or necrosis (cells stained with both 7AAD and Annexin V).

As expected, the percentage of islet viability decreased in ≅ 40% after treatment with cytokines compared to islets of the control condition (group 2: 61.8 ± 16.3% *vs*. group 1: 96.2 ± 1.0%; P = 0.066; **Figure 2**). EXE treatment was not able to protect islets against proinflammatory cytokines (group 4: 62.5% ± 11.1% *vs*. group 2: 61.8% ± 16.3%; P = 0.952; **Figure 3A**). Moreover, the percentages of early apoptosis (24.99 ± 8.29 *vs*. 26.16 ± 16.88; P = 0.919; **Figure 3B**) and necrosis + late apoptosis (12.5 ± 2.9 *vs*. 12.1 ± 3.4; P = 0.881; **Figure 3C**) were similar between groups 4 and 2, respectively.

Viability also did not differ significantly between islets treated with LIRA compared to islets treated only with cytokines (group 6: 54.2 ± 9.9% *vs*. group 2: 61.8% ± 16.3%; P = 0.529; **Figure 3A)**. The percentages of early apoptosis (P = 0.552) and necrosis + late apoptosis (P = 0.788) were also not affected by LIRA treatment (*vs*. group 2; **Figure 3C**). Of note, viability was similar between islets of the control (group 1), EXE (group 3) and LIRA (group 5) conditions (**Figure 3A**).

Effects of EXE and LIRA treatment on insulin stimulation index (SI)

In agreement with the viability data, cytokine treatment also significantly decreased SI (in ≅ 7 folds) compared to the control group (group 2: 0.6 ± 0.2% *vs*. group 1: 4.7 ± 2.6%; P = 0.048; **Figure 2**). Interestingly, EXE treatment improved in two folds the SI of islets exposed to inflammation compared to the cytokine-treated group (group 4: 1.2 ± 0.4 *vs.* group 2: 0.6 ± 0.2; P = 0.037; **Figure 4**). LIRA, however, failed to significantly improve SI in the presence of inflammation (group 6: 0.9 ± 0.4 *vs*. group 2: 0.6 ± 0.2; P = 0.469). In addition, the SI was similar between islet from the control (group 1), EXE (group 3) and LIRA (group 5) conditions (**Figure 4**).

Discussion

Successful islet transplantation as a therapy for T1DM is not only dependent on the number of transplanted islets, but also on their quality (2, 38). Therefore, preserving islet mass and quality during culture period could improve the clinical outcomes of islet transplantation, preventing early graft failure, and may also decrease the requirement for multiple organ donors (2). In this context, the addition of different additives to the culture medium has been shown optimistic results regarding the preservation of islet quality during culture (9). Accordingly, here, we showed that EXE addition to the culture medium seems to protect rat pancreatic islets against a decrease of function (SI) triggered by a direct inflammatory stress. EXE treatment had no significant effect on islet viability.

A number of *in vitro* and *in vivo* studies have shown that EXE has protective effects on beta-cells against insults triggered by inflammation (21, 25, 39), lipotoxicity (24, 40), glucocorticoids (16, 23), hypoxia (41, 42), STZ treatment in mice (22), and BD of the rat pancreas donor (20). The improvement in SI values by EXE treatment observed here is in accordance with previous studies in human islets (15, 16, 26-28), rat

and mice islets (20, 41) and beta-cell lines (41, 42). It is suggested that for product release for transplantation, isolated islets should reach SI values >1.0 (43). Therefore, in a clinical transplanting setting, a two-fold increase in SI values, as observed here, would improve islet function, reaching the SI values suggested as minimum for transplantation.

The exact mechanism by which EXE improves SI by stimulating GSIS is not fully understood. Lupi *et al*. (26, 27) showed that treatment with EXE improved SI after 48 h of culture of islets isolated from non-diabetic and T2DM donors. Interestingly, these authors demonstrated that EXE increased the expression of several genes involved in insulin secretion and regulation of glucose utilization, and beta-cell differentiation and proliferation, such as *INS*, *GLUT2*, *GCK*, *PDX-1*, *E2F1*, *cyclin D1*, and *KI67* (27). Hence, GSIS improvement in EXE-treated islets is mediated, at least in part, by an action at the transcriptional level (27). Moreover, GLP-1 signaling was found to elevate cytosolic Ca_2 (44), cAMP/PKA (45, 46), and PKB (also known as AKT) (39) pathways in target cells, which might contribute to increase GSIS. The increase in cytosolic Ca_2 concentration is an important trigger of the secretion of insulin-containing granules (47). It has been suggested that the activation of CREB, a cAMP-dependent transcription factor, may provide a direct link between GLP-1R activation and the PI3K-PKB pathway through the enhanced synthesis of IRS-2, which potentiates insulin/IGF signaling (48). Furthermore, EXE has been shown to induce activation of ERK1/2, which play important roles in insulin secretion as well as cell survival, proliferation, and protection against apoptosis (20, 25). Padmasekar *et al*. (41) reported that EXE treatment was also able to reduce oxidative stress in INS-1 cells (a rat-derived beta-cell line); thus, increasing GSIS.

Several mechanisms have been suggested for EXE protection against apoptosis, such as counter-regulation of the decrease in electron transport chain proteins (49), reduction in the proapoptotic *TXNIP* expression (50), reduction of endoplasmic reticulum stress (20, 51), down-regulation of the NFκB-INOS-nitric oxide pathway (52), and inhibition of the c-Jun N-terminal kinase pathway (21) and activated caspase 3 (25). Accordingly, several studies have shown that the addition of EXE to the culture medium of islets or beta-cell lines improves viability (15, 16, 21, 22, 24, 25, 41, 42). In contrast, in the present study, we were not able to observe any significant effect of EXE treatment on viability of islet isolated from rats.

Here, we also did not observe any effect of LIRA treatment on islet viability and function after 72 h of culture in presence or absence of proinflammatory cytokines. This result is in agreement with a previous study performed with human islets that showed that LIRA addition to the culture medium had no effect on islet viability after 48 h of culture, although it reduced the apoptosis of insulin-positive cells after 24 h of culture (10). Moreover, this study also demonstrated that LIRA treatment during culture did not improve the SI (10). Nevertheless, the addition of LIRA to the static incubation medium during GSIS test increased the release of insulin after stimulation with high glucose concentration compared to the control condition, suggesting that this drug should be continuously administered in order to observe the metabolic effect associated to it (10). These data could also explain our negative results on the effect of LIRA treatment. Toso *et al*. (10) also reported the islet treated with LIRA during culture demonstrated similar post-transplant engraftment in nude mice to controls. In contrast, the *in vivo* treatment with LIRA significantly improved the rate of engraftment, suggesting that the *in vitro* treatment with LIRA should be followed by a post-transplant LIRA administration to the recipient. Accordingly to our data, the study by Zhao *et al*.

(53) also did not report an effect of LIRA treatment on viability of islet isolated from human pancreas; however they observed that LIRA was able to increase non-stimulated insulin secretion.

In the other hand, islets isolated from rats showed improved viability and insulin secretion when treated with LIRA during culture compared to non-treated islets, and this effect was due to anti-inflammatory, antioxidative and angiogenic properties of this drug (54, 55). Miao *et al*. (56) found that LIRA also protected INS-1 cells against apoptosis after 72 h of culture, which was mediated through the AMPK/mTOR signaling. Emamaullee *et al*. (57) reported that addition of LIRA to the culture medium improved porcine islet quality, particularly during the first 24 h, where a significant reduction in apoptosis and an increase in SI were observed. However, after 48 h of culture, the percentage of apoptosis and SI were similar between vehicle- and LIRA-treated conditions, also suggesting that the effect of LIRA requires continuous addition to the culture medium. Interestingly, Xu *et al*. (58) reported that LIRA treatment had different effects on pancreatic alfa- and beta-cells: LIRA improved both viability and insulin secretion of mice beta-cells (β-TC-tet cell line) while decreased glucagon secretion and increased apoptosis of murine alfa-cells (α-TC1-6 cells), mainly due to the respective activation and inhibition of the cAMP-PKA pathway. Hence, it is possible that we did not observe any effect of LIRA on islet viability, and possible of EXE, due to the differential effect of this drug on islet cells, which were analyzed together during flow cytometry analysis.

In conclusion, our results demonstrated that EXE treatment during culture is able to preserve islet function in the presence of a cytokine-induced inflammatory stress. Additional studies are needed in order to confirm EXE and LIRA effects on islet viability and function during culture.

Conflicts of interest:

The authors declare no conflict of interest.

Ethical approval:

This study was approved by the Ethics Committee for the Use of Animals (CEUA) from Hospital de Clínicas de Porto Alegre (project number: 13-0166), Rio Grande do Sul, Brazil.

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Figure legends:

Figure 1. Representative image of the gates strategy used for flow cytometry viability analysis. In the first line are the controls. In the second line are the exenatide groups. In the third line are the liraglutide groups. EXE: exenatide; LIRA: liraglutide.

Figure 2. Viability and SI only among the control – control (group 1) and cytokines (group 2) groups, demonstrating the effectiveness of the *in vitro* experimental model used. Bars represent mean ± SD. **(A):** mean viability (%) results between groups (group 1 = 96.2% ± 1.01 and group 2 = 61.8% ± 16.3). * P = 0.066. **(B):** mean SI results between groups (group 1 = 4.7 ± 2.6 a group 2 = 0.60 ± 0.22). * P = 0.048.

Figure 3. Viability results assessed by flow cytometry. Bars represent mean (%) ± SD (n = 3 in all groups). P > 0.05. **(A):** mean viability results between groups. **(B):** mean early apoptosis results between groups. **(C):** mean necrosis and late apoptosis between groups.

Figure 4. Stimulation index (SI) results assessed by glucose-stimulated insulin secretion (GSIS). Bars represent mean ± SD (n = 4 in all groups). Mean SI results between groups (P = 0.037).

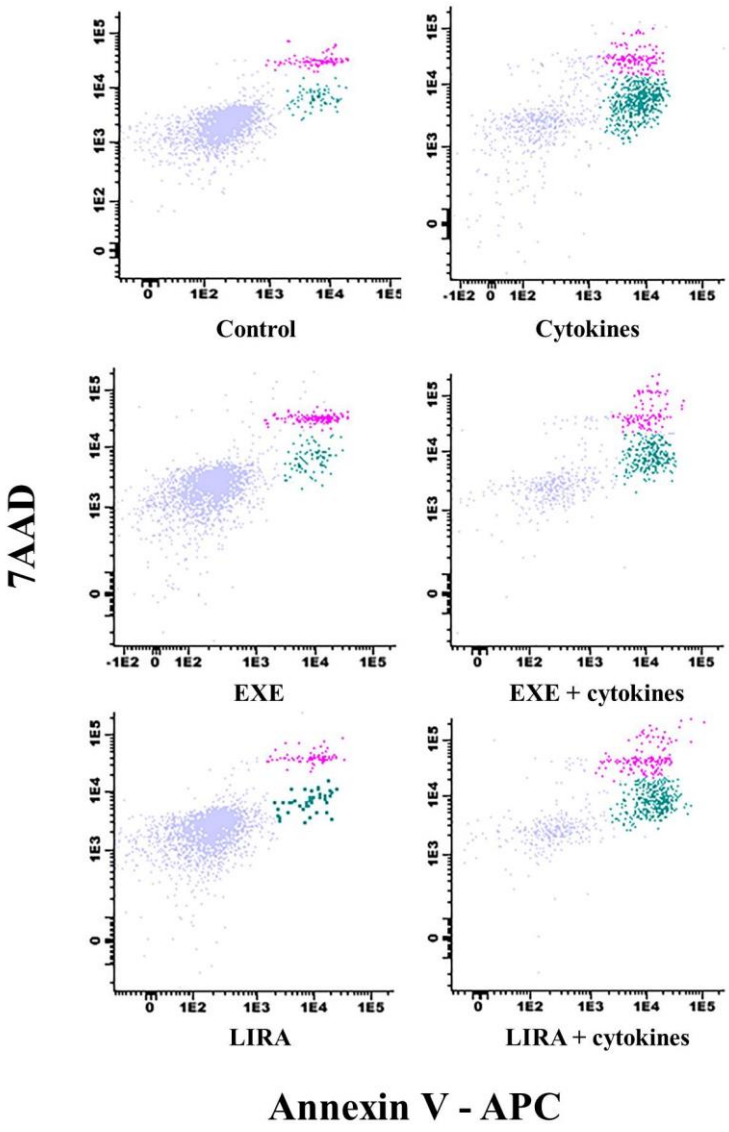


Figure 1. Representative image of the gates strategy used for flow cytometry viability analysis. In the first line are the controls. In the second line are the exenatide groups. In the third line are the liraglutide groups. EXE: exenatide; LIRA: liraglutide.

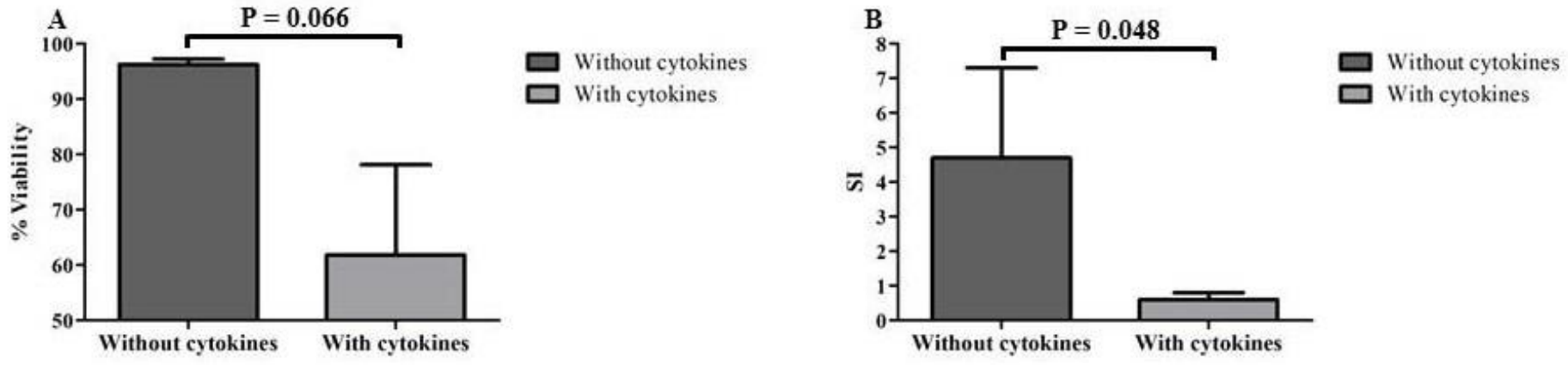
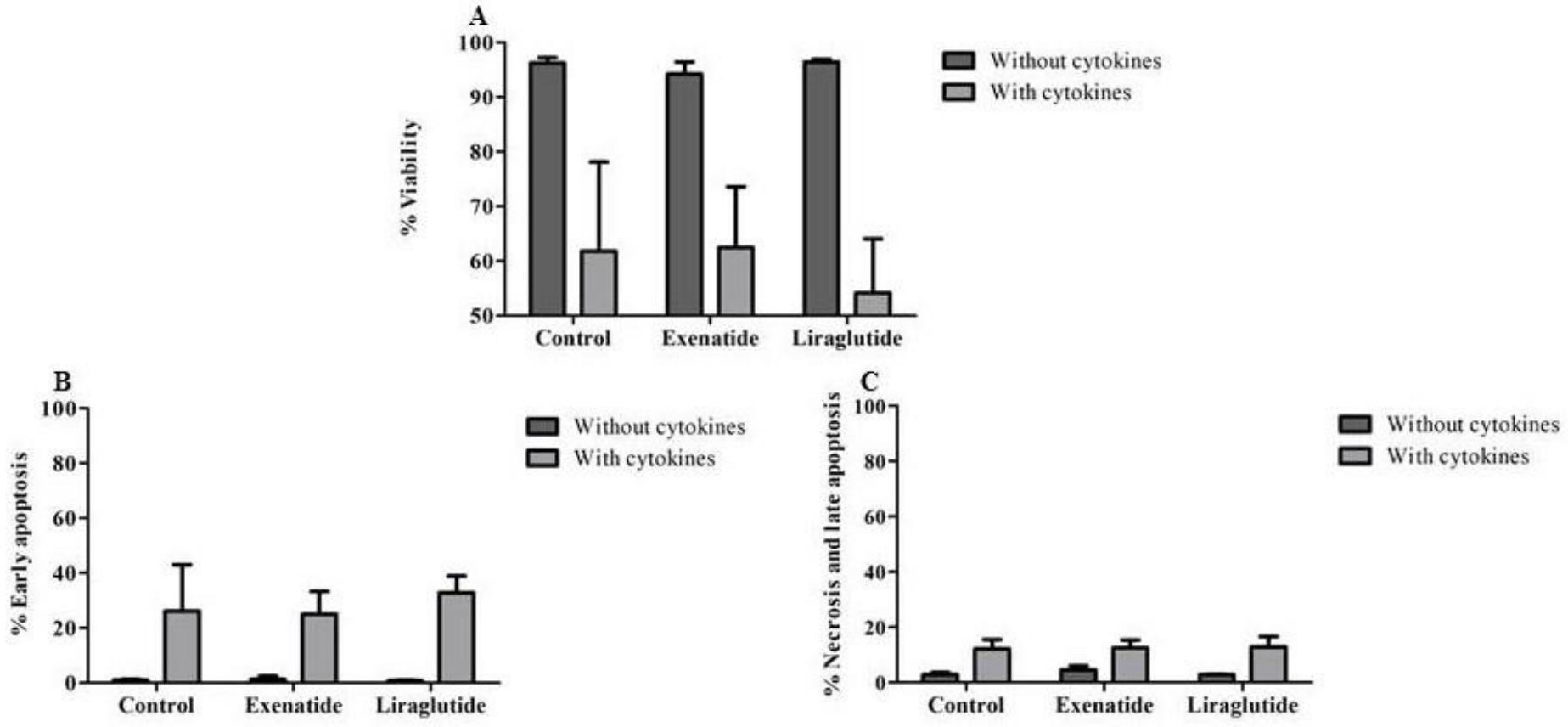


Figure 2. Viability and SI only among the control – control (group 1) and cytokines (group 2) groups, demonstrating the effectiveness of the *in vitro* experimental model used. Bars represent mean ± SD. **(A):** mean viability (%) results between groups (group 1 = 96.2% ± 1.01 and group 2 = 61.8% ± 16.3). * P = 0.066. **(B):** mean SI results between groups (group 1 = 4.7 ± 2.6 a group 2 = 0.60 ± 0.22). * P = 0.048.



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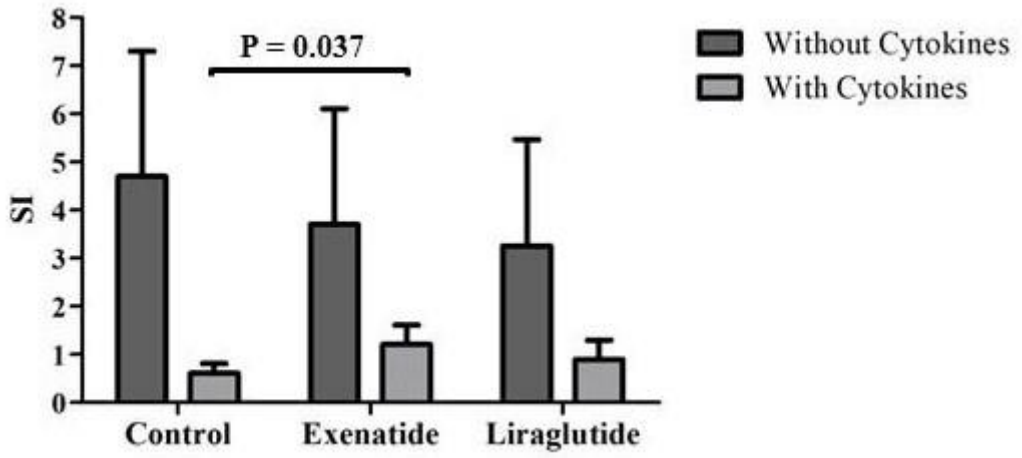

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ARTIGO 4

Renal effects of exendin-4 in an animal model of brain death

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Renal effects of exendin-4 in an animal model of brain death.

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Abstract

Background: Organ transplantation is the gold standard therapy for the majority of patients with terminal organ failure. However, it is still a limited treatment due, specially, to the low number of brain death (BD) donors in relation to the number of waiting list recipients. Strategies to increase the quantity and quality of donor organs have been studied, and the administration of Exendin-4 (Ex-4) to the donor may be a promising approach. **Methods:** Male Wistar rats were randomized into 3 groups: 1) control, without central nervous system injury; 2) BD induced experimentally, and 3) BD induced experimentally + Ex-4 administrated immediately after BD induction. After BD induction, animals were followed during 6 h before collection of blood and kidney biopsy. Kidney function was assessed by biochemical quantification of plasma kidney markers. Gene and protein expressions of inflammation and stress-related genes were evaluated by RT-qPCR and Immunoblot. **Results:** Animals treated with Ex-4 had lower levels of creatinine and urea compared with controls. BD induced oxidative stress in kidney tissue through increased expression of *Ucp2*, *Sod2* and *Inos*, and Ex-4 administration was able to reduce expressions of these genes. Ex-4 also induced an increase in the antiapoptotic *Bcl2* gene. *Nlrp3* and *Tnf* expressions were upregulated in the BD group compared with controls, but Ex-4 treatment had no effect on these genes. **Conclusions:** Our findings suggest that Ex-4 administration to BD rats reduces BD-induced kidney damages by decreasing oxidative stress genes and increasing *Bcl2*.

Keywords: brain death; kidney transplantation; renal tissue; exendin-4.

Introduction

Organ transplantation is currently the gold standard therapy for the majority of patients with terminal organ failure. However, it is still a limited treatment due, specially, to the low number of brain death (BD) donors in relation to the number of waiting list recipients. Because of this, a rising number of "expanded criteria" BD donors have been accepted for transplantation, even though this approach is related to increased risk for primary graft non-function and/or primary delayed function, in addition to worse long term graft survival [1]. There are currently over 120,000 patients waiting for lifesaving organ transplants in the United States of America. The majority of those patients (n= 100,791) is waiting for kidney transplants [2].

Therefore, strategies to increase the quantity and quality of donor organs have been studied and are of great interest [3-5]. One of the target points is the management of donor candidates with interventions to reduce damage caused by BD itself. It is known that BD is associated with a cascade of inflammatory reactions that can lead to donor organ dysfunction and has been associated with risk for rejection [6-9]. Despite the progressive improvement in kidney transplant graft survival rates in the last years, delayed graft function and rejection are still a reality on the field of organ transplantation, and BD environment is pointed out as a key factor involved in these negative outcomes [10-12].

In this context, our group has investigated, *in vivo*, strategies to improve the quality of organs from BD donors [13,14]. Using a rat model of BD, Carlessi *et al.* [14] showed that administration of exendin-4 (Ex-4), a glucagon like peptide-1 (GLP-1) analogue, to BD rats was able to increase both pancreatic islet viability and glucose-stimulated insulin secretion. These effects were mediated by modifications in genes related to oxidative stress, endoplasmic reticulum (ER) stress, and inflammation [14].

Furthermore, our group showed that administration of Ex-4 to rats alleviated the impact of BD on liver damage markers (aspartate aminotransferase and lactate dehydrogenase), which was accompanied by a reduction in hepatocyte apoptosis [13]. Accordingly, Chen *et al*. [15] demonstrated that Ex-4 was able to protect the renal tissue from damage caused by ischemia-reperfusion, reducing the oxidative stress and inflammatory reaction [15].

GLP-1 is a 30-amino-acid peptide hormone secreted from the L-cells of the intestinal epithelium in response to a meal [16]. Its analogue Ex-4 was approved as a therapy for type 2 diabetes since it enhances glucose-stimulated postprandial insulin release, inhibits inadequate glucagon secretion and delays gastro-intestinal motility [16,17]. Lately, anti-inflammatory, antiapoptotic and cytoprotective properties of GLP-1 analogues have been revealed, opening a new therapeutic perspective for this class of drugs [18-20,13,14]. Based on the previous findings, we hypothesized that administration of Ex-4 in BD donors would ameliorate markers of renal injury after organ procurement, which might, in turn, lead to a better renal graft outcome. Thus, the aim of the present study was to evaluate the effect of Ex-4 administration to a rat model of BD on kidney function markers, kidney cells apoptosis, and expression of genes related to oxidative stress, inflammation and proliferation.

Material and methods

Animals and experimental design

Male Wistar rats fed with standard laboratory diet *ad libitum*, weighing 300 to 350 g, were used in the study. All animals were kept in the animal facility of Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) and were cared following the guidelines

for use and care of laboratory animals [21,22]. The study was approved by the local ethics committee for the use of animals in research.

Animals were randomized into 3 experimental groups: 1) control, without central nervous system injury; 2) BD induced experimentally; and 3) BD induced experimentally + Ex-4 (BD + Ex-4). Animals from group 3 received, immediately after BD induction, an intraperitoneal administration of Ex-4 (Exenatide; AstraZeneca, London, UK) in the concentration of 5 µg/kg of body weight (**Fig. 1A**).

BD induction was done as previously reported by Contreras *et al*. [23]. Briefly, animals were anesthetized using a mixture of 2% isoflurane (Biochimico, Itatiaia, Brazil) in oxygen using a precision vaporizer (Takaoka, model 1415, Sao Paulo, Brazil). Then, they were intubated with a plastic cannula from a 16-gauge catheter (Abbocath; Abbott Laboratories, Abbott Park, IL, USA) and mechanically ventilated using a volumetric ventilator (Harvard Rodent Ventilator model 683; Harvard Apparatus Co., Millis, MA, USA). For BD induction, a hole was drilled at the left front-lateral region of the animal's skull to permit the passage of a No. 4 Fogarty catheter (Fogarty Arterial Embolectomy Catheter, 4F; Edwards Lifesciences, Irvine, CA, USA) without causing cerebral damage. Then, the balloon was inflated with 0.5 mL of saline for 1 minute. Animals in the control group (sham-operated) also had the catheter inserted but not inflated. Maximally dilated pupils, apnea, and absence of palpebral reflexes confirmed BD induction. Arterial blood pressure was monitored through cannulation of the left femoral artery, attached to a Biopac MP100 data acquisition system (Biopac Systems Inc., Goleta, CA, USA). Normotension was achieved by fluid administration of 2 mL saline solution immediately after BD induction. No norepinephrine administration was used to achieve normotension. Animals were maintained for 6 additional hours before cardiac puncture for total blood collection and kidney biopsy.

Plasmatic measurements of renal function

For all animals, 4 mL of total blood was collected, and plasma was obtained by centrifugation at 2000 rpm for 15 minutes at 4ºC. Creatinine was determined by Jaffe kinetic colorimetric method and urea was determined by the kinetic urease and glutamate dehydrogenase method, both in the ADVIA 1800 Clinical Chemistry System (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) equipment. Results are expressed as mg/dL.

Relative quantification of gene expressions

Kidney biopsies were excised, snapped-frozen in liquid nitrogen, and kept at -80°C until analysis. Renal tissue was homogenized in phenol-guanidine isothiocyanate (Trizol®, Thermo Fisher Scientific, Waltham, MA, USA). Then, RNA was extracted with chloroform and precipitated with isopropanol by centrifugation (12,000 rpm at 4°C). RNA pellets were washed with 75% ethanol and resuspended in 10 μL of diethylpyrocarbonate-treated water. Concentration and quality of RNA samples were evaluated using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and agarose gel electrophoresis, respectively.

Real-time reverse transcription polymerase chain reaction (PCR) was performed into 2 separated steps: first, RNA was reversed-transcribed into cDNA and, then, cDNA was amplified by quantitative real-time PCR (RT-qPCR). Reverse transcription of RNA into cDNA was performed using SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific), following the manufacturers protocol.

RT-qPCR reactions were performed in a 7500 RT-PCR system (Thermo Fisher Scientific), and are described according to Minimum Information for Publication of

Quantitative Real-Time PCR Experiments (MIQE) guidelines [24]. Analyzed genes and primers sequences are shown in **Table 1**. RT-qPCR experiments were done using 10 μL of Fast SYBR Green Master Mix (Thermo Fisher Scientific), 7 μL of H_2O, 1 μL (1 ng/μL) of forward and reverse primers, and 1 μL of cDNA template (200 ng/μL), in a total volume of 20 μL. Each sample was assayed in triplicate, and a negative control was included in each experiment. Relative expressions were calculated using the ΔΔCt method [25], and expressed in relation to the reference gene (cyclophilin A - *CypA*). Of note, *CypA* did not vary across different samples. Target and reference genes showed similar amplification efficiencies. RT-qPCR specificity was determined using melting curve analyses, and all primers generated amplicons that produced a single sharp peak during the analyses. Data are shown as n fold changes in relation to the control group.

Immunoblot and caspase-Glo 3/7 assay

Total protein extracts from frozen kidney biopsies were resolved on 12% polyacrylamide gels, transferred to Immobilon-P^{SQ} membranes (Millipore, Billerica, MA, USA), and incubated with monoclonal antibodies to total extracellular signal-regulated kinases 1/2 (ERK1/2, also called MAPK) or phosphoERK1/2 (Thr202/Tyr204, pERK1/2) (Cell Signaling Technology, Beverly, MA, USA). The secondary antibody consisted of horseradish peroxidase (HRP) conjugated goat anti-rabbit (Millipore). Detection was done using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Images were acquired in an ImageQuant LAS 500 digital imaging system (GE Healthcare, Piscataway, NJ, USA), and band densitometry analysis was performed using ImageJ v.1.47 (National Institutes of Health, Bethesda, MD, USA). Intensity values for the target proteins (pERK1/2)

were corrected by values of the respective reference proteins (ERK1/2), and are shown as n fold changes in relation to the control group.

Additionally, 10 μg of total protein extracts were used for quantification of caspase 3 and 7 activities using the Caspase-Glo 3/7 assay (Promega Corporation, Madison, WI, USA), following the manufacturer's instructions.

Statistical analysis

Variables are described as mean ± standard error (SE). Differences across groups were tested using student's *t*-tests. P values <0.05 (two-tailed) were considered as statistically significant. All analyses were performed using SPSS 18 (SPSS Inc., Chicago, IL, USA).

Results

Ex-4 effect on plasma markers of renal function

Renal function was evaluated by measurements of creatinine and urea levels in plasma. Creatinine levels were not significantly different between animals that underwent BD and sham-operated controls (P= 0.326), but BD animals treated with Ex-4 showed lower levels of this maker compared to controls (**Fig. 1B**; P <0.001). Moreover, urea levels did not differ between control and BD rats (116.5 ± 5.4 *vs*. 94.7 ± 31.3 mg/dL; P= 0.223); however, Ex-4 treatment reduced urea levels (65.8 ± 20.4) in BD animals when compared to the control group (P= 0.003).

Ex-4 prevents BD-induced increase in expression of oxidative stress-related genes

Oxidative stress seems to have a role in BD-induced organ damage [26,14,13,27], and Ex-4 is believed to have antioxidant properties [28,13]. Thus, to explore if Ex-4 treatment influences oxidative stress in renal tissue, we analyzed the expression of

uncoupling protein 2 (*Ucp2*), superoxide dismutase 2 (*Sod2*), and inducible nitric oxide synthase (*Inos*), which are known to be up-regulated in situations of increased oxidative stress [29,30].

Ucp2 expression was ≅ 3 fold-increased in the BD group compared to control animals (**Fig. 2A**, P= 0.006), and the Ex-4 treatment partially prevented BD-induced increase in *Ucp2* (P= 0.026). Accordingly, *Sod2* expression was increased in BD group compared to controls (**Fig. 2B**, P= 0.029), and decreased after Ex-4 treatment; although, this comparison did not reach conventional statistical significance (P= 0.088). Moreover, *Inos* expression that was 4 fold-increased in the BD group compared to control group (**Fig. 2C**; P= 0.065), returned to control values in the Ex-4 treated group (P= 0.021 *vs*. BD group).

Afterwards, we evaluated the expression of hypoxia-inducible factor 1-α (*Hif-1α*) (**Fig. 2D**), a transcription factor involved in the cellular response to hypoxia [31]. *Hif-1α* expression was up-regulated in the BD-group compared to the control group (P= 0.050), but treatment with Ex-4 was not able to significantly decrease the BD-induced alteration in this gene (P= 0.179).

BD induces up-regulation of inflammation- and anti-apoptotic-related genes

BD has been shown to induce gene expression of proinflammatory cytokines in different organs [14,13,23]. Therefore, we evaluated expressions of inflammation-related genes in renal tissue: interleukin-1β (*Il-1β*), tumor necrosis factor (*Tnf*), interleukin-6 (*Il-6*), and NLR family, pyrin domain containing 3 (*Nlrp3*). Both *Nlrp3* and *Tnf* were significantly up-regulated by BD compared to control animals (P= 0.023 and P= 0.014, respectively; **Fig. 3A and B**); however Ex-4 treatment was not able to

decrease the expression of these genes (P >0.50). *Il-1β* and *Il-6* expressions did not differ significantly among the three experimental groups (**Fig. 3C and D**).

Next, we evaluated the expression of the anti-apoptotic B cell lymphoma 2 (*Bcl-2*) gene in renal tissue (**Fig. 4A**). *Bcl-2* expression was similar between control and BD-groups, but was significantly increased after Ex-4 treatment (P= 0.041 *vs*. BD-group). Expression of the Nf-kappa-B inhibitor-α (*Nfkb1a*) gene was similar among the 3 experimental groups (P >0.05; **Fig. 4B**). This gene codes for the IκBα protein, an inhibitor of the Nf-κB transcription factor, which is associated with inflammation and apoptosis [32].

Finally, kidney protein extracts from all experimental groups were assayed for activated caspase-3 and caspase-7 activities using the Caspase-Glo 3/7 assay as a measurement of apoptosis (**Fig. 4C**). Caspase 3/7 activity seems to be increased in the BD group compared to controls (P= 0.092), but the Ex-4 treatment was not able to reduce caspase 3/7 levels (P= 0.640).

Proliferation

Studies have reported that GLP-1 analogues promote ERK1/2 phosphorylation/activation [33,34,14], which are widely expressed protein kinase intracellular signaling molecules involved in many functions, including regulation of cell proliferation. Thus, we also assessed the phosphorylation status of ERK1/2 in renal tissue from rats of the 3 experimental groups through immunoblot (**Fig. 5A**). Both pERK1 (**Fig. 5B**) and pERK2 (**Fig. 5C**) quantities were not significantly different among BD, BD + Ex4 and control groups (P <0.05).

Discussion

Significant better outcomes in graft survival after kidney transplantation occur when the kidney is retrieved from living donors compared to BD donors. Multiple mechanisms may justify this difference. Among them, BD-related injury seems to have a significant role in diminishing the quantity and quality of the organs available for transplantation [35,36,7]. BD is associated with a cascade of inflammatory reactions leading to serious pathophysiologic changes that may result in donor organ dysfunction [6,11,10,27]. Degenerative changes of renal structure and necrosis of renal proximal and distal tubules were found in BD donors [37]. Also, immune cells are found in higher quantities in kidney donors during BD, and the amplification of cytokines, chemokines, and adhesion molecules causes a chemotactic gradient that promotes the influx of leukocytes to the kidney, contributing to the BD-related injury [38,39].

Oxidative stress is one of the primary triggers of organ injury during BD, contributing to the development of complications following organ transplantation, specially ischemia-reperfusion (IR) injury, delayed graft function and primary allograft dysfunction [27,40,12]. In the present study, BD up-regulated *Ucp2* and *Sod2* expressions, while Ex-4 treatment was able to partially prevent BD-induced increase in their expressions. UCP2 is a mitochondrial protein that uncouples substrate oxidation from adenosine triphosphate (ATP) synthesis, thereby decreasing ATP production by mitochondrial respiratory chain. The uncoupling then leads to tissue-specific functions, including the reduction of reactive oxygen species (ROS) formation by mitochondria [29]. SOD2 codes for the mitochondrial superoxide dismutase 2 (MnSOD2), an important antioxidant enzyme that scavenges mitochondrial superoxide [30]. Based on our results, we therefore hypothesize that *Ucp2* and *Sod2* up-regulation in renal tissue of BD rats may be a compensatory response to counterbalance the increased oxidative

stress environment created by BD-induced injury. The fact that these two genes were not up-regulated in the BD + Ex-4 group may reflect the effective antioxidant properties of this medication, which is possibly preventing the up-regulation of Ucp2 and MnSod2.

In agreement with the present data, our group previously reported that *Ucp2* was increased in pancreas and liver of rats with BD [14,13]. Interestingly, Ex-4 treatment was able to reduce *Ucp2* expression in pancreatic tissue but not in liver [14,13]. Regarding *Sod2* expression, we showed that BD had no significant influence in *Sod2* expression in pancreatic tissue, while it up-regulated the expression of this gene in liver. Moreover, Ex-4 treatment increased *Sod2* expression in pancreas compared to both BD and control groups, but had no effect on *Sod2* expression in liver [13,14]. In humans, both *UCP2* and *SOD2* genes were showed to be up-regulated in pancreas from BD organ donors [41]. To our knowledge, no other study has evaluated *Ucp2* expression in human or animal models of BD. *Sod2* was also reported to be induced by BD in murine kidneys [42] and in liver from pigs [27].

Here, BD also induced an increase in *Inos* expression, which was prevented by Ex-4 treatment. iNOS is one of the three key nitric oxide (NO) synthases that generate the signaling molecule NO from L-arginine [43]. This enzyme is induced by pro-inflammatory cytokines, leading to massive NO production and, consequently, cytotoxic effects [32]. Hoeksma *et al*. [40] found an increase in *Inos* expression in renal tissue following BD induction. These authors suggested that although NO production by Inos could have a protective effect by increasing blood flow to different ischemic organs after BD induction, it will probably exert a negative effect because excess of NO will quickly react with oxidants, such as superoxide, forming a potent reactive oxidant, peroxynitrite, which may contribute to BD-induced renal dysfunction [40]. Although

other *in vivo* studies also have reported antioxidant actions of Ex-4 in renal tissue of murine models submitted to different experimental conditions [15,44,45], the full mechanism behind this effect still needs to be clarified.

HIF-1α is a master regulator of a broad range of genes that facilitate adaptation to low O_2 conditions [31]. We found that BD is associated with increased *Hif-1α* expression in renal tissue, but Ex-4 treatment had no effect on its expression. Carlessi *et al*. [13] previously showed that BD induced *Hif-1α* expression in liver of rats; however, Ex-4 treatment further increased *Hif-1α* expression in this tissue. Interestingly, it has recently been unveiled that *Hif-1α* is the transcription factor responsible to mediate metabolic adaptations in pancreatic β-cells chronically exposed to Ex-4 [46]. In this scenario, however, Hif-1α protein accumulates in response to prolonged exposure to Ex-4 through a post-transcriptional mechanism involving enhanced translation of *Hif-1α* mRNA. Therefore, it remains to be elucidated whether Ex-4 can control Hif-1α protein levels in the kidney in a similar way. In contrast, Saat *et al*. [47] did not observe any effect of BD injury on *Hif-1α* expression in renal tissue of rats. The role of HIF-1α on BD-induced renal injury needs to be confirmed in additional studies.

Cytokine up-regulation after BD has been reported for many authors in both clinical and experimental studies [14,13,38,23,12,47]. Saat *et al*. [47] reported that *Il-1β*, *Il6* and *Tnf* expressions were increased in kidneys of BD-induced rats compared to controls. However, two other studies only observed *Il6* [12,42] and *Il-1β* [42] up-regulations in renal tissue of BD rats. In our BD model, we did not observe any difference in *Il-1β* and *Il-6* expressions in renal tissues of the different experimental groups. *Tnf* expression was increased in BD rats compared to the control group, but Ex-4 treatment had no effect on its expression. The present data is in agreement with results from our previous study in liver of BD rats [13]. In pancreatic tissue, BD induced both

Il-1β and *Tnf* up-regulations, but Ex-4 only protected against BD-induced increase in *Il-1β* expression [14]. In other murine studies analyzing renal tissue, Ex-4 was able to reduce *Il-1β* and *Tnf* expressions after IR injury [15,44] or in streptozotocin-induced diabetic mice with tubular injury [45].

The inflammasome NLRP3 is a polyprotein complex that is activated by several exogenous and endogenous stimuli, leading to the sequential cleavage of caspase-1 and IL-1β, followed by secretion of active IL-1β; thus, playing a key role in the inflammatory process and innate immunity [48]. Here, BD was associated with an increase in *Nlrp3* expression; nevertheless, Ex-4 treatment was not able to decrease the expression of this gene. To our knowledge, no other study has evaluated *Nlrp3* expression after BD-induction. Although it is unclear what the priming step for Nlrp3 activation is in kidneys of BD rats, it may be possible that danger-associated molecular patterns (DAMPs) released after BD-induced renal injury could be activating the inflammasome. Interestingly, in a renal IR injury murine model, *Nlrp3* knockout protected mice against renal dysfunction [49]. This study also showed that in B16 cells (a murine melanoma cell line), Nlrp3 was capable of sensing cellular damage induced by hypoxic conditions [49]. Therefore, it is plausible that BD-induced hypoxia might be activating the Nlrp3 inflammasome in our BD model.

Currently, the gold standard marker of apoptotic cell death is activation of caspase-3, which is involved in the execution phase of both intrinsic and extrinsic apoptosis pathways [50]. A number of studies have suggested that BD is associated with increased apoptosis in different tissues [27,51,52,13], including the kidneys [53-55]. Accordingly, our study showed that apoptosis seems to be increased in kidney of BD rats. Ex-4 treatment did not decrease apoptosis of renal cells, which is in contrast to the study by Carlessi *et al*. [13] that reported that Ex-4 was able to decrease BD-induced

apoptosis in liver. Regarding the anti-apoptotic *Bcl-2* gene, results are more inconsistent, with some studies reporting *BCL-2* down-regulation in tissues from BD donors [53,56], while other studies did not show any difference in the expression of this gene between study groups [55,52,13]. Here, *Bcl-2* expression was similar between BD and control rats, but Ex-4 caused an up-regulation of this gene.

In our experimental model, BD induction did not significantly change creatinine and urea levels compared to control animals. Interestingly, these markers were decreased in BD rats after Ex-4 treatment. A number of studies have shown that BD is associated with decreased creatinine [27,42,40] and urea levels [27], contrasting with our data. Rebolledo *et al*. [57] observed that a slower speed of BD induction elicited more renal (measured by increased creatinine and urea levels) and hepatic effects compared to a faster speed of BD, as used in our BD model. Therefore, there is the possibility that our model of BD induction was not sufficient to cause significant changes in these renal markers, at least at 6 hours of BD. Ex-4 was previously shown to decrease creatinine levels [58] and renal tubular injury [45] in diabetic rats or mice compared to untreated controls. Moreover, Ex-4 therapy protected murine glomerulus from IR, decreasing creatinine levels [44].

The present study indicates that Ex-4 protects against BD-induced oxidative stress in renal tissue, as well as up-regulated the anti-apoptotic *Bcl-2* expression. Further studies are needed to clarify the mechanism by which Ex-4 acts in the kidneys. Although our group has already demonstrated the beneficial effects of this medication on liver, pancreatic islets and now in kidneys, clinical trials in human donors of multiple organs are necessary to investigate if Ex-4 administration might be a satisfactory strategy to protect multiple organs against BD-induced injury.

Compliance with ethical standards

Conflicts of interest:

The authors declare no conflict of interest.

Ethical approval:

This study was approved by the Ethics Committee for the Use of Animals (CEUA) from Hospital de Clínicas de Porto Alegre (project number: 11-0623), Rio Grande do Sul, Brazil.

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Table 1. Oligonucleotides used for RT-qPCR analyses.

Target	Forward and Reverse Sequences
Il-1β	5' CAGAACATAAGCCAACAAGTGGTATT 3'
	5' CACAGGGATTTTGTCGTTGCT 3'
Tnf	5' TGATCGGTCCCAACAAGGA 3'
	5' TGGGCTACGGGCTTGTCA 3'
Il-6	5' ATCTGCCCTTCAGGAACAGC 3'
	5' GAAGTAGGGAAGGCAGTGGC 3'
Sod2	5' GCCAAGGGAGATGTTACAACTCA 3'
	5' CCCCGCCATTGAACTTCA 3'
Ucp2	5' TCAACTGTACTGAGCTGGTGACCTA 3'
	5' GGAGGTCGTCTGTCATGAGGTT 3'
Hif-1α	5' TGTTTGATTTTACCCATCCATGTG 3'
	5' TTCTCACTGGGCCATTTCTGT 3'
Bcl2	5' GGCATCTGCACACCTGGAT 3'
	5' GGGCCATATAGTTCCACAAAGG 3'
Inos	5' GGCTCACGGTCAAGATCCA 3'
	5' ACTCGTACTTGGGATGCTCCAT 3'
Nlrp3	5' ACCAGCCAGAGTGGAATGATG 3'
	5' GCAAACCTATCCACTCCTCTTCA 3'
Nfkb1a	5' TTGGTCAGGTGAAGGGAGAC 3'
	5' GTCTCGGAGCTCAGGATCAC 3'
CypA	5' GTCAACCCCACCGTGTTCTTC 3'
	5' ACTTGCCACCAGTGCCATTATG 3'

IL-1β: Interleukin-1β; *Tnf*: Tumor necrosis factor; *IL-6:* interleukin-6; *Sod2*: Superoxide dismutase 2; Ucp2: uncoupling protein 2; *Hif-1α*: Hypoxia-inducible factor 1-α; *Bcl2:* B cell lymphoma 2; *Inos*: inducible nitric oxide synthase; *Nlrp3:* NLR family, pyrin domain containing 3; *Nfkb1a:* nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α; *CypA:* cyclophilin A.

Figure Legends

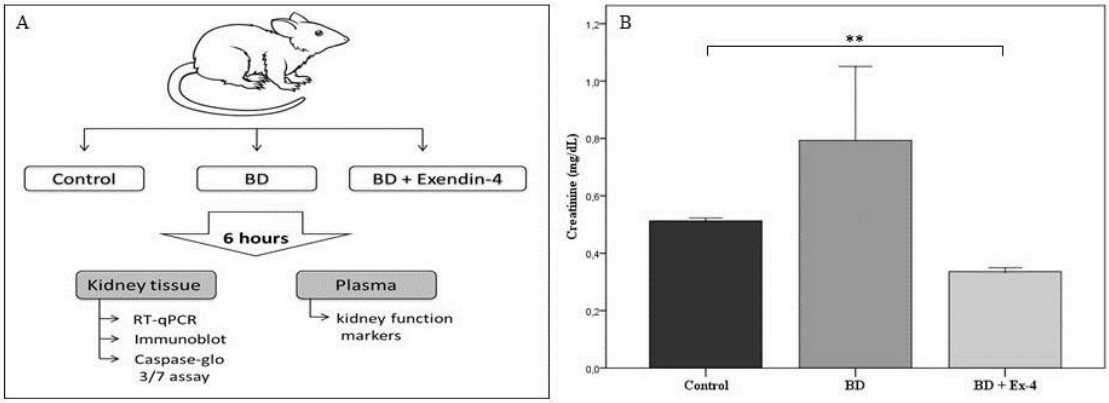
Figure 1. (A) Representative scheme of the methodology used in this study; **(B) Plasma creatinine level**s. Bars represent mean (mg/dL) ± SE (control group: n = 4; BD group: n = 6; and BD + Ex-4 group: n = 5). **P < 0.01.

Figure 2. Oxidative stress-related genes on renal tissue. Relative mRNA expressions of **(A)** *Ucp2*, **(B)** *Sod2*, **(C)** *Inos* and **(D)** *Hif-1α* were assessed in kidneys from all experimental groups by quantitative RT-qPCR. Results are expressed as mean n fold changes ± SE in comparison with control animals. *Cyclophilin A* was used as reference gene. Control group: n = 4; BD and BD + Ex-4 groups: n = 3. *P < 0.05, **P < 0.01. †: P > 0.05 but < 0.09.

Figure 3. *Nlrp3* inflammasome and proinflammatory cytokine genes on renal tissue. Relative mRNA expressions of **(A)** *Nlrp3*, **(B)** *Tnf*, **(C)** *Il-1β* and **(D)** *Il-6* were assessed in kidneys of animals from all experimental groups by RT-qPCR. Results are expressed as mean n fold changes ± SE in comparison with control animals. *Cyclophilin A* was used as reference gene. Control group: n = 4; BD and BD + Ex-4 groups: n = 3. *P < 0.05, **P < 0.01.

Figure 4. Apoptosis markers in renal tissue. Relative mRNA expressions of **(A)** *Bcl2* and **(B)** *NfκBia* were assessed in kidneys of animals from all experimental groups by RT-qPCR. Results are expressed as mean n fold changes ± SE in comparison with control animals. *Cyclophilin A* was used as reference gene. Control group: n = 4; BD and BD + Ex-4 groups: n = 3. *P < 0.05, †: P > 0.05 but < 0.09. **(C)** Caspase Glo 3/7 levels in renal tissue. N = 6 in each group. †: P > 0.05 but < 0.09.

Figure 5. ERK1/ERK2 phosphorilation in renal tissue. (A) Representative immunoblot for total and phospho ERK1/2, (**B)** Relative quantification of phospho ERK1 (pERK1)/total ERK1 ratio, **(C)** Relative quantification of phospho ERK2 (pERK2)/total ERK2 ratio. Results are expressed as mean n fold change ± SE in comparison with control animals. Control group: n = 3; BD and BD + Ex-4 groups: n = 5.



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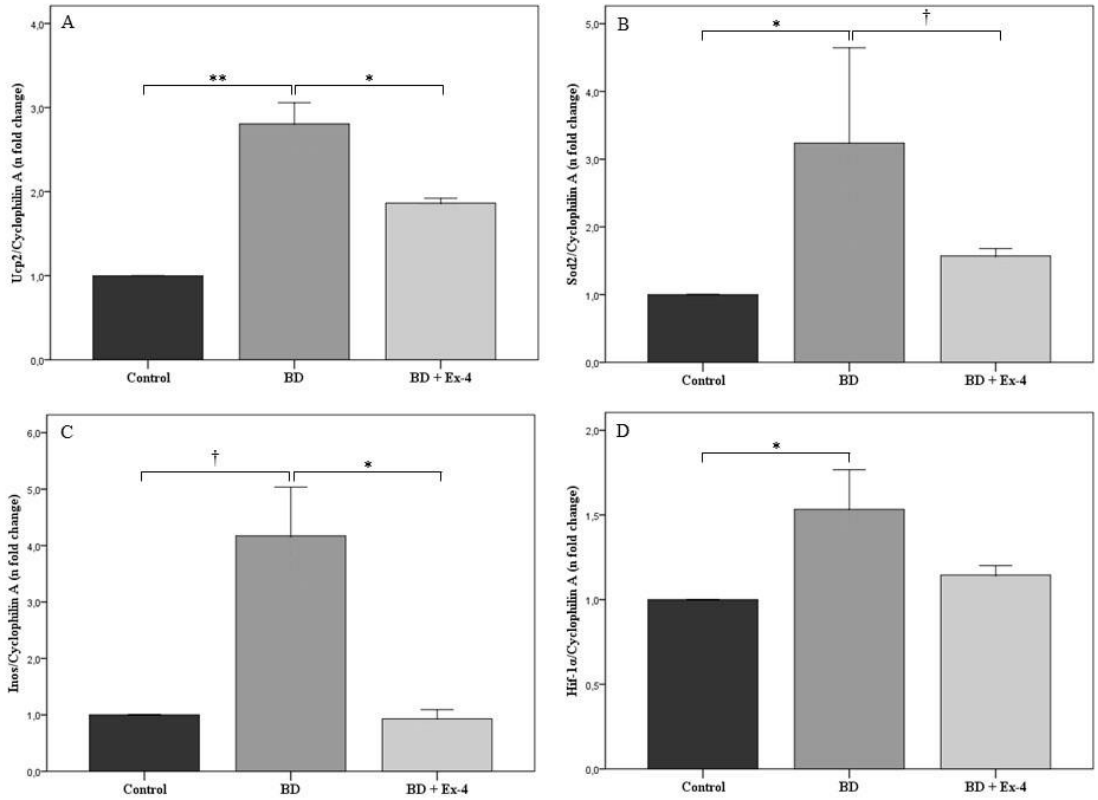

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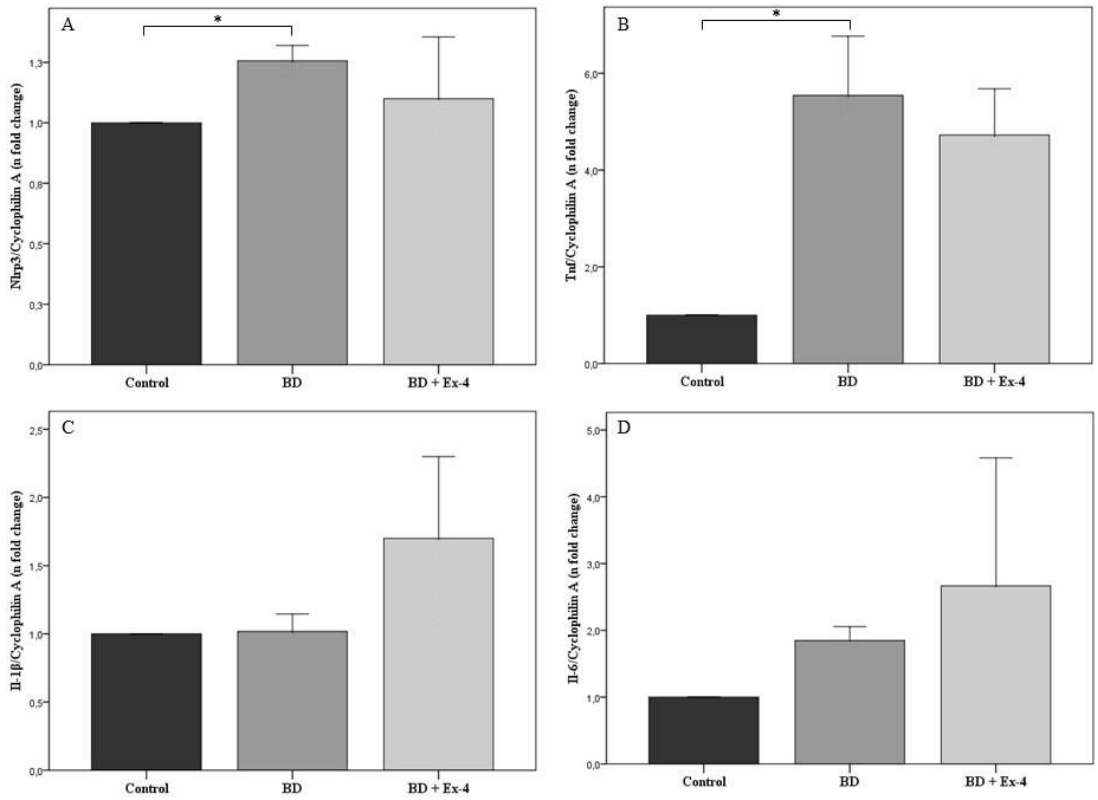

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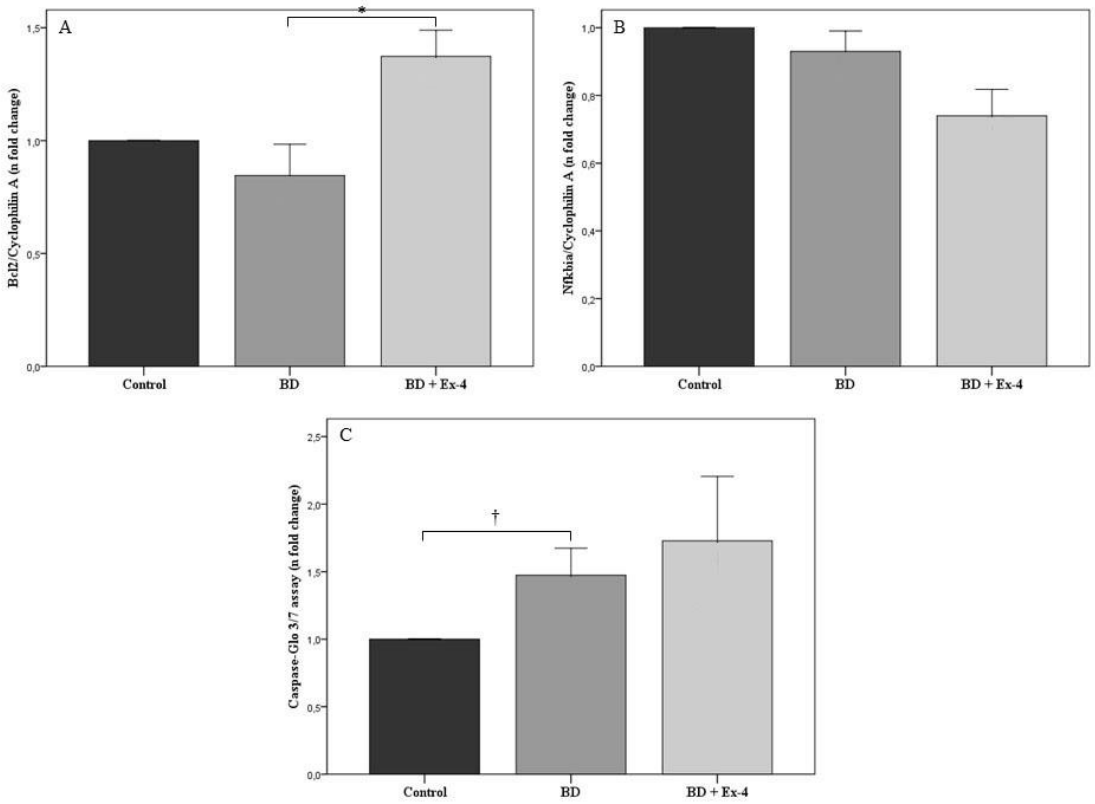

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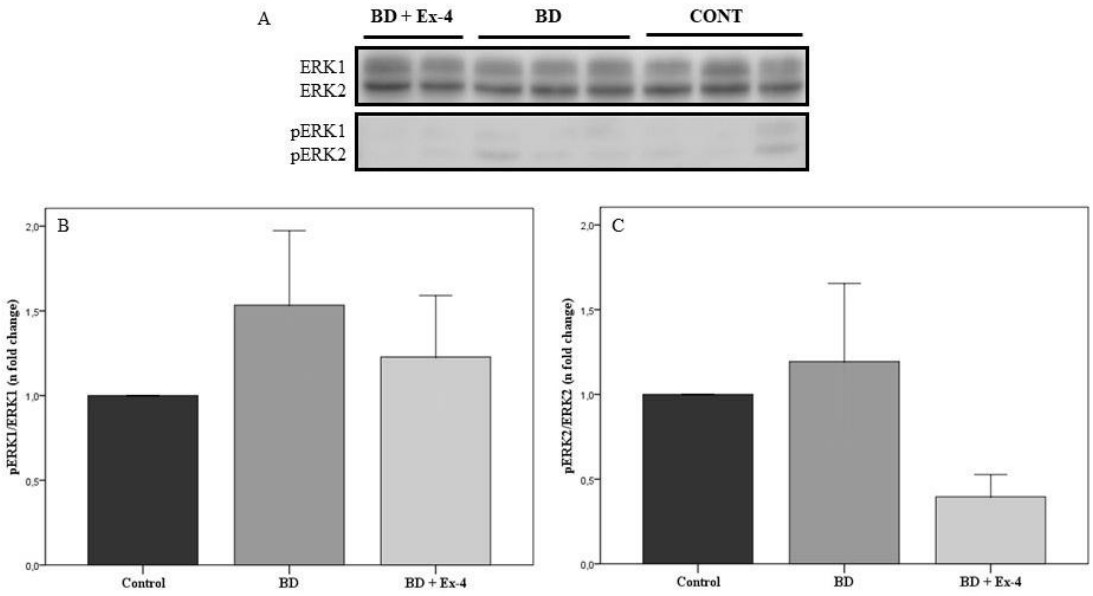

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CONCLUSÕES GERAIS

Podemos concluir com o presente estudo:

- De uma forma geral, aditivos do grupo "antiapoptóticos/anti-inflamatórios/antioxidantes" parecem diminuir a apoptose das ilhotas e melhorar o SI, calculado após estimulação com concentrações normal e alta de glicose. Além disso, a cultura de ilhotas em *scaffolds* ou sobre componentes de ECM foi capaz de melhorar o SI. Os efeitos dos outros grupos de aditivos sobre os desfechos analisados foram heterogêneos, tornando difícil uma conclusão. Dessa forma, novos estudos são necessários para definir o real impacto desses aditivos na qualidade das ilhotas isoladas e nos desfechos do transplante.

- Embora a citometria de fluxo seja mais cara e mais demorada do que a coloração FDA/PI, é uma técnica quantitativa e não subjetiva. Sendo assim, deve ser a técnica de escolha para uma determinação mais eficaz da viabilidade das ilhotas.

- A EXE parece possuir um papel protetor sobre a função das ilhotas de ratos frente a um estresse inflamatório direto. Já a LIRA não influenciou o SI das ilhotas na presença ou ausência de citocinas. Estamos realizando experimentos adicionais de expressão gênica para avaliar por quais mecanismos essa melhora do SI acontece.

- A administração de EXE a ratos com ME pode reduzir danos renais induzidos pela ME através da diminuição da expressão de genes relacionados ao estresse oxidativo e aumento de *Bcl2*.

OUTRAS PRODUÇÕES BIBLIOGRÁFICAS NO PERÍODO DO DOUTORADO

Além dos artigos que fazem parte da presente tese, ao longo do período do doutorado foram desenvolvidos os seguintes manuscritos:

1. Lemos NE, Dieter C, Dorfman LE, Assmann TS, Duarte GCK, Canani LH, Bauer AC, Crispim D. The rs2292239 polymorphism in ERBB3 gene is associated with risk for type 1 diabetes mellitus in a Brazilian population. Gene. 2018;644:122-8.

2. Carlessi R, Lemos NE, Dias AL, Brondani LA, Oliveira JR, Bauer AC, Leitao CB, Crispim D. Exendin-4 attenuates brain death-induced liver damage in the rat. Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society. 2015;21(11):1410-8.

3. Rheinheimer J, Bauer AC, Silveiro SP, Estivalet AA, Boucas AP, Rosa AR, Souza BM, Oliveira FS, Cruz LA, Brondani LA, Azevedo MJ, Lemos NE, Carlessi R, Assmann TS, Gross JL, Leitao CB, Crispim D. Human pancreatic islet transplantation: an update and description of the establishment of a pancreatic islet isolation laboratory. Archives of endocrinology and metabolism. 2015;59(2):161-70.

4. Carlessi R, Lemos NE, Dias AL, Oliveira FS, Brondani LA, Canani LH, Bauer AC, Leitao CB, Crispim D. Exendin-4 protects rat islets against loss of viability and function induced by brain death. Molecular and cellular endocrinology. 2015;412:239-50.

5. Lemos NE, Farias MG, Kubaski F, Scotti L, Onsten TGH, Brondani LA, Wagner SC, Sekine L. Quantification of peripheral blood CD34(+) cells prior to stem cell harvesting by leukapheresis: a single center experience. Hematology, transfusion and cell therapy. 2018;40(3):213-8.

Manuscritos desenvolvidos no período do doutorado e em fase de finalização:

1. Dieter C, Lemos NE, Dorfman LE, Assmann TS, Duarte GCK, Canani LH, Bauer AC, Crispim D The rs11755527 polymorphism in *BACH2* gene and the Type 1 Diabetes Mellitus: case control study in a Brazilian population.

2. Dieter C, Assmann TS, Lemos NE, Souza BM, Crispim D. 866G/A and Ins/Del polymorphisms in the *UCP2* gene and diabetic kidney disease: case-control study and meta-analysis.