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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

MARCOS DIVINO FERREIRA JUNIOR

SUSCETIBILIDADE DE FASES PERINATAIS NO DESENVOLVIMENTO DE DOENÇAS CARDIOMETABÓLICAS: a puberdade como período de manifestação de sintomas

GOIÂNIA
2023

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MARCOS DIVINO FERREIRA JUNIOR

SUSCETIBILIDADE DE FASES PERINATAIS NO DESENVOLVIMENTO DE DOENÇAS CARDIOMETABÓLICAS: a puberdade como período de manifestação de sintomas

SUSCEPTIBILITY OF PERINATAL PHASES IN THE DEVELOPMENT OF CARDIOMETABOLIC DISEASES: puberty as a period of symptoms onset

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas do Instituto de Ciências Biológicas da Universidade Federal de Goiás, como requisito para obtenção do título de Doutor em Ciências Biológicas.

Área de Concentração: Farmacologia e Fisiologia.

Orientador: Prof. Dr. Rodrigo Mello Gomes

Coorientador(es): Prof. Dr. Paulo Matafome

Prof. Dr. Carlos Henrique Xavier Custódio

GOIÂNIA

2023

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Tese (Doutorado) - Universidade Federal de Goiás, Instituto de Ciências Biológicas (ICB), Programa de Pós-graduação em Ciências Biológicas, Goiânia, 2023.
Bibliografia. Anexos.
Inclui siglas, lista de figuras, lista de tabelas.

1. Puberdade. 2. Doenças Cardiometabólicas. 3. Glicotoxinas. 4. LEAP2. 5. DOHaD. I. Gomes, Rodrigo Mello, orient. II. Título.

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ATA DE DEFESA DE TESE

Ata Nº 116 da sessão de Defesa de Tese do Programa de Pós-graduação em Ciências Biológicas que confere a **Marcos Divino Ferreira Junior** o título de Doutor em Ciências Biológicas, na área de concentração em Farmacologia e Fisiologia.

Aos dezoito dias do mês de setembro de 2023, a partir das 08:30 horas, no Anfiteatro do Instituto de Ciências Biológicas 2, realizou-se a sessão pública de Defesa de Tese intitulada "Suscetibilidade de fases perinatais no desenvolvimento de doenças cardiometabólicas: a puberdade como período de manifestação de sintomas". Os trabalhos foram instalados pelo Orientador, Professor Doutor Rodrigo Mello Gomes (ICB - UFG) com a participação dos demais membros da Banca Examinadora: Professor Doutor Antonio Carlos Boschero (UNICAMP), membro titular externo; Professor Doutor Paulo Cezar de Freitas Mathias (UEM), membro titular externo; Professora Doutora Patrícia Cristina Lisbôa da Silva (UERJ), membro titular externo, cuja participação ocorreu através de videoconferência; Professor Doutor Paul David Taylor (King's College London), membro titular externo, cuja participação ocorreu através de videoconferência. Durante a argüição os membros da banca **não** fizeram sugestão de alteração do título do trabalho. A Banca Examinadora reuniu-se em sessão secreta a fim de concluir o julgamento da Tese tendo sido o candidato **aprovado** pelos seus membros. Proclamados os resultados pelo Professor Doutor Rodrigo Mello Gomes, Presidente da Banca Examinadora, foram encerrados os trabalhos e, para constar, lavrou-se a presente ata que é assinada pelos Membros da Banca Examinadora, aos dezoito dias do mês de setembro de 2023.

TÍTULO SUGERIDO PELA BANCA

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DECLARAÇÃO

Informamos que o coordenador do Programa de Pós-Graduação em Ciências Biológicas, Prof. Dr. Manoel Francisco Biancardi, assinou a ata de defesa (Documento SEI 4020939) em substituição aos membros externos Dr. Antonio Carlos Boschero, Dr. Paul David Taylor e Dr. Paulo Cezar de Freitas Mathias, conforme Item 04 da Instrução Normativa PRPG 001, de 27 DE março de 2020.

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SUMÁRIO

DEDICATÓRIA

Dedico este trabalho, em primeiríssimo lugar, à minha mãe Idaides Pereira dos Santos, à minha avó Geraldina Pereira dos Santos, e à minha tia Wenya dos Santos Cabral. Poucas pessoas neste mundo terão a sorte de contar com um trio de mulheres tão fortes e guerreiras nesta vida. Para mim, verdadeiras amazonas, a começar pela matriarca.

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“Um pássaro que repousa numa árvore nunca teme que o galho quebre, porque a sua confiança não é no galho, ***mas nas suas próprias asas.”***

Autor desconhecido

“Life's tragedy is that we get ***old too soon*** *and* ***wise too late****.”*

Benjamin Franklin

LISTA DE ABREVIATURAS E SIGLAS

ACH	Acetilcolina
AUC	Área Sob a Curva
DOHaD	Origens desenvolvimentistas da saúde e da doença
GD	Dia gestacional
GHSR	Receptor do Hormônio Secretagogo do GH
GK	Rato Goto-Kakizaki
GTT	Teste de tolerância à glicose
IVDP	Pressão Intraventricular Diastólica
IVSP	Pressão Intraventricular Sistólica
LEAP2	Peptídeo Antimicrobiano Expresso no Fígado - 2
LGA	Grande para a Idade Gestacional
MG	Metilglioxal
PHE	Fenilefrina
PND	Dia pós-natal
PVAT	Tecido Adiposo Perivascular
SNP	Nitroprussiato de Sódio
VEH	Veículo

LISTA DE FIGURAS

RESUMO

Compreender como a suscetibilidade de fases iniciais da vida podem predispor o indivíduo a um maior risco de desenvolvimento de doenças a longo-prazo é o foco dos estudos em DOHaD. Esta tese tem por objetivo principal evidenciar a importância da avaliação precoce dos efeitos de estímulos danosos sofridos ainda nas fases iniciais de vida. Dada a sutileza destes efeitos a curto-prazo, a maioria dos estudos se concentra no estudo destes efeitos quando estes já estão consolidados. Aqui, abrimos uma nova discussão sobre a puberdade como o período inicial do aparecimento dos sintomas, e também como um período propício para estabelecer estratégias para mitigar os danos já conhecidos na fase adulta. Para isso, avaliamos os efeitos da exposição materna ao metilglioxal em parâmetros metabólicos e cardiovasculares na prole jovem de ratos Wistar, e também avaliamos a contribuição da sinalização do receptor do hormônio secretagogo do hormônio do crescimento (GHSR) durante fases perinatais, no neurodesenvolvimento e metabolismo energético de animais jovens. Em ambos os estudos, o enfoque foi dado à avaliação de parâmetros diversos durante a fase puberal. Como principais achados, vimos que a exposição precoce às glicotoxinas levou a deficiências cardíacas e vasculares, e que a modulação do receptor GHSR em estágios perinatais pode afetar a homeostase da glicose. Ao longo da vida, estes efeitos podem se tornar mais danosos, levando à insuficiência cardíaca ou doenças metabólicas graves como a doença hepática gordurosa não alcoólica. Assim, fica evidente a importância da puberdade como fase de vigilância e intervenção.

Palavras-Chave: Puberdade; Doenças Cardiometabólicas; Glicotoxinas; LEAP2; DOHaD.

ABSTRACT

Understanding how susceptibility in the early stages of life can predispose an individual to a greater risk of developing long-term diseases is the focus of DOHaD studies. The main aim of this thesis is to highlight the importance of early assessment of the effects of harmful stimuli suffered in the early stages of life. Given the subtlety of these short-term effects, most studies focus on studying these effects when they have already been consolidated. Here, we open up a new discussion on puberty as the initial period of symptom onset, and also as a favourable time to establish strategies to mitigate the damage already known in adulthood. To this end, we evaluated the effects of maternal exposure to methylglyoxal on metabolic and cardiovascular parameters in young Wistar rat offspring, and also assessed the contribution of growth hormone secretagogue hormone receptor (GHSR) signalling during perinatal stages on neurodevelopment and energy metabolism in young animals. In both studies, the focus was on evaluating several parameters during the pubertal phase. As main findings, we demonstrated that early exposure to glycotoxins led to cardiac and vascular deficiencies, and that modulation of the GHSR receptor in perinatal stages can affect glucose homeostasis. Throughout life, these effects can become more harmful, leading to heart failure or serious metabolic diseases such as non-alcoholic fatty liver disease. This highlights the importance of puberty as a stage for surveillance and intervention.

Keywords: Puberty; Cardiometabolic Diseases; Glycotoxins; LEAP2; DOHaD.

IMPACTO E RELEVÂNCIA DA PESQUISA PARA SOCIEDADE

É frequente a associação entre diversas doenças e a exposição a elevados níveis de glicose, bem como a presença de glicotoxinas, compostos resultantes da glicação avançada. Além disso, a desregulação na sinalização do receptor para o hormônio da fome, a grelina, tem sido importante para a compreensão de doenças que envolvem o metabolismo.

Entretanto, a compreensão das doenças que surgem na vida adulta tem sido associada aos estímulos e exposições vivenciados durante as fases iniciais do desenvolvimento humano.

Recentemente, glicotoxinas têm emergido como fatores contribuintes para a inflamação crônica, resistência à insulina e outras anomalias metabólicas que podem influenciar o surgimento de doenças cardiometabólicas. No mesmo sentido, a modulação da sinalização do receptor GHSR, responsável pela mediação dos efeitos da grelina, poderia ser uma chave para desvendar os mecanismos responsáveis por desencadear a desregulação do metabolismo como um todo.

Portanto, investigar como a exposição precoce a glicotoxinas e alterações na sinalização do receptor da grelina afetam o desenvolvimento pós-natal, e como isso pode levar a efeitos duradouros, é crucial para o entendimento da predisposição a doenças cardiometabólicas da idade adulta.

1. INTRODUÇÃO

1.1. PERÍODOS PERINATAIS E SUA IMPORTÂNCIA PARA O CONCEITO DOHAD

O desenvolvimento de um evento importante passa por diversas etapas que visam o funcionamento sinérgico entre diversas variáveis. Como em um manual de instruções, a cada novo passo uma nova informação é agregada para que um objetivo final seja alcançado, a funcionalidade. Entretanto, basta que se adicionem algumas distrações ou instruções erradas para que o objetivo final não saia como o esperado. Nosso organismo funciona desta mesma forma, desde sua a concepção ao seu fim.

Durante a gestação, processos que vão desde o dobramento dos folhetos embrionários até a migração de células para os locais corretos, levam à formação dos principais órgãos e sistemas do corpo, e influências externas de origem química ou física podem atrapalhar estes processos, gerando malformações fetais e perda da viabilidade fetal (Ong and Guest, 2018). Além disso, o ambiente intrauterino é o primeiro contato do novo organismo com as instruções às quais obedecerá, incluindo a expressão diferencial de genes e a readequação dos sistemas biológicos para o que está por vir (Ozaki *et al.*, 2001; Vickers *et al.*, 2011; Saad *et al.*, 2016; Ferreira-Junior *et al.*, 2023). Por sua vez, o ambiente intrauterino é o reflexo de quem o conduz. Neste sentido, o estado de saúde, comportamento alimentar, bem como os hábitos de vida maternos podem tornar o embrião/feto preparado para responder aos estímulos que recebeu. Contudo, na maioria dos casos às custas da própria saúde a longo prazo (Jackson *et al.*, 2012; Kawaharada *et al.*, 2018; Larsen *et al.*, 2019).

Após o nascimento, o contato com o ambiente externo é desafiador, e também pode condicionar o neonato aos estímulos recebidos. No entanto, ao menos em mamíferos, o contato direto com a genitora ainda é mantido por meio do leite materno. O leite materno é uma fonte essencial de nutrição e imunidade nos primeiros momentos de vida e desempenha um papel crítico na formação de um sistema imunológico competente e na maturação do sistema digestivo (Nozhenko *et al.*, 2015; Vieira Borba, Sharif and Shoenfeld, 2018; Yu *et al.*, 2018). Desequilíbrios nutricionais e doenças metabólicas

como o diabetes ou a obesidade podem alterar a composição do leite materno, forçando adaptações metabólicas no neonato que podem perdurar durante toda a vida (Knowles, 1974; Von Kries *et al.*, 1999; Savino *et al.*, 2011; Shamir and Shehadeh, 2013; Nozhenko *et al.*, 2015; Vieira Borba, Sharif and Shoenfeld, 2018; Yu *et al.*, 2018).

O termo DOHaD (Origens Desenvolvimentistas da Saúde e da Doença; do inglês *Developmental Origins of Health and Disease*), se refere a um campo de pesquisa que avalia como os eventos e condições durante os períodos perinatais podem ter um impacto significativo na saúde e no desenvolvimento futuro de um indivíduo (Silveira *et al.*, 2007; Lacagnina, 2020). Este campo de pesquisa busca compreender os efeitos de diversas intercorrências que ocorrem durante o desenvolvimento do organismo nas respostas futuras ao ambiente.

Portanto, compreender como os períodos perinatais podem influenciar o desenvolvimento de doenças na vida adulta é fundamental para a promoção da saúde a longo prazo.

1.2. PREDISPOSIÇÃO A DOENÇAS DEVIDO A EXPOSIÇÃO PRECOCE À GLICOTOXINAS

As glicotoxinas, assim chamadas por serem derivadas do metabolismo incompleto da glicose, são moléculas altamente instáveis e reativas. Dada a sua instabilidade, as glicotoxinas tendem a formar complexos, ou adutos, com outras moléculas biológicas de forma não-enzimática. O metilglioxal (MG) é uma das glicotoxinas mais abundantes, e deriva do piruvato. A formação de adutos de MG pode afetar proteínas citoplasmáticas, circulantes, e também da matriz extracelular (Goldin *et al.*, 2006; Bento *et al.*, 2010; Guerin-Dubourg *et al.*, 2012; Carlsson and Törnqvist, 2016; Matafome *et al.*, 2017). Os produtos finais de glicação-avançada (AGEs, *Advanced Glycation End-products*).

Efeitos comuns do aumento das concentrações de MG incluem a maior geração de espécies reativas de oxigênio (ROS), estresse do retículo endoplasmático e disfunção mitocondrial (Padival, Crabb and Nagaraj, 2003; Bento *et al.*, 2010; Palsamy *et al.*, 2014; Nam *et al.*, 2015a). Em partes, o círculo vicioso de formação de ROS e maior formação

de MG pode ser explicado pela dependência da Glutationa (GSH) para a detoxificação do MG pelas Glioxalases (GLO) (Pun *et al.*, 2014).

Os AGEs por sua vez, medeiam efeitos danosos ao ativar os receptores de AGEs (RAGE), que reconhecem basicamente dois tipos de ligantes: imidazolonas (derivados de MG) e adutos de Nε-(carboximetil)lisina (CML) (Yan, Ramasamy and Schmidt, 2009). Os principais efeitos decorrentes da ativação do RAGE incluem a ativação da NADPH-oxidase e aumento da transcrição de NF-κB, levando novamente ao aumento do estresse oxidativo e ativação de respostas pró-inflamatórias (Du *et al.*, 2003; Goldin *et al.*, 2006; Xue *et al.*, 2011, 2014).

Estudos pioneiros relacionaram as injúrias a diferentes órgãos, outrora atribuídos somente à hiperglicemia, ao aumento sustentado do nível de glicotoxinas circulantes (Chang, Wang and Wu, 2005; Sena *et al.*, 2012; Crisóstomo *et al.*, 2013; Blackburn *et al.*, 2017). Além dos efeitos no tecido adiposo (Rodrigues *et al.*, 2013, 2017; Rodrigues, Matafome and Seiça, 2013), os efeitos do aumento sustentado de glicotoxinas circulantes em parâmetros cardiovasculares foram intensamente estudados (Chang, Wang and Wu, 2005; Sena *et al.*, 2012; Crisóstomo *et al.*, 2013; Su *et al.*, 2013; Nam *et al.*, 2015b; Blackburn *et al.*, 2017). No mesmo sentido, estudos prévios de nosso grupo de pesquisa mostraram que a exposição precoce a altos níveis de glicotoxinas pode desencadear efeitos duradouros, mesmo muito tempo após o término da exposição (Francisco *et al.*, 2018, 2022). Entretanto, estes estudos deixaram uma questão em aberto que o Artigo 1 desta tese buscou avaliar: “A exposição precoce à glicotoxinas durante a fase de lactação também leva a efeitos cardiovasculares, tal qual ocorre em modelos de exposição crônica?”.

1.3. SINALIZAÇÃO GHSR, GRELINA, LEAP2 E DOENÇAS METABÓLICAS

O receptor de hormônios secretagogos do hormônio do crescimento (sigla em inglês, GHSR), descoberto por Howard e colaboradores (1996), teve sua busca motivada pela necessidade de fornecer tratamentos mais eficazes para distúrbios do crescimento baseada em peptídeos liberadores do GH. No ano seguinte, as duas isoformas do GHSR foram descobertas, a GHSR-1a (366 aminoácidos e sete domínios transmembrana) e a

sua forma truncada não-funcional GHSR-1b (289 aminoácidos e cinco domínios transmembrana) (Guan *et al.*, 1997; McKee *et al.*, 1997). Além disso, foi constatada a ampla distribuição do GHSR no sistema nervoso central, com localizações compatíveis com os efeitos conhecidos na liberação de GH, e outras localizações em que foram hipotetizadas funções fisiológicas adicionais (Guan *et al.*, 1997). Mais tarde, foi constatada a ampla distribuição de GHSR em tecidos periféricos (Gnanapavan *et al.*, 2002).

A grelina, hormônio descoberto por Kojima e colaboradores (1999), é um ligante endógeno do receptor para o hormônio secretagogo do hormônio do crescimento. A grelina possui 28 aminoácidos, em humanos e roedores, e sua principal função é relacionada à secreção do hormônio do crescimento. A forma tida como bioativa da grelina é a sua forma acilada, ou seja, com uma molécula de ácido octanoico esterificada ao terceiro aminoácido (serina). A enzima responsável por ativar a grelina se chama GOAT (do inglês, *Ghrelin O-acyltransferase*). A forma mais abundante da grelina é a forma desacilada (*desacyl-ghrelin*), chegando a 60% do total circulante. Ainda assim a forma desacilada, apesar de ter sido correlacionada a uma potencialização da secreção de insulina dependente de glicose (Gauna *et al.*, 2007), não possui papel bem descrito na literatura.

Com o propósito de estudar os efeitos da sinalização GHSR, inibidores de diversas classes foram desenvolvidos. Os primeiros antagonistas foram desenvolvidos a partir da estrutura do GHRH, com substituições que tornam uma das ligações com o receptor inerte (Robberecht *et al.*, 1985; Zarandi *et al.*, 1994; Schally and Varga, 1999). Vários desses antagonistas foram desenvolvidos para o tratamento da secreção aumentada de GH, a acromegalia. Além disso, outros antagonistas como o [D-Lys^{3}]-GHRP-6 e a [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]-Substância P já tinham papéis bem conhecidos, e serviram como antagonistas de referência em alguns estudos (Everard *et al.*, 1992; Patchett *et al.*, 1995; Bennett *et al.*, 1997).

O peptídeo antimicrobiano expresso no fígado 2 (do inglês *Liver-Expressed Antimicrobial Peptide* 2), foi descoberto em 2003 e identificado primeiramente por sua função antimicrobiana (Krause, 2003; SANG *et al.*, 2006). Em um estudo pioneiro na

utilização do LEAP2, Xuecai Ge e colaboradores (2018), utilizando-se de dados bioinformáticos, fizeram a varredura de diversos genes regulados pela gastrectomia vertical, cirurgia que já é relacionada a diminuição da secreção de grelina. M'Kadmi e colaboradores (2019), utilizando-se de análises conformacionais no GHSR, avaliaram a capacidade de ligação do LEAP2 no referido receptor. O grande achado deste estudo foi que o LEAP2 não é somente um antagonista, mas dada a atividade tônica do GHSR, é um agonista inverso.

Para além de seus efeitos relacionados à fome, a grelina também realiza ajustes no metabolismo em sinergia com outros hormônios, fazendo com que haja disponibilidade de glicose para o metabolismo. Um destes hormônios é o próprio GH, liberado em resposta a ativação de GHSR em somatotrofos, na hipófise (Broglio *et al.*, 2001). Outro hormônio estimulado pela ativação de GHSR pela grelina é o glucagon, que já possui efeitos conhecidos na mobilização de estoques de glicogênio hepático (Gauna *et al.*, 2005; Chuang *et al.*, 2011).

Por ser o "hormônio da fome", e também ser o agonista endógeno de GHSR, a grelina já foi alvo de diversos estudos que buscaram avaliar os efeitos de diversas intercorrências maternas nos níveis de grelina em neonatos (Hayashida *et al.*, 2002; Nakahara *et al.*, 2006; Yousheng Jia *et al.*, 2008) e também os efeitos do aumento dos níveis de grelina durante as fases perinatais no metabolismo de sua prole (Nakahara *et al.*, 2006; Cesur *et al.*, 2012; Torres *et al.*, 2021; Sato *et al.*, 2022). Não obstante, já foi demonstrado que a grelina exerce papel fundamental na maturação do sistema nervoso central (Torres *et al.*, 2021).

A questão em aberto acerca da sinalização GHSR, cujo Artigo 2 desta tese buscou esclarecer, é: "Quais os impactos da exposição perinatal ao LEAP2, ou seja, da modulação de GHSR nesta fase, no neurodesenvolvimento e na homeostase da glicose em animais jovens?".

1.4. A PUBERDADE COMO PERÍODO DE MANIFESTAÇÃO DE SINTOMAS

As transformações hormonais e físicas distinguem a puberdade, que é uma fase fundamental do crescimento humano. Esta fase também pode servir como um ponto de checagem para que as respostas compensatórias à estímulos vivenciados durante a gestação e lactação venham à tona. No início da adolescência, alterações metabólicas e hormonais podem desencadear o surgimento de distúrbios predispostos, como a resistência à insulina e doenças cardiovasculares.

O impacto a longo prazo de estímulos prejudiciais vividos durante o desenvolvimento inicial pode ser reduzido através da identificação precoce dos seus efeitos, juntamente com o rastreio do motivo pelo qual estes efeitos se manifestaram. Este reconhecimento é vital na elaboração de estratégias que limitem futuros problemas de saúde. Para melhorar os métodos de prevenção e intervenção, é necessária investigação contínua para compreender como doenças programadas podem manifestar-se durante a puberdade, proporcionando aos indivíduos uma possibilidade de intervenção cada vez mais precoce, e uma vida adulta mais saudável e estável.

2. HIPÓTESES

2.1. ARTIGO 1

A exposição precoce a glicotoxinas durante a fase de lactação por meio do leite materno, nomeadamente ao metilglioxal, leva a deficiências cardiovasculares devido ao acúmulo de glicotoxinas no coração em desenvolvimento e alterações na influência do PVAT sobre o endotélio vascular na puberdade.

2.2. ARTIGO 2

O antagonismo/agonismo inverso do GHSR em fêmeas durante a lactação promove efeitos negativos no neurodesenvolvimento e no comportamento alimentar de sua prole.

O antagonismo/agonismo inverso do GHSR em fases perinatais promove inibição a longo prazo da sinalização da grelina, resultando em aumento da secreção de insulina estimulada pela glicose na puberdade.

3. OBJETIVOS

3.1. ARTIGO 1

3.1.1. Objetivo geral

Avaliar os efeitos da exposição materna ao metilglioxal durante a fase de lactação em parâmetros metabólicos e cardiovasculares na prole de ratos Wistar na puberdade.

3.1.2. Objetivos específicos

Avaliar na prole púbere de ratas Wistar expostas ou não ao metilglioxal durante a fase de lactação:

- parâmetros de ingestão alimentar, e evolução da massa corporal;
- as concentrações plasmáticas em jejum de glicose, colesterol total e HDL, triglicerídeos e frutosamina;
- parâmetros cardíacos *ex vivo*, através dos experimentos de coração de langendorff;
- parâmetros de reatividade vascular *ex vivo*, através dos experimentos de vaso isolado com anéis de aorta torácica;
- a morfologia do coração e aorta;

3.2. ARTIGO 2

3.2.1. Objetivo geral

Avaliar a contribuição da sinalização do receptor do hormônio secretagogo do hormônio do crescimento (GHSR) durante fases perinatais, no neurodesenvolvimento e metabolismo energético de animais recém-nascidos.

3.2.2. Objetivos específicos

Avaliar os efeitos da administração do peptídeo truncado do inibidor endógeno de GHSR, o LEAP2(1-14)-NH_2, em ratas Wistar prenhes, na:

- Evolução da massa corporal e ingestão alimentar;
- Em parâmetros reprodutivos e de desenvolvimento fetal;

Analisar na prole jovem (PND45) de mães administradas com o peptídeo truncado do inibidor endógeno de GHSR, LEAP2[1-14]-NH_2:

- Parâmetros de neurodesenvolvimento, como reflexos e comportamentos relacionados a sobrevivência.
- A evolução da massa corporal e a ingestão alimentar ao longo da vida;
- A dinâmica glicêmica, por meio do teste oral de tolerância à glicose;
- A morfologia pancreática, com foco na área de ilhotas, e a marcação de células positivas para insulina.
- A expressão hepática de PEPCK, enzima envolvida na gliconeogênese.

Analisar em animais jovens (PND45) expostos ao LEAP2[1-14]-NH_2 ou ao agonista de GHSR (MK677) durante a fase de lactação:

- A evolução da massa corporal e a ingestão alimentar ao longo da vida;
- A dinâmica glicêmica, por meio do teste oral de tolerância à glicose;
- A morfologia pancreática, com foco na área de ilhotas, e a marcação de células positivas para insulina.
- A expressão hepática de PEPCK.

4. MATERIAIS E MÉTODOS

Neste estudo, ambos os trabalhos foram de caráter experimental, um voltado para a avaliação, em animais jovens, das respostas cardiovasculares à exposição precoce a glicotoxinas e também voltados para a avaliação dos efeitos metabólicos devido à modulação da atividade do receptor GHSR em animais jovens.

Resumidamente foram utilizados experimentos *in vivo*, *ex vivo* e *in vitro* para avaliar os parâmetros supracitados. Brevemente, foram utilizados dois modelos experimentais:

1. no primeiro, cujo objetivo foi avaliar os efeitos da exposição precoce a glicotoxinas, um modelo animal onde um precursor da formação de AGEs foi administrado em ratas Wistar durante a fase de lactação (60 mg/kg/dia; Via Oral) foi concebido, sendo a prole macho avaliada aos 45 dias de vida quanto a parâmetros cardiovasculares e metabólicos.

2. no segundo, cujo objetivo foi avaliar o papel da modulação do receptor GHSR por seu antagonista endógeno LEAP2, dois modelos animais foram desenhados para avaliar tanto o papel indireto quando o papel direto desta modulação:

- para avaliar o papel indireto da modulação de GHSR pelo LEAP2, ratas Wistar prenhes foram injetadas com LEAP2[1-14]-NH_2 durante a segunda metade da gestação (72 nmol/kg/dia; Via Subcutânea), sendo a prole de ambos os sexos avaliada aos 45 dias de vida quanto a parâmetros metabólicos;

- para avaliar o papel direto da modulação de GHSR pelo LEAP2, ratos Wistar machos e fêmeas recém-nascidos foram injetados com o antagonista endógeno de GHSH (LEAP2[1-14]-NH_2) nas doses de 0,1 ou 1 µmol/kg (Via Subcutânea) ou com o agonista farmacológico de GHSR (MK677) na dose de 750 µmol/kg do dia pós-natal (PND) 3 ao PND14, sendo a estes animais avaliados aos 45 dias de vida quanto a parâmetros metabólicos.

As principais técnicas *in vivo*, *ex vivo,* histológicas e moleculares utilizadas nestes estudos, a serem explicadas em detalhes nas seções de materiais e métodos dos artigos correspondentes foram:

- Coração de langendorff – técnica que permite avaliar a funcionalidade cardíaca *ex vivo* por meio da manutenção da viabilidade do órgão pela perfusão retrógrada pela artéria aorta com solução nutridora, e pelo monitoramento dos parâmetros de contratilidade por meio de sensores posicionados nas câmaras cardíacas e nas tubulações que conduzem a solução nutridora.

- Banho de órgãos com anéis de aorta – técnica ex vivo que permite avaliar a contratilidade isométrica de anéis de artérias, excisados e mantidos em solução nutridora, em resposta a diversos agentes farmacológicos indutores de relaxamento ou contração.

- Teste oral de tolerância à glicose – técnica in vivo que permite avaliar a dinâmica da glicemia em resposta a uma sobrecarga glicêmica administrada por via oral. Possui como principais fatores confundidores a sensibilidade à insulina endógena, a secreção de insulina endógena, bem como a absorção intestinal de glicose. Todavia, é uma técnica de alto custo/benefício para se avaliar a dinâmica da glicose sanguínea.

- Imunohistoquímica (IHC) – técnica que permite avaliar de forma semiquantitativa a expressão de alvos moleculares em cortes histológicos. Através da marcação com anticorpos e posterior revelação por métodos colorimétricos, é possível identificar a localização e intensidade da expressão dos alvos. Útil na identificação de vias moleculares que possam explicar os resultados obtidos em experimentos *in vivo* ou *ex vivo*.

- Western Imunoblotting – técnica que permite avaliar de forma semiquantitativa a expressão de proteínas em tecidos-alvo, por meio de interações antígeno-anticorpo e revelação do complexo formado por quimiluminescência ou métodos colorimétricos. De forma diferente da IHC, a identificação de vias moleculares é realizada com foco em sua intensidade relativa a alvos expressos de forma regular.

5. ARTIGOS EM FORMATO PARA PUBLICAÇÃO

5.1. ARTIGO 1 – EFEITOS DA EXPOSIÇÃO PRECOCE A GLICOTOXINAS DURANTE A FASE DE LACTAÇÃO NA SAÚDE CARDIOVASCULAR DE ANIMAIS JOVENS

EARLY METHYLGLYOXAL EXPOSURE LEADS TO WORSENED CARDIAC FUNCTION IN YOUNG RATS

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Running title: Glycotoxins leads to cardiac dysfunction in juvenile rats.

ABSTRACT

The number of diabetic or obese women of childbearing age grow every year. Also, maternal diabetes effects are well described in the literature. However, the effects of maternal diabetes in postnatal phases are often overlooked. Given that diabetic individuals have higher levels of circulating glycotoxins, and that there is a positive correlation between maternal derived glycotoxins and circulating glycotoxins in their progeny, is necessary to evaluate the effects of this exposure in the lactation. Previous studies evaluated the metabolic effects of high glycotoxins exposure during lactation in adult animals. This study aimed to evaluate the effects of early methylglyoxal exposure in the cardiovascular system of juvenile rats, to evaluate if the observed effects are time-dependent or not. For this, pregnant Wistar rats were separated into two groups: CO (Veh); and MG (Methylglyoxal; 60 mg/kg/day). At the postnatal day (PND) 3, the litters were standardized to eight rats, preferentially males. The treatment was carried out from PN3 to PN14. The offspring were evaluated at PND45. No differences were detected in the body composition and plasma biochemical parameters between the groups. MG offspring presented cardiac dysfunction, and subtle vascular vasomotor responses in the presence of PVAT, however, without morphological alterations in these tissues. In conclusion, our data suggest that early glycotoxins exposure led to cardiac and vascular impairments, which tends to worsen and develop end-organ injury.

Keywords: Glycotoxins; Methylglyoxal; Lactation; Left ventricular dysfunction;

1. INTRODUCTION

Despite easy control, the number of diabetic or obese women of childbearing age with respective projections of increased prevalence by almost 10% for diabetes and 57% for obesity or overweight (Kelly *et al.*, 2008; Cho *et al.*, 2018). Some of the factors related to higher birth weight are the increased nutritional supply due to the maternal lifestyle and the increased energy available to the foetus due to hyperglycaemia, combined with increased plasma insulin. A classic example of this impact is that children of diabetic mothers, as well as those of obese mothers, tend to become large for gestational age (LGA) (Plagemann *et al.*, 2012; Bashir *et al.*, 2019).

It is also known that due to higher glycemia, diabetic individuals have higher levels of circulating glycotoxins derived from non-canonical or incomplete glucose metabolism (Schalkwijk and Stehouwer, 2020). One of the main glycotoxins is methylglyoxal (MG), which has its main mechanism of action in the formation of adducts with other molecules, these called Advanced Glycation End-products (AGEs) (Matafome *et al.*, 2017; Schalkwijk and Stehouwer, 2020). The link between diabetes-associated comorbidities and the AGEs is well described, and the major end-organ injury are associated with oxidative stress and glycation of important proteins (Brouwers *et al.*, 2011; Sena *et al.*, 2012; Crisóstomo *et al.*, 2013; Pei *et al.*, 2018; Francisco *et al.*, 2022). In addition to oxidative stress, methylglyoxal-induced cardiac injury is mainly associated with the decreased mitochondrial function, even in the absence of hyperglycaemia (Brouwers *et al.*, 2011; Sena *et al.*, 2012; Crisóstomo *et al.*, 2013; Tikellis *et al.*, 2014; Nam *et al.*, 2015; Wang *et al.*, 2018).

Also important to the cardiovascular system health, endothelial function is worsened in cells exposed to MG (Sena *et al.*, 2012; Blackburn *et al.*, 2017; Azul *et al.*, 2020; Lee *et al.*, 2020). Lee and colleagues (2020) demonstrated that MG-induced endothelial dysfunction acts via increased oxidative stress and lower autophagy, which leads to apoptosis. Additionally, the perivascular adipose tissue (PVAT), which surround large blood vessels, has proven to impact endothelial function in as an effect of glycotoxins exposure (Azul *et al.*, 2020; Wang *et al.*, 2020).

The influence of maternal diabetes in the health of the progeny at adulthood is the aim of several studies, and also the aim of DOHaD (Developmental Origins of Health and Disease) concept. Gestational diabetes is of great importance due to its association with large gestational weight at birth and adverse health outcomes at adulthood (Lowe *et al.*, 2019; Moon and Jang,

2022). Maternal diabetes increases both cardiomyocyte apoptosis and macrophage infiltration in the heart of their offspring (Cerychova *et al.*, 2018). Also, diabetic pregnancy leads to impaired mitochondrial dynamism in the offspring, which implies in lower adaptation to environmental nutrient changes (Larsen *et al.*, 2019). In addition, exposure of the foetus/neonate to metabolites via the placenta or breast milk, such as glycotoxins, has been linked to short- and long-term disorders in their development (Toop *et al.*, 2017; Csongová *et al.*, 2018; Francisco *et al.*, 2018).

It is well known that mother and foetus share nutrients, metabolites, and other biomolecules. Moreover, it has also been proved that maternal glycotoxins were shared with the newborn through the blood (Roest *et al.*, 2009; Mericq *et al.*, 2010). However, an important period, such as lactation, is often overlooked. In this sense, Francisco et al. (2018, 2022) previously showed that early exposure to MG during lactation can induce metabolic disturbances at adulthood. The authors also shown that MG, or at least advanced glycation end-products were delivered through breast milk, since in both direct exposure through injection in the offspring and indirect exposure through oral administration in the mother leads to insulin resistance (Francisco *et al.*, 2018, 2022). Furthermore, Amaro et al. (2023) have shown that early glycotoxins exposure, induced by the inhibition of Glyoxalase-1 with BBGC (S-p-Bromobenzylglutathione cyclopentyl diester), causes sex-specific neurodevelopmental changes in juvenile rats.

However, despite higher incidence of cardiovascular disease and the knowledge about the effects of early glycotoxins exposure, little is known about the effects on the cardiovascular system. Thus, the aim of this study is to evaluate the effects of early methylglyoxal exposure in the cardiovascular system of juvenile rats, to evaluate if the observed effects are time-dependent or not. The main hypothesis is that early methylglyoxal exposure leads to cardiovascular impairments due to the accumulation of glycotoxins in the developing heart and changes in PVAT influence in the vascular endothelium.

2. MATERIAL AND METHODS

All experimental protocols were approved by the Ethics Committee on the Use of Animals of the Federal University of Goiás (007/21) and followed the current legal standards.

2.1. Animal model

Male (n=6) and female (n=12) 70-day-old virgin Wistar rats from the Centre for production and science in biomodels (CPCBio) of the Federal University of Goiás were housed in the sectoral animal facility of the Department of Physiological Sciences of the Federal University of Goiás, Campus Samambaia, Goiânia, Goiás. Throughout the experimental period the animals remained in polypropylene cages (45x30x15 cm) under controlled conditions of luminosity (12/12h – lights on 07:00) and temperature (23 ± 2 °C). Mating was performed at a ratio of 2 females to 1 male. Pregnancy was confirmed in the presence of vaginal plug or vaginal smear analysis (GD1; Gestational Day 1) and females were allocated to individual cages.

Pregnant Wistar rats were separated into two groups: CO (n=6), in which vehicle was administered orally; and MG (n=6), in which methylglyoxal was administered orally (60 mg/kg). At the postnatal day (PND) 3, the litters were standardized to eight pups, preferentially males. The treatment was carried out from PN3 to PN14. Figure 1A represents the experimental design.

The dams and their offspring were kept in their respective cages until PND21, at the weaning, when the pups were placed in collective cages, in a maximum number of 3 rats per cage. The offspring had their body weight and food intake monitored weekly until PND45. All animals had free access to tap water and standard rodent chow.

At PND45, end of the experimental period, 12-hour fasted animals were anesthetized with Sodium Thiopental (i.p., 40 mg/kg. Thiopentax, Cristália, São Paulo, Brazil) and euthanized for blood collection through inferior vena cava with sterile needle and syringe (Hepamax, Blau, Cotia, SP, Brazil). Blood samples were deposited in microtubes and centrifuged for plasma collection (Microcentrifuge, 7000 rpm, 15 min). Plasma was stored at -20 °C for later analysis.

After blood collection, the heart, interscapular brown adipose tissue and white adipose tissue stocks (mesenteric, retroperitoneal, inguinal) were dissected and weighed. An intermediate part of the ventricles and a fragment of the thoracic aorta was dissected for fixation in 10% formaldehyde solution for histological analysis.

2.2. Plasma biochemical measurements

Plasma levels of Blood Glucose, Fructosamine, Total and HDL cholesterol and Triglycerides, were measured by enzymatic-colorimetric methods (Bioclin, Minas Gerais, Brazil)

with commercial kits, following the manufacturer's information. The results, except for fructosamine which is expressed in μmol/L, were expressed as mg/dL.

2.3. *Ex vivo* experiments

Langendorff Heart

Another batch of adult rat offspring (n = 5 animals from different litters per group) were decapitated, and the hearts were perfused according to Langendorff technique. After trunk opening, the heart was excised and perfused through the aortic stump with Krebs-Ringer solution (118 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2 \cdot 2H_2O$, 1.20 mM $MgSO_4 \cdot 7H_2O$, 1.20 mM KH_2PO_4, 26.5 mM $NaHCO_3$ and 11.7 mM glucose) at 37°C with constant oxygenation (5% CO_2 and 95% O_2). A water filled balloon was inserted into the left ventricle and connected to a pressure transducer coupled to a data acquisition system (DataQ Instruments, USA), to acquire intraventricular pressure during systole (IVSP) and diastole (IVDP). The maximal rate of left ventricular pressure rise (max dP/dt) and maximal rate of left ventricular pressure decline (min dP/dt) were calculated from IVP. The AUC of all parameters were calculated from pre- and post-ischemia periods. The perfusion flow was adjusted to keep the perfusion pressure between 60 – 110 mmHg. The perfusion pressure was monitored through a transducer connected in parallel to the perfusion system. The sample rate was 1 KHz, and the data passed through a low-pass filter of 50 Hz to minimize interferences from power source. After a basal period (30 to 40 minutes), the hearts were perfused for an additional 15 minutes with Krebs-Ringer solution to IVP, Maximum and Minimum dP/dt assessment. Then, circumflex artery was ligated using a silk suture, and local ischemia was maintained for 30 minutes. After suture removal, the hearts were normally perfused during another 30 minutes to assess post-ischemia responses. The results of IVP were expressed in mmHg, the results of dP/dt were expressed in mmHg/s, and the results of AUC were expressed in arbitrary units.

Isolated aorta rings

Thoracic aorta rings (4 mm) from animals used in the isolated heart experiment, were placed in 10 mL organ baths at 37 °C containing gassed (95% O_2 and 5% CO_2) Krebs-Hanseleit solution (118 mM NaCl, 4.6 mM KCl, 3.3 mM $CaCl_2 \cdot 2H_2O$, 2.4 mM $MgSO_4 \cdot 7H_2O$, 0.9 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 11.1 mM Glucose). The set of rings were assessed as follow: PVAT+ E+; PVAT+

E-; PVAT- E+; and PVAT- E-. Isometric force was evaluated using a force transducer connected to a data acquisition system (DataQ Instruments, USA). After preparation, the rings were initially stretched until the resting tension reached 1.5 g and allowed to equilibrate for 1 h. Endothelium-dependent relaxation was performed with Acetylcholine (ACh; 10 μM) after pre-contraction with Phenylephrine (Phe; 0.1 μM) to evaluate the endothelium viability. The integrity of the endothelium was observed in the rings that achieved contraction more than 60% of basal value, and relaxation above 80% of maximum contraction. After viability test, a 30-minute period for the stabilization of the preparation and exchange of nutrient solution, a pre-contraction of aorta rings using phenylephrine was performed (Phe; 0.1 μM). Then, the E+ rings were submitted to the concentration curve of acetylcholine (Ach; -9; -8.5; -8; -7.5; -7; -6.5; -6; -5.5 and -5 Log mol/L), and the E- rings were submitted to the concentration curve of Sodium Nitroprusside (SNP; -11; -10.5; -10; -9.5; -9; -8.5; -8; -7.5; -7; -6.5; -6.5.5 and -5 Log mol/L). Relaxation responses were analyzed individually. Following the evaluation of relaxation, after a new period of 30 minutes for stabilization of the preparation and exchange of the nutrient solution, E- contractile response was assessed thought a concentration curve of Phe (-9; -8.5; -8; 7.5; -7; -6.5; -6; -5.5; -5 Log mol/L).

2.4. Morphological analysis

Samples of the heart and aorta, were fixed in formalin solution (10%), dehydrated in series of increasing alcohol concentrations (70% to 100%), and diaphanized in xylene, were embedded in histological paraffin. Subsequently, the materials were sectioned in a microtome (RM2245, Leica Microsystems, Wetzlar, Germany) in non-serial cuts of 6 μm thickness that were placed on glass slides and dried in an oven at 37 °C for subsequent staining with Haematoxylin and Eosin or Picrosirius-red (heart).

Photomicrographs were taken in a light microscope coupled to a digital camera (DM500 + ICC50 HD, Leica Microsystems, Wetzlar, Germany), at 1000x magnification to measure left ventricular cardiomyocytes (n= 60/group), at 400x to measure aortic medial thickness (n=30/group), perivascular fibrosis (n=30/group), and at 100x to measure interstitial fibrosis (n=60/group).

178 ***Measurement of cardiomyocyte diameter and intima–media thickness in aortas***

179 Photomicrographs of cross-sections of left ventricular cardiomyocytes and thoracic aortas
180 were analyzed. The distance between the upper and lower parts of the membrane was measured at
181 the height of the nucleus of each cardiomyocyte. Measurements were made at 4 different points in
182 the middle layer of each cut, in three different cuts, to assess the thickness of the intima-media
183 layer. The mean and standard error of mean of cardiomyocyte diameter and aortic intima-media
184 thickness for each animal were calculated, and the results compared between groups.

185 ***Perivascular fibrosis measurement***

186 Photomicrographs containing collagen markings in fields where arterioles were observed
187 in cross-sections were analyzed manually with the ICY software (Institut Pasteur, Paris, France.
188 http://icy.bioimageanalysis.org/). The area of perivascular fibrosis was determined, divided by the
189 area of the lumen of the vessel, resulting in the perivascular fibrosis index, expressed in arbitrary
190 units. The mean and standard error of the mean of the perivascular fibrosis for each animal were
191 calculated, and the results compared between groups.

192 ***Interstitial fibrosis measurement***

193 Interstitial fibrosis was analyzed by a macro processing using FIJI (ImageJ). Briefly, the
194 images were processed using colour deconvolution to split haematoxylin staining component and
195 picrosirius red staining component. Picrosirius red channel was submitted to threshold for evidence
196 real staining areas. Then, marked areas were quantified. The percentage of interstitial fibrosis was
197 estimated by the ratio between the marked areas and total area of the image. The mean and standard
198 error of the mean of the percentage of Interstitial Fibrosis were calculated for each animal and the
199 results compared between groups.

200 **2.5. Statistical analysis**

201 Data was expressed as Mean ± Standard Error of Mean (M ± SEM). Two-way ANOVA
202 followed by Sidak's post-hoc test was used for the analysis of time-dependent parameters.
203 Student's t-test was used for the analysis of time-independent parameters. The significance level

was set at $p<0.05$. For the analyses and graphical representation, GraphPad Prism (v 9.01; GraphPad, San Diego, CA, USA) was used.

3. RESULTS

3.1. Effects of early methylglyoxal exposure during lactation on the phenotype of young offspring

Exposure to glycotoxins during lactation does not impact body weight gain during the lactation (Figure 1B) or after weaning (Figure 1C). In addition, body composition and plasmatic biochemical parameters, namely liver weight (Figure 1D), blood glucose (Figure 1E), fructosamine (Figure 1F), total and HDL cholesterol (Figure 1G-H), triglycerides (Figure 1I) and adipose tissue fat pads (Figures 1J and 1M), were similar in both groups.

3.2. Effects of early methylglyoxal exposure during lactation on left ventricular cardiac function

Regarding cardiac function, early exposure to glycotoxins impacts the left ventricular contractility. Our results shown that hearts from MG offspring developed lower intraventricular systolic pressure (Pre-Ischaemia; CO 516.30 ± 56.89 mmHg vs MG 368.83 ± 12.48 mmHg; Figures 2A and 2B; $p<0.05$), although no changes were observed after local ischaemia. No changes were observed in intraventricular diastolic pressure (Figures 2C and 2D). As a consequence of lower developed IVSP, both positive and negative dP/dt were different before local ischaemia induced by circumflex artery ligation (dP/dt+ Pre-Ischaemia; CO 14736.63 ± 1716.83 mmHg/s vs MG 10780.65 ± 253.37 mmHg/s; $p<0.05$) (dP/dt- Pre-Ischaemia; CO 9514.79 ± 1419.16 mmHg/s vs MG 6577.72 ± 420.11 mmHg/s; $p<0.05$).

3.3. Effects of early exposure to methylglyoxal during lactation on vasomotricity

We sought to evaluate vasorelaxation and vasoconstriction in the presence or the absent of PVAT. In PVAT- preparations the results were similar for endothelium-mediated relaxation (Figures 3A and 3C) and NO induced smooth muscle relaxation (Figures 3D and 3F). However when PVAT was present in the preparation, despite visual similarity between the curves, EC_{50} were different in both endothelium-mediated relaxation (Figures 3B and 3C; CO $1.778 \times 10^{-7} \pm 2.77 \times 10^{-8}$ [ACh] M vs MG $2.784 \times 10^{-7} \pm 4.58 \times 10^{-8}$ [ACh] M; $p<0.05$) and NO induced smooth muscle

relaxation (Figures 3E and 3F; CO 3.310 $\times 10^{-9} \pm 4.42 \times 10^{-10}$ [SNP] M vs MG 8.156 $\times 10^{-9} \pm 1.21 \times 10^{-9}$ [SNP] M; $p<0.05$), which suggests the negative effect of PVAT on relaxation responses.

In opposite way, exerting negative regulation in the phenylephrine-induce constriction, PVAT+ preparations presents high EC_{50} values in both CO (Figures 3G-I; CO PVAT- 4.210 $\times 10^{-8} \pm 1.30 \times 10^{-8}$ [PHE] M vs CO PVAT+ 2.845 $\times 10^{-7} \pm 5.66 \times 10^{-8}$ [PHE] M; $p<0.05$) and MG groups (Figures 3G-I; MG PVAT- 8.083 $\times 10^{-8} \pm 1.41 \times 10^{-8}$ [PHE] M vs MG PVAT+ 4.820 $\times 10^{-7} \pm 6.66 \times 10^{-8}$ [PHE] M; $p<0.05$). However, early glycotoxins exposure increases PVAT negative regulation for vasoconstriction (Figure 3I; CO PVAT+ 2.845 $\times 10^{-7} \pm 5.66 \times 10^{-8}$ [PHE] M vs MG PVAT+ 4.820 $\times 10^{-7} \pm 6.66 \times 10^{-8}$ [PHE] M; $p<0.05$).

3.4. Effects of early exposure to methylglyoxal during lactation on cardiac and aorta morphology

We evaluated the morphology of the heart and aorta of MG offspring. However, no differences were detected in heart weight (Figure 4A), cardiomyocyte diameter (Figure 4B), perivascular (Figure 4C) and interstitial (Figure 4D) collagen deposition, or aorta thickness (Figure 4E).

4. DISCUSSION

In this study we aimed to evaluate the cardiovascular effects of early glycotoxins exposure during the lactation on juvenile rats. We demonstrated that MG exposure leads to cardiac dysfunction and PVAT-mediated subtle vascular dysfunction, however, with no apparent morphological remodelling. To the best of our knowledge, this study shows for the first time the cardiac functional loss in juvenile rats due to glycotoxins exposure in the lactation, which can lead to heart failure in adulthood.

The outcomes of diabetic pregnancies are well described in the literature, leading to LGA offspring (Ornoy *et al.*, 2015). However, little is known about the effects of maternal diabetes during the lactation (Ornoy *et al.*, 2015; Peila *et al.*, 2020). In this sense, corroborating experimental data that correlates high circulating glycotoxins and diabetes-induced end-organ damage (Uribarri *et al.*, 2007), approaches that address the effects of glycotoxins in the progeny are crucial to understand the phenotype of the newborns exposed to high glycotoxins environment (Mericq *et al.*, 2010; Francisco *et al.*, 2018, 2022). Mericq and colleagues (2010) addressed the

effects of maternal diabetes on breast milk and demonstrated that levels of circulating glycotoxins in the infants not only correlates with circulating glycotoxins in the mother, but also increases with age. In addition, the levels of adiponectin, an anti-inflammatory adipokine and marker of white adipose tissue health, were negative correlated with the increased circulating glycotoxins (Mericq *et al.*, 2010). Francisco and colleagues (2018) evaluate the effects of high maternal glycotoxins exposure during the lactation on the phenotype of the adult offspring. The authors demonstrated that both plasma and milk composition of MG-injected mothers was rich in fructosamine, an indicator of AGE formation. Also, the plasma of the offspring of MG-injected mother was rich in fructosamine. The main phenotype of the offspring from MG-injected mothers at adulthood is the insulin resistance and adipose tissue accumulation. In another study Francisco and colleagues (2022) demonstrated that direct exposure to glycotoxins during the same period of previous study leads to insulin resistance, end-organ oxidative stress and liver lipid accumulation at adulthood. Here, corroborating these studies we do not observe significant differences on the body weight and food intake (data not shown). Also, plasma biochemical parameters, such as fructosamine levels and dyslipidaemia, was not different between the groups. We hypothesised that metabolic effects of glycotoxins exposure are time-dependents and will appear later in life.

Despite the low impact on the metabolic profile, early glycotoxins exposure promotes cardiac dysfunction, leading to lower developed intraventricular pressure, but with no differences on the response to local ischemia. Previous studies have been evaluated the effects of glycotoxins exposure on cardiac health throughout the life. Crisóstomo and colleagues (2013) demonstrated that chronic administration of MG induces diabetes-like cardiomyopathy in the hearts of Wistar rats, and leads to lower AKT activation on ischaemic-reperfusion hearts, however, despite no increases in tissue MG the plasmatic MG was increased. In the other hand, the inhibition of AGE circulation through administration of aminoguanidine, a scavenger of reactive carbonyl groups, ameliorates cardiac dysfunction on streptozotocin-induced diabetic mice based on autophagic flux normalization and prevention of ER-stress (Pei *et al.*, 2018). The same outcome was observed through Glyoxalase 1 overexpression in the heart of mice, which preserves post-ischaemia function and morphology (Blackburn *et al.*, 2017). Taken together, this data suggests that the cardiomyocytes of MG offspring have metabolic impairments, which impacts on the contractile function and could lead to apoptosis later in life.

Together with cardiac alterations which often induces perceptive symptoms, vascular dysfunction is an important problem to cardiovascular system, leading to increased peripheral resistance and vascular hypertension due to increased contractile and decreased relaxation responses. Sena and colleagues (2012) proved that methylglyoxal increases oxidative stress, attracts immune cells and promotes endothelial dysfunction in Wistar rats, in addition to worsening the same phenotype in Goto-Kakizaki (GK) rats. MG also promotes increased autophagic flux in several endothelial cell lineages, leading to anti-angiogenic and apoptotic signals, which means low vascularization and increased tissue hypoxia (Lee *et al.*, 2020). Another factor that contributes to endothelial dysfunction is perivascular adipose tissue health. PVAT can impact smooth muscle responses to endothelium-derived substances and changes endothelial cells viability through the differential release of adipokines (Molica *et al.*, 2015; Azul *et al.*, 2020; Wang *et al.*, 2020). Here, we evaluated the endothelium-mediated relaxation using Acetylcholine and relaxation via vascular smooth muscle cells through NO donor Sodium Nitroprusside, both in the presence or absence of PVAT. In juvenile rats, the Ach-mediated relaxation remains the same between the groups in PVAT- aorta rings, however in PVAT+ rings the EC_{50} of MG aortas was slight increased in comparison to PVAT- MG rings. Also, the effects of PVAT in the NO-induced smooth muscle relaxation is subtle, given that PVAT+ CO rings presents lower EC_{50} than MG counterparts. In contrast, PVAT+ rings from MG decrease the magnitude of the phenylephrine-induced constriction, evidenced by the raised EC_{50} compared to their counterparts. Similar to our hypothesis for metabolic alterations, we believe that the vascular dysfunction via PVAT still buffered by the plasticity attributed to the puberty, however, increasing the effects in a time-dependent manner. Azul and colleagues (2020) proved that the thoracic aorta PVAT from non-obese type 2 diabetic rats (GK) contributes to endothelial dysfunction through increased macrophage infiltration, proinflammatory cytokines release, and reduction of antioxidant enzymes. The same conditions were observed in aorta from obese type 2 diabetic mice (Wang *et al.*, 2020).

Given the observed functional impairments, we sought to evaluate the morphological alterations in the heart and aorta due to early glycotoxins exposure. In this study we evaluated the cardiomyocyte diameter, perivascular and interstitial collagen deposition, and aorta wall thickening. None of these parameters have been changed in MG offspring, showing that there is a time-dependent component to progression of cardiovascular disease, which is assessed by other studies. Hypertrophied cardiomyocyte is a marker of diabetic cardiomyopathy, however the

inhibition of AGE formation using aminoguanidine has been shown to inhibit cardiomyocyte hypertrophy in adult animals, revealing the role of glycotoxins in this disease (Pei *et al.*, 2018). Regarding inflammation and collagen deposition, Fransisco and colleagues (2022) have shown that the direct exposure to MG during lactation leads to increased collagen deposition in the liver and kidney of adult rats, in addition to increased oxidative stress in these tissues. These results shown that the progression of glycotoxins-derived cardiovascular disease is time-dependent, and in the puberty the symptoms begin to appear.

5. CONCLUSION

In conclusion, our data suggest that early glycotoxins exposure led to cardiac and vascular impairments. Conversely, the aforementioned studies conducted by Francisco and colleagues shown that the effects observed in this study tend to get worse and develop end-organ injury. In this sense, knowing the effects that occur during a phase of phenotypic plasticity as relevant as puberty can open doors to new interventions that can reduce or even abolish the effects that would manifest in adult life, becomes of great importance for the studies conducted in the progeny of diabetic mothers.

Declaration of interest

The authors declare that there are no conflicts of interest.

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FIGURE CAPTIONS

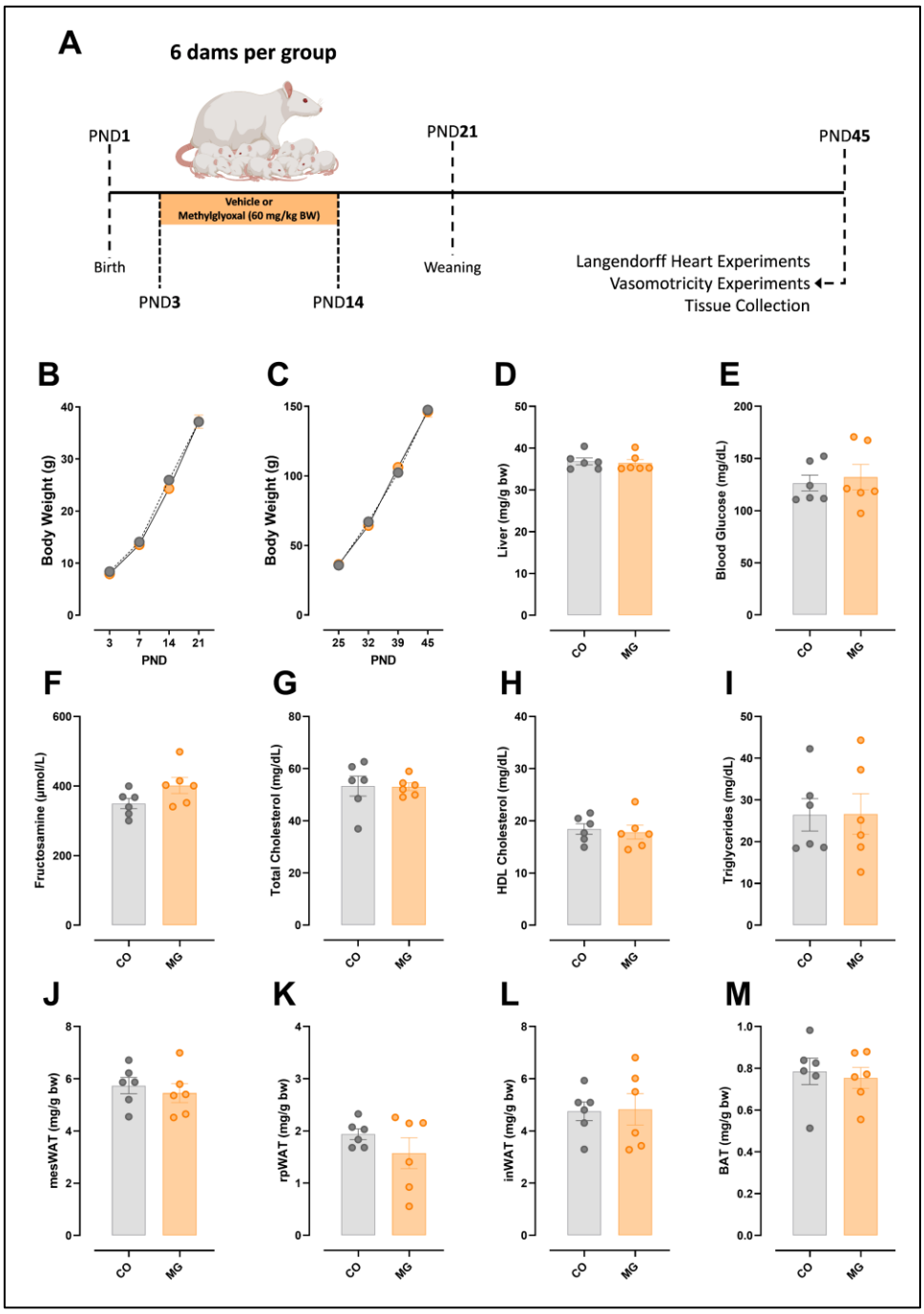
Figure 1. Effects of early methylglyoxal exposure during lactation on the phenotype of young offspring. Experimental design (A). Body weight of CO and MG offspring remains the same before (B) and after weaning (C). The weight of the most affected tissue, the liver, in MG injected animals was the same in MG than CO animals. Early MG exposure was not able to impact plasmatic biochemical parameters, namely blood glucose (E), fructosamine (F), total (G) and HDL (H) cholesterol, and triglycerides (I). The weight of mesenteric (J), retroperitoneal (K), inguinal (L) white adipose tissues were the same between the groups. In the same sense, the weight of interscapular brown adipose tissue (M) was similar between the groups. Two-way ANOVA followed by Sidak's post-hoc test was used for the analysis of time-dependent parameters. Student's t-test was used for the analysis of time-independent parameters.

Figure 2. Effects of early methylglyoxal exposure during lactation on left ventricular cardiac function. During *ex vivo* assessment of basal left ventricular intraventricular pressure MG offspring developed lower systolic pressure (A-B), despite no changes in diastolic pressure (C-D). However, MG hearts also developed lower both positive (E-F) and negative (G-H) dP/dt. No changes were observed in all evaluated parameters in response to local ischemia promoted by circumflex artery ligature. Two-way ANOVA followed by Sidak's post-hoc test was used for the analysis of time-dependent parameters. *$p<0.05$ vs CO.

Figure 3. Effects of early exposure to methylglyoxal during lactation on vasomotricity. During the vasomotricity experiments MG animals presented similar responses to CO group in the absent of PVAT for Ach (A, C) or SNP (D, F) mediated relaxation, and in PHE (G, I) mediated constriction. However, in the presence of PVAT the responses were altered in the vessels of MG animals for Ach (B-C) or SNP (E-F) mediated relaxation, and in PHE (H-I) mediated constriction. Two-way ANOVA followed by Sidak's post-hoc test was used for the analysis of time-dependent parameters. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ vs CO.

Figure 4. Effects of early exposure to methylglyoxal during lactation on cardiac and aorta morphology. Despite functional impairments, the morphology of MG hearts and aorta remains

similar to the hearts of CO offspring, evidenced by the similar heart weight (A), cardiomyocyte diameter (B), perivascular (C) and interstitial (D) fibrosis, and aorta thickness (E). Representative images of each parameter are positioned at the right side of respective graph. Black lines represent, from top to bottom, 2 mm, 25 μm, 40 μm, 100 μm and 25 μm. Student's t-test was used.



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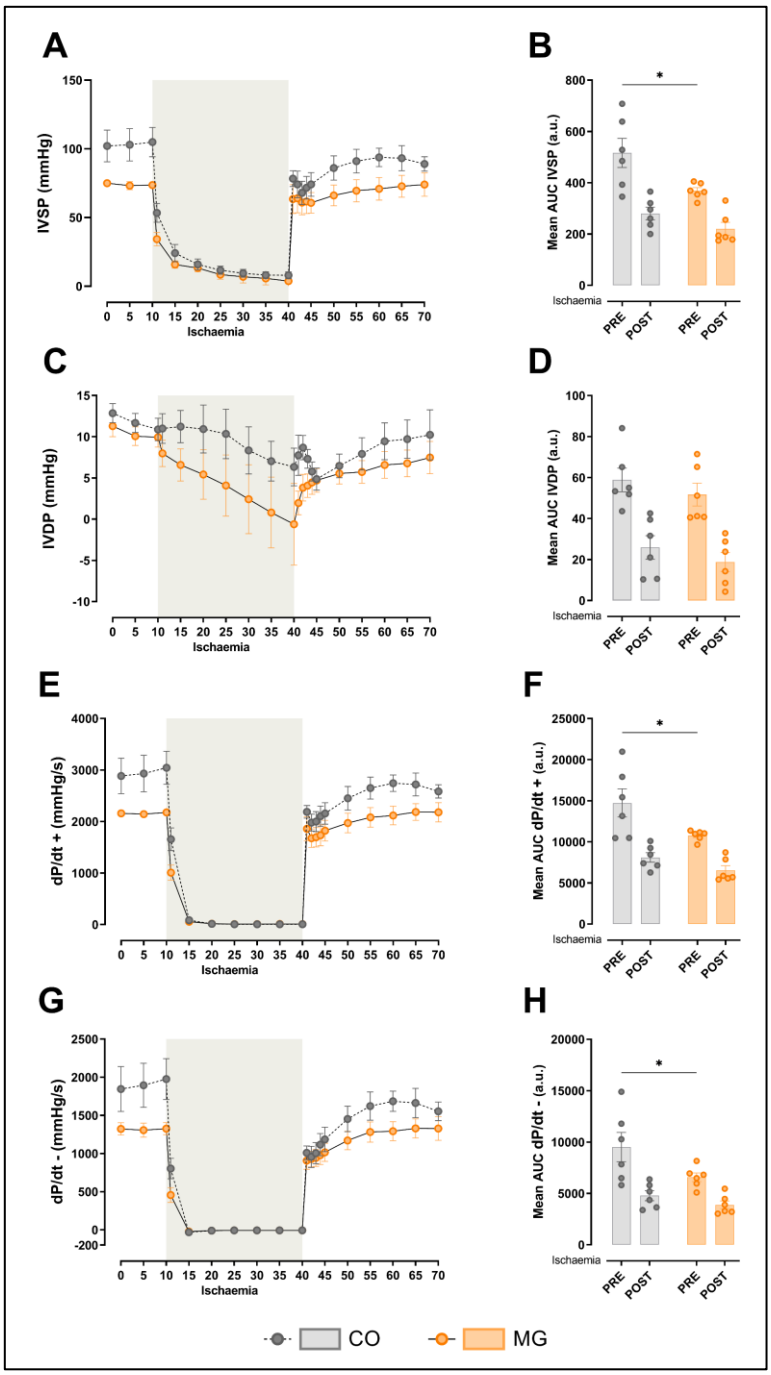

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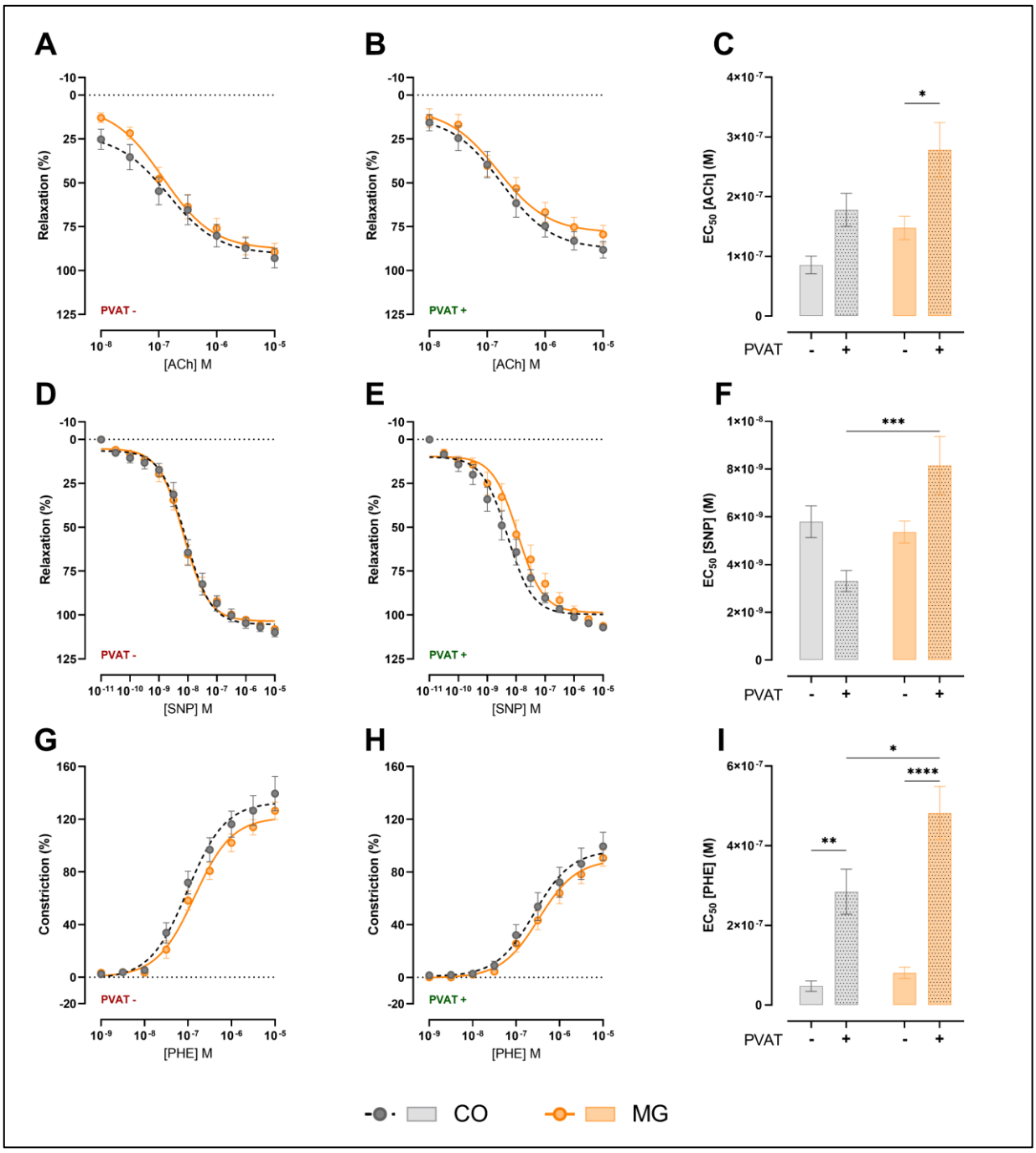

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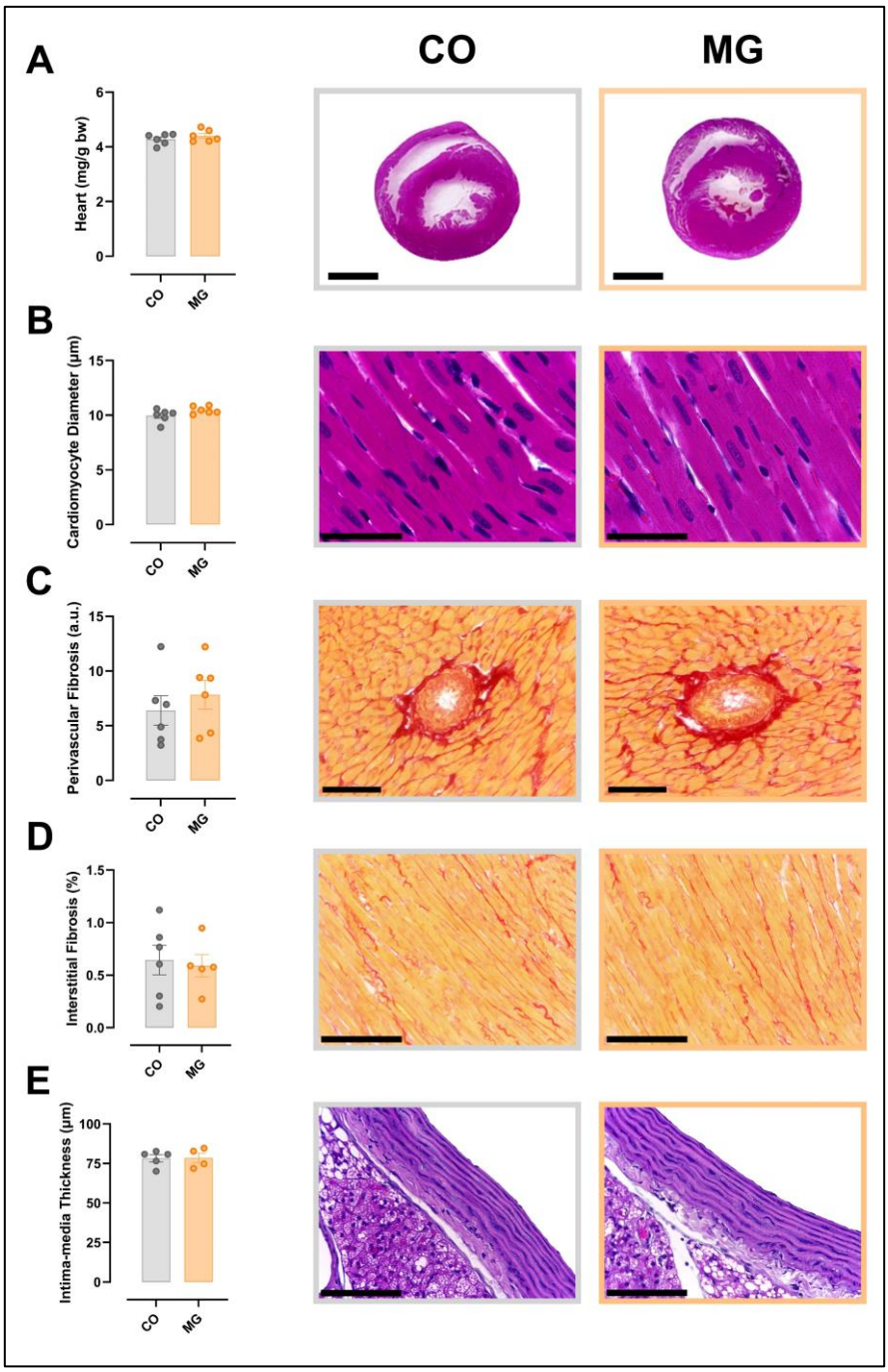

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5.2. ARTIGO 2 – EFEITOS DA ADMINISTRAÇÃO DE LEAP2 EM FASES PERINATAIS NA SAÚDE METABÓLICA DE ANIMAIS JOVENS

GHSR SIGNALLING IN PERINATAL PHASES IS INVOLVED IN THE LIVER ENERGY METABOLISM AND GLUCOSE TOLERANCE IN YOUNG ANIMALS

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Running title: Perinatal GHSR modulation affects metabolic parameters

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ABSTRACT

The normal growth and maturation of organs and systems is influenced by several hormones, especially during pregnancy and lactation. Ghrelin has effects that range from the maturation of the central nervous system to the regulation of energy balance, being an important hormone for pre- and postnatal development. The production of ghrelin increases significantly during the first weeks of life, and its production occurs not only in the stomach, but also in the pancreas of neonates up to first two weeks of life in mice. Conformational analyses demonstrated the ability of LEAP2 to bind to GHSR as an inverse agonist. Studies have addressed the metabolic effects of LEAP2 in inhibiting the effects evoked by ghrelin, mainly in glucose homeostasis, insulin resistance and lipid metabolism. Despite the known roles of ghrelin in the postnatal development, little is known about the long-term metabolic influences of modulation with the endogenous expressed GHSR inverse agonist LEAP2. Thus, this study aimed to evaluate the contribution of GHSR signalling during perinatal phases, to neurodevelopment and energy metabolism in young animals, under inverse antagonism by LEAP2. For this, two experimental models were used: 1. LEAP2 injections in female rats during the pregnancy. 2. Postnatal modulation of GHSR with LEAP2 or MK677. Perinatal GHSR modulation by LEAP2 impacts glucose homeostasis in a sex and phase dependent manner, despite no effects on body weight gain or food intake. Interestingly, liver PEPCK expression was markable impacted by LEAP2 injections. Taken together the observed results suggests that perinatal LEAP2 exposure can modulate liver metabolism and systemic glucose homeostasis. In addition, these results, although not expressive, may just be the beginning of the metabolic imbalance that will occur in adulthood.

Keywords: DOHaD; GHSR; LEAP2; Glucose Tolerance; Liver metabolism.

1. INTRODUCTION

The normal growth and maturation of organs and systems is influenced by several hormones, especially during pregnancy and lactation. Some of these hormones are still released by the mother, exerting effects on the foetus. Others help in the development of the newborn, exerting their influence through breast milk.

Ghrelin, a hormone first reported by Kojima et al. (1999), is an endogenous ligand of the growth hormone secretagogue receptor (GHSR). In addition to the effects on eating behaviour (Wren, 2001), ghrelin has effects that range from the maturation of the central nervous system to the regulation of energy balance, being an important hormone for pre- and postnatal development. Ghrelin is also important to development and nutrient sensing of the foetus and milk production in lactating rats (Nakahara *et al.*, 2003, 2006; Torsello *et al.*, 2003; Torres *et al.*, 2021).

Ghrelin production increases significantly during the first weeks of life, and its production occurs not only in the stomach, but also in the pancreas of neonates up to first two weeks of life in mice (Hayashida *et al.*, 2002; Wierup *et al.*, 2002; Steculorum and Bouret, 2011). Circulating ghrelin levels in newborns decrease with age, reinforcing the role of activation of GHSR-dependent pathways in maturation and growth (Soriano-Guillén *et al.*, 2004), and corroborates the decrease in the pancreatic secretion of ghrelin (Wierup *et al.*, 2002).

GHSR has basal constitutive activity, different from other G-protein-coupled receptors (Damian *et al.*, 2012). Conformational analyses demonstrated the ability of LEAP2 (Liver-expressed antimicrobial peptide 2) to bind to GHSR as an inverse agonist, in view of the change in conformation compared with the activation of the receptor by ghrelin (Damian *et al.*, 2015; M'Kadmi *et al.*, 2019). The effects of dose-dependent LEAP2 injections were evaluated on GH release and food intake, showing that LEAP2 inhibits these two GHSR-influenced parameters even in the presence of ghrelin (Ge *et al.*, 2018).

Aiming to evaluate the metabolic effects of the interaction between LEAP2 and ghrelin, and corroborating data from previous studies on eating behaviour (Ge *et al.*, 2018; M'Kadmi *et al.*, 2019), Mani et al. (2019) evaluated the expression and correlation between LEAP2, ghrelin, body mass and food intake in humans and rodents, also showing the anorexigenic action of LEAP2 and the positive correlation between LEAP2 and body weight. Furthermore, the inhibitory activity of

LEAP2 on the depolarization of NPY+ neurons in the arcuate nucleus has been reported, even in the presence of ghrelin (Mani *et al.*, 2019).

Studies have addressed the metabolic effects of LEAP2 in inhibiting the effects evoked by ghrelin, mainly in glucose homeostasis, insulin resistance and lipid metabolism (Aslanipour, Alan and Demir, 2020; Fittipaldi *et al.*, 2020; Ma *et al.*, 2021; Spann, Welch and Grayson, 2021). Address this effects of GHSR signalling, in special on liver metabolism, could help to understand long-term effects of LEAP2 exposure early in life, since ghrelin activates both glucagon secretion in pancreatic alpha cells, and regulates the liver gluconeogenesis through increased PEPCK and G6Pase content (Chuang *et al.*, 2011; Lin *et al.*, 2019).

In the light of DOHaD concept, some studies have addressed the role of GHSR signalling in perinatal development through pharmacological and nutritional interventions (Hayashida *et al.*, 2002; Sun *et al.*, 2020; Spann, Welch and Grayson, 2021). However, little is known about the long-term metabolic influences of modulation with the endogenous expressed GHSR inverse agonist LEAP2. Thus, this study aimed to evaluate the contribution of GHSR signalling during perinatal phases, to neurodevelopment and energy metabolism in young animals, under inverse antagonism by LEAP2.

2. MATERIALS AND METHODS

All experimental protocols were approved by the Ethics Committee on the Use of Animals of the Federal University of Goiás (72/23) and followed the current legal standards.

2.1. Drugs

Based on full length LEAP2 peptide, which consists of 40 amino acids, LEAP2[1-14]-NH2 was synthetized to assess the minimal active sequence within LEAP2. In the study of M'Kadmi and colleagues (2019), LEAP2[1-14]-NH2 exhibits satisfactory actions through GHSR, with similar values for full length peptide for binding constants (Ki), and receptor inhibition (EC50 and Emax for inositol 1-phosphate formation).

MK677, also known as Ibutamoren, is a potent GHSR agonist used in this study to be a positive control for GHSR activation.

2.2. Animal models

Experimental model 1 – Intrauterine

Male (n=6) and female (n=12) 70-day-old virgin Wistar rats from the Central Animal Facility of the Federal University of Goiás were housed in the sectoral animal facility of the Department of Physiological Sciences of the Federal University of Goiás, Campus Samambaia, Goiânia, Goiás. Throughout the experimental period the animals remained in polypropylene cages (45x30x15 cm) under controlled conditions of luminosity (12/12h – lights on 07:00) and temperature (23 ± 2 °C). Mating was performed at a ratio of 2 females to 1 male. Pregnancy was confirmed in the presence of vaginal plug or vaginal smear analysis (GD1; Gestational Day 1) and females were allocated to individual cages.

Pregnant females were separated into Veh (Vehicle) and LEAP2 groups. The injections of vehicle (PBS; 1ml/kg of bw, s.c.) or LEAP2 (72 nmol/kg of bw, s.c.) were performed from GD10 to GD20. The dose of LEAP2 was chosen based on previous studies, in order to do not impact food intake, but only interfere with GH secretion (Ge *et al.*, 2018; Islam *et al.*, 2020).

At birth PND1 (Postnatal Day 1), litters had their mass and male/female ratio assessed. At PND3 litters were standardised to 8 pups per dam, with male/female equity preserved whenever possible. The remaining pups were euthanised by decapitation. At PND21, the animals were weaned and allocated to separate boxes containing two animals of the same sex and treatment. Body weight and food intake were assessed until PND45, when the experiments were done. Figure 1A shows the schematic representation of experimental model 1.

Experimental model 2 – Lactation

Male (n=7) and female (n=14) 70-day-old virgin Wistar rats from the Central Animal Facility of the Federal University of Goiás were housed in the sectoral animal facility of the Department of Physiological Sciences of the Federal University of Goiás, Campus Samambaia, Goiânia, Goiás. The conditions of housing and matting were similar to the Experimental Model 1.

After birth, in the PND3, litters were standardised to 8 pups per mother, preserving equity between males and females whenever possible.

One animal of each offspring, which contained eight animals (four males and four females whenever possible), received daily injections of vehicle, the GHSR antagonist LEAP2[1-14]-NH_2 (0.1 and 1 μmol/kg), or the GHSR agonist MK677 (750 μmol/kg).

Injections of vehicle (1ml/kg of bw), LEAP2, or MK677 were performed subcutaneously from PND3 to PND14. Figure 4A shows the schematic representation of experimental model 2.

2.3. Oral glucose tolerance test (oGTT)

At PND45, six-hour fasted animals, from both experimental models, were subjected to glucose tolerance test (oGTT). A glucose solution (1 g/ml) diluted in normal saline (0.9% NaCl) was intragastric administered (2 ml/kg of bw). Blood samples were collected at a time point prior to administration of glucose solution, and after 15, 30, 60 and 120 minutes. Blood samples were collected via a little puncture at the tail end and the blood glucose measured by commercial glucometer with disposable strips (On Call Plus II, San Diego, California, USA).

2.4. Euthanasia and sample collection

At PND46, animals from both groups were separated into three subgroups for tissue collection: *Standard Fasting* (SF), in which animals were euthanised after a six-hour fast; *Postprandial* (PP), in which for six-hour fasted animals were euthanised one hour after administration of a mixed meal (NAN SciencePro S.L., 300 mg/animal). Supplementary Figure S1 schematises the experimental design of sample collection.

The mixed meal was composed by 59% carbohydrates, 11% proteins and 25% fats. The dose of the mixed meal was calculated based on the average hourly caloric intake of an animal in the Veh group. The average calorific intake was approximately 1.5 Kcal per hour, which was proportional to 0.3 g of the mixed meal. The 0.3 g was then diluted to a volume of 0.5 ml and administered by gavage to the PP animals. Before administration, and one hour after administration of the mixed meal, the blood glucose of the PP animals was measured by a commercial glucometer (On Call Plus II, San Diego, California, USA).

After the specific protocol for each subgroup, the animals had their blood glucose measured by a commercial glucometer (On Call Plus II, San Diego, California, USA), and then were

anaesthetised with Sodium Thiopental (i.p.; 40 mg/kg BW; Thiopentax, Cristália, São Paulo, Brazil) and euthanised by exsanguination. Then, the liver, pancreas, brown (BAT) and white (retroperitoneal and perigonadal) fat pads were collected, dissected, and weighed.

From animals in the SF subgroup, samples of the pancreas were fixed in Buffered Formalin (4% formaldehyde diluted in PBS, pH 7.4) for histological evaluations. Samples of the liver were frozen to perform molecular analysis.

2.5. Histological analysis

Formalin fixed pancreas samples were dehydrated in a series of increasing alcohol concentrations (70% to 100%), diaphanized in xylene and embedded in histological paraffin (Synth, Diadema, São Paulo, Brazil). Subsequently, the materials were sectioned on a microtome (RM2245, Leica Microsystems, Wetzlar, Germany) in non-serial sections of 6 µm thickness that were placed on glass slides for subsequent staining with Haematoxylin and Eosin.

Photomicrographs were taken under a light microscope coupled to a digital camera (DM500 + ICC50 HD, Leica Microsystems, Wetzlar, Germany), at 400x magnification to measure pancreatic islet area. Results were expressed in μm^2.

Immunohistochemistry

A commercial kit (Histostain-Plus, Invitrogen, Carlsbad, CA, USA) was used as previously described (Gomes *et al.*, 2018). Anti-Insulin mouse monoclonal primary antibody were used to label insulin positive cells (1:250 dilution - catalogue # I2018 from Sigma-Aldrich Inc., St. Louis, MO, USA). Then, sections were counterstained with hematoxylin. Photomicrographs were taken under a light microscope coupled to a digital camera (DM500 + ICC50 HD, Leica Microsystems, Wetzlar, Germany), at 400x magnification to measure insulin staining. Colorimetric staining was analyzed by a macro processing using FIJI (ImageJ). Briefly, the images were processed using colour deconvolution to split haematoxylin staining component and AEC staining component. AEC channel was submitted to threshold for evidence real staining areas. Then, marked areas were quantified. Insulin staining was calculated by the ratio between integrated optical density and their respective marked areas. Results were first expressed in intensity/area, and then normalized to control group.

2.6. Western blot

Liver samples (n = 4 per group) were homogenized in RIPA lysis buffer using a glass homogenizer at 4 °C. Tissue extracts were centrifuged, and the supernatant collected. After centrifugation, supernatant total protein content was quantified by the Bradford method (Ernst and Zor, 2010). The samples were then denatured in Laemmli buffer. Aliquots of 40 μg of proteins were subjected to separation by SDS-PAGE. Separated proteins on the gel were transferred to nitrocellulose membranes (Amersham Protran, GE Healthcare, Little Chalfont, BUX, UK) in a wet transfer system, soaked in transfer buffer. The membranes were blocked with skim milk solution and were incubated with primary antibodies for PEPCK (catalogue SC-271029). Then, the membranes were washed and incubated with appropriate HRP-conjugated secondary antibody. The chemiluminescence was detected by an image documentation system (ImageQuant LAS 4000 series, GE Healthcare, Chicago, IL, USA), and images were captured. The intensity of the bands was quantified by relative optical density using FIJI software (ImageJ, NIH, Cambridge, MA, USA). B-actin (catalogue MA5-15739) was used as load control. The results were normalized to loading control, and the results expressed in % of control.

2.7. Statistical analysis

Data were expressed as the Mean ± Standard Error of the Mean (M ± SEM). For comparison of the effects of LEAP2 administration in pregnancy or lactation on time-dependent parameters, two-way analysis of variance followed by Sidak's post-hoc test was used, with the significance level set at $p<0.05$. For non-time-dependent comparisons, Student's t-test was used for comparisons between groups in the pregnancy protocol, and one-way analysis of variance was used for comparisons between groups in the lactation protocol (Tukey's post-hoc test), with the significance level set at $p<0.05$. Prism 9 software (v 9.01; GraphPad, San Diego, CA, USA) was used for the analyses and graphical representation.

3. RESULTS

3.1. Effects of GHSR inhibition by the administration of LEAP2 during the pregnancy on the pregnant female and in the young offspring

Effects of GHSR inhibition by the administration of LEAP2 during the pregnancy on the pregnancy outcome, offspring health and neurodevelopment-related parameters

Administration of LEAP2 during pregnancy did not cause significant changes in body weight gain, food intake nor water intake in the mothers (Supplementary Figure S2). As expected, administration of LEAP2 during pregnancy was not able to impact in the pregnancy outcome nor influence with sex of live-born foetuses (Supplementary Figure S3).

Administration of LEAP2 during pregnancy did not influence postnatal development, with no differences observed in the assessments of reflexes related to survival (Supplementary Figure S5), locomotor activity and auditory maturation (Supplementary Figure S6).

Effects of GHSR inhibition by the administration of LEAP2 during the pregnancy on body weight, food intake of their young offspring

Administration of LEAP2 during pregnancy was also unable to alter the eating behaviour of the offspring, as evidenced by breast milk intake (Supplementary Figure S4A-D). Breast milk composition at PND12 was also not modified by LEAP2 administration during pregnancy (Supplementary Figure S4E).

LEAP2 administration during pregnancy was not able to affect body weight gain of the offspring (Figure 1B, 1L), which also did not affect food intake after weaning (Figure 1C, 1M).

Intrauterine exposure to LEAP2 was unable to alter the final body weight or nasoanal length (Figure 1D-E, N-O), fasting blood glucose (Figure 1F, P) most morphological parameters (Figure 1G-K, R-U) including adipose tissue fat pads and pancreas weight, with the exception of liver mass corrected for body weight in females (Figure 1Q).

Effects of GHSR inhibition by the administration of LEAP2 during the pregnancy on glucose dynamics and pancreatic morphology

Intrauterine exposure to LEAP2, was able to alter glucose dynamics in females (Figure 2D-E), without altering the response to oral glucose administration on blood glucose (Figure 2A), area under the blood glucose curve during the test (Figure 2B) and the response to mixed meal administration (Figure 2C) in the male offspring. In the female offspring, the lower response to oral glucose administration was evidenced by the lower area under the curve (Figure 2E; Veh 7260 ± 326 a.u. vs LEAP2 6249 ± 209 a.u.; $p<0.05$ vs Veh) during the oGTT, and at 60 min into the test (Figure 2D; Veh 110.9 ± 3.3 mg/dL vs LEAP2 96.4 ± 2.4 mg/dL; $p<0.01$ vs Veh). In common with males, the administration of mixed meal was unable to affect the glycaemic response in females (Figure 2F).

Additionally, intrauterine exposure to LEAP2 was unable to significantly alter pancreatic islet morphology in the offspring, despite higher mean pancreatic islet area in males and females (Figure 2G, 2H).

Even though no alterations in islet area or insulin staining in male LEAP2 offspring (Figure 2I), there is a trend to lower insulin staining in the pancreatic islets of female LEAP2 offspring (Figure 2H; Veh 100.0 ± 3.04 vs LEAP2 93.1 ± 0.52 % of control; $p=0.0866$ vs Veh).

Effects of GHSR inhibition by the administration of LEAP2 during the pregnancy on hepatic PEPCK expression

We sought to also evaluate hepatic gluconeogenesis, through liver PEPCK expression. Male LEAP2 offspring present similar levels of PEPCK content (Figure 3A), whereas female LEAP2 offspring had a strong trend to overexpress PEPCK in the liver (Figure 3B; Veh 100.0 ± 9.28 vs LEAP2 174.3 ± 34.34 % of control; $p=0.0816$ vs Veh).

3.2. Effects of modulation of GHSR signalling during lactation on metabolic parameters in young animals

Effects of GHSR signalling modulation during lactation on body weight and food intake in young animals

GHSR signalling modulation during lactation was not able to affect body weight gain of the animals (Figure 4A, 4K), which also did not affect food intake after weaning (Figure 4C, 4L). In the same sense, final body weight (Figure 4D, 4M), fasting blood glucose (Figure 4E, 4N), liver (Figure 4F, 4O), adipose tissue fat pads (Figure 4G-I, 4P-R) and pancreas weight (Figure 4J, 4S) were similar in all groups compared to Veh.

Effects of GHSR signalling modulation during lactation on glucose dynamics in young animals

Modulation of GHSR signalling during lactation was not able to cause major changes in glucose dynamics. Postnatal administration of LEAP2 was able to change peak response during the GTT in males significantly in the first fifteen minutes of the test (Figure 5A; Veh 157.64 ± 7.53 mg/dL vs LEAP2[0.1] 122.15 ± 6.52 mg/dL and LEAP2[1] 121.10 ± 5.05 mg/dL; $p<0.05$ vs Veh). There is a slight trend to decrease the AUC of the test in animals administered with MK677 compared to the Veh group (Figure 5B; Veh 8241 ± 572.5 a.u. vs MK677 6875 ± 244.9 a.u.; $p=0.0614$ vs Veh). There were no changes in glucose dynamics in females (Figures 15F and 15G), nor in the response to mixed meal in both sexes (Figures 5C and 5H).

Despite the alterations on glucose dynamics, postnatal injections of LEAP2 tends to reduce pancreatic islet area in males (Figure 5D; Veh 27979 ± 4962 μm^2 vs LEAP2[1] 3830 ± 481.3 μm^2; $p=0.0915$ vs Veh), but not in females (Figure 5I). However, insulin staining was similar in the pancreatic islets between all the groups (Figures 5E and 5J).

Effects of GHSR signalling modulation during lactation on hepatic PEPCK expression

We also tried to evaluate hepatic gluconeogenesis in the liver, through PEPCK expression. Male LEAP2 present reduced levels of PEPCK content (Figure 6A; Veh 100.0 ± 1.48 vs LEAP2[0.1] 71.50 ± 5.26 and LEAP2[1] 64.90 ± 8.75 % of control; $p<0.05$ vs Veh) in addition to lower PEPCK content in MK677 injected animals (Figure 6A; MK677 70.92 ± 2.41

277 % of control; p<0.05 vs Veh). Female rats injected with LEAP2 also presented lower levels of
278 liver PEPCK (Figure 6A; Veh 100.0 ± 0.15 vs LEAP2[0.1] 49.24 ± 12.09 and LEAP2[1] 55.95 ±
279 9.57 % of control; p<0.05 vs Veh).

280 **4. DISCUSSION**

281 In our study, we sought to understand the role of GHSR signalling in the evolution of body
282 weight and energy metabolism in young rats exposed early in life to GHSR modulators. Perinatal
283 modulation of GHSR receptor signalling was not able to generate relevant effects on body weight
284 gain, food intake or the weight of important organs for the metabolism. However, early exposure
285 to LEAP2, a GHSR receptor inhibitor, was able to change the glycaemic response during the oral
286 glucose tolerance test, and PEPCK enzyme in the liver.

287 Here, maternal exposure to LEAP2 did not interfere with body weight gain, food intake or
288 water intake in pregnant rats. In fact, the dose of LEAP2 used was thought to only affect GH
289 release, with no planned effects on food intake, corroborating Xuecai Ge et al. (2018). Furthermore,
290 even if LEAP2 injection influenced food intake, the caloric deficit could be reversed throughout
291 the day, abolishing the nutrient deficit that our experimental design sought to avoid. However, due
292 to GH and ghrelin signalling being of extreme importance for offspring survival and foetal
293 maturation (Torsello *et al.*, 2003; Hietaniemi *et al.*, 2009; Steculorum and Bouret, 2011; Sato *et al.*,
294 2022), in this study, we were unsuccessful in assessing the offspring of females that underwent
295 50% calorie restriction in pregnancy in parallel with daily LEAP2 injections (data not shown). A
296 study involving pregnant rats undergoing vertical gastrectomy also do not reported loss of foetal
297 viability, which may be an indication that it is not the lack of ghrelin, but rather the inverse agonism
298 of GHSR associated with calorie restriction that may disrupt pregnancy (Spann, Welch and
299 Grayson, 2021). In order to assess possible abortifacient effects of GHSR signalling inhibition, we
300 evaluated the progression of pregnancy and the sex of live-born foetuses. The administration of
301 LEAP2 also did not interfere with the evolution of pregnancy, nor did it affect the ratio of
302 male/female pups born alive, corroborating other studies (Torsello *et al.*, 2003; Torres *et al.*, 2018;
303 Spann, Welch and Grayson, 2021). A limitation of our study was the lack of assessment of foetal
304 viability, which would not only require a larger number of pregnant females but would also
305 extrapolate our hypotheses.

Ghrelin, and consequently GH, are critical for proper neurodevelopment in rodents (Katayama *et al.*, 2000; Nakahara *et al.*, 2006). Torres and colleagues (2021) showed that the offspring of ghrelin-injected pregnant mothers, had precocious physical and neurobiological development. However, maternal injections of LEAP2 were not able to induce changes in the neurodevelopment of their offspring.

In the offspring of LEAP2-injected mothers, there was no decrease in milk intake by the offspring as hypothesised, nor was there a difference in body weight gain in the lactation phase or after weaning. In addition, there was no difference in breast milk composition in mothers injected with LEAP2 during pregnancy, compared to their counterparts. Studies have shown that ghrelin release is critical for eating behaviour (Nakahara *et al.*, 2003; Yanagi *et al.*, 2018; Sun *et al.*, 2020). Therefore, it is appropriate for other studies to evaluate whether it is the inverse agonism of GHSR by increased plasma LEAP2 or the lower production of ghrelin that influences the eating behaviour of the offspring. We hypothesise that unlike models in which increased ghrelin levels promote increased food intake, higher LEAP2 levels may have a regulatory role, but without detrimental effects, corroborating the study of Torres and colleagues (2018) in which the GHSR blockade by (D-Lys^3)GHRP-6 during pregnancy does not impact the body weight gain in the offspring. Similar results on body weight and food intake were observed when the injections of LEAP2 or MK677 were done in the lactation. In fact, the expected behaviour would be a decrease in food intake in LEAP2-injected rats, and high food intake in the MK677 group. However, it should be noted that the injections were performed once a day, with limited drug bioavailability, which makes room for the readjustment of metabolism in the remaining period.

Male and female LEAP2 offspring, as well as animals injected with LEAP2 or MK677 during lactation, showed no differences in body adiposity, liver weight or pancreas weight. LEAP2 only decreased the liver weight of female offspring of LEAP2-injected mothers. Further studies should evaluate liver morphology and perform molecular analysis of pathways responsible for lipid metabolism to elucidate this effect.

It is well known that ghrelin has actions beyond the control of eating behaviour, being important for development and exerting effects on organs and systems, including the endocrine pancreas (Broglio *et al.*, 2001; Nakahara *et al.*, 2006). LEAP2 has been shown to counteract the effects of ghrelin on glucose homeostasis (Bayle *et al.*, 2022; Stark *et al.*, 2023). It is also known

that alterations in signalling pathways during perinatal phases can remain for a long time, as an effect of metabolic programming. Studies not involving metabolic programming have shown that under ghrelin regulation, glucose-stimulated insulin secretion was reduced (Broglio *et al.*, 2001; Dezaki *et al.*, 2004; Dezaki, Kakei and Yada, 2007). However, when the GHSR was blocked by [D-Lys^3]-GHRP-6, blood glucose decreased remarkably as a result of increased insulin release (Dezaki *et al.*, 2004). In this regard, LEAP2 expression has been correlated with enhanced insulin secretion in overweight humans (Stark *et al.*, 2023) and antagonised the actions of ghrelin in isolated pancreatic islets (Bayle *et al.*, 2022). In our study, there were global changes in glycaemia of female offspring of LEAP2-injected mothers during the glucose tolerance test. In males, the effect of LEAP2 injections in the mother were less pronounced, with statistical differences not detected. Interestingly, although female LEAP2 offspring showed lower blood glucose values during GTT, which may indicate higher insulin sensitivity or higher insulin secretion, the response to mixed meal administration did not interfere with the increase in blood glucose after 60 min between groups. This may be explained by the high percentage of complex sugars in the mixed meal or the presence of insulinotropic amino acids than in glucose overload necessary to perform the oGTT. In animals injected with LEAP2 or MK677 during lactation, only males showed a difference in glycaemia during the glucose tolerance test. The two doses of LEAP2 used in this study were able to promote effects on peak glycaemic response in the first fifteen minutes of the test. Injections of MK677 did not promote noticeable effects on the GTT, however a slope towards lower AUC values during the test is observed. Conversely, females exhibited no effects due to GHSR modulation on the GTT. Similar to the offspring of mothers injected with LEAP2 during pregnancy, GHSR modulation during lactation did not influence the response to the mixed meal.

Despite the effects during GTT, LEAP2 administration in mothers did not interfere with pancreatic islet size in their offspring. In females, despite the larger pancreatic islet size compared to males, there were no differences between groups. Taken together, these results did not explain the differences observed in the GTT, thus we performed insulin labelling in pancreatic islets. We do not observed differences in the insulin staining in all groups, however in the female offspring of LEAP2-injected mothers there is a strong tendency to reduced insulin marking regardless of the results observed in GTT.

An indicative of hepatic insulin resistance is the upregulation of gluconeogenic pathways. It is well known that ghrelin exerts coordinated actions to improve glucose release from the liver, which mainly includes glucagon release in the pancreatic islets, and glucagon stimulates gluconeogenesis in the liver (Gauna *et al.*, 2005; Esler *et al.*, 2007; Chuang *et al.*, 2011; Brial *et al.*, 2015; Lin *et al.*, 2019). In this study we evaluate liver PEPCK content through western blot. The female offspring of LEAP2-injected mothers tends to present increased levels of PEPCK in the liver, whereas male offspring had normal expression of this enzyme, in comparison to Veh offspring. Further studies need to assess whether the higher expression of PEPCK in the liver of female offspring of LEAP2-injected mothers contributes to the depletion of glycogen content, and consequently reduce liver weight. On the other hand, in the animals injected with LEAP2 during lactation, the expression of PEPCK was markable reduced in both sexes, however only males injected with MK677 presented the same phenotype, compared to Veh counterparts.

5. CONCLUSION

Our results demonstrate that exposure to LEAP2 at perinatal stages can impact glucose homeostasis. To the best of our knowledge, perinatal GHSR antagonism promoted a long-term inhibition of ghrelin signalling, resulting in increased glucose-stimulated insulin secretion. However, the observed results may just be the beginning of the metabolic imbalance that will occur in adulthood. Future studies should assess whether the phenotype observed here is maintained or worsened throughout life.

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FIGURE CAPTIONS

Figure 1. Effects of GHSR inhibition by the administration of LEAP2 during the pregnancy on body weight, food intake of their young offspring. Experimental design (A). The phenotype of LEAP2 offspring was not changed by the treatment, as evidenced by the similarity between body weight gain (B, L) and food intake (C, M) throughout experimental period, and the body weight (D, N), nasoanal length (E, O), blood glucose (F, P), liver (G, Q), retroperitoneal (H, R) and perigonadal (I, S) white adipose tissues, interscapular brown adipose tissue (J, T) and pancreas (K, U) weight at the end of the experimental period in males and females, respectively. Two-way ANOVA followed by Sidak's post-hoc test was used for the analysis of time-dependent parameters. Student's t-test was used for the analysis of time-independent parameters. *$p<0.05$ vs Veh.

Figure 2. Effects of GHSR inhibition by the administration of LEAP2 during the pregnancy on glucose dynamics and pancreatic morphology. During the oral glucose tolerance test, despite male LEAP2 offspring present no changes in the response (A) or AUC (B), female offspring presented increased glucose tolerance during the test (D) evidenced by the lower AUC (E). The response of a mixed meal injection was the same in both groups (C, F). No differences were observed in the pancreatic islets area (G, H) or insulin staining in the pancreatic islets (I, J). Two-way ANOVA followed by Sidak's post-hoc test was used for the analysis of time-dependent parameters. Student's t-test was used for the analysis of time-independent parameters. *$p<0.05$, **$p<0.01$ vs Veh.

Figure 3. Effects of GHSR inhibition by the administration of LEAP2 during the pregnancy on hepatic PEPCK expression. Phosphoenolpyruvate carboxykinase (PEPCK) expression in male (A) and female (B) livers from Veh or LEAP2 offsprings. Student's t-test was used. Representative immunoblots of PEPCK and Actin.

Figure 4. Effects of GHSR signalling modulation during lactation on body weight and food intake in young animals. Experimental design (A). The phenotype of the offsprings was not changed by LEAP2 or MK677 injections during the lactation, as evidenced by the similarity between body weight gain (B, K) and food intake (C, L) throughout experimental period, and the body weight (D, M), blood glucose (E, N), liver (F, O), retroperitoneal (G, P) and perigonadal (H, Q) white adipose tissues, interscapular brown adipose tissue (I, R) and pancreas (J, S) weight at

the end of the experimental period in males and females, respectively. Two-way ANOVA followed by Sidak's post-hoc test was used for the analysis of time-dependent parameters. One-way ANOVA followed by Tukey's post-hoc test was used for the analysis of time-independent parameters.

Figure 5. Effects of GHSR signalling modulation during lactation on glucose dynamics in young animals. During the oral glucose tolerance test, male LEAP2 offsprings ([0.1] and [1]) present increased glucose tolerance during the test, evidenced by the lower peak response to glucose overload (A), despite no changes in glucose tolerance in female offsprings (F). However, there are no changes in the AUC of the test for both sexes (B, G). The response of a mixed meal injection was the same in both groups (C, H). No differences were observed in the pancreatic islets area (D, I) or insulin staining in the pancreatic islets (E, J). Two-way ANOVA followed by Sidak's post-hoc test was used for the analysis of time-dependent parameters. One-way ANOVA followed by Tukey's post-hoc test was used for the analysis of time-independent parameters. *$p<0.05$ LEAP2 [0.1] and [1] vs Veh.

Figure 6. Effects of GHSR signalling modulation during lactation on hepatic PEPCK expression. Phosphoenolpyruvate carboxykinase (PEPCK) expression in male (A) and female (B) livers from Veh, LEAP2 and MK677 injected animals. One-way ANOVA followed by Tukey's post-hoc test was used. *$p<0.05$, **$p<0.01$. Representative immunoblots of PEPCK and Actin.

Supplementary Figure 1. Experimental design of postprandial blood glucose assessment. Experimental design of standard fasting (A) and postprandial (B) blood collection.

Supplementary Figure 2. Effects of LEAP2 injections during pregnancy. Effects of LEAP2 injections on raw and corrected body weight (A, D), food (B, E) and water intake (C, F).

Supplementary Figure 3. Effects of LEAP2 injections in pregnant females on pregnancy outcome. Ratio of matted rats to successful pregnancies (A) and sex of the pups (B).

Supplementary Figure 4. Effects of maternal LEAP2 injections on the food intake of their offspring and milk composition. Raw and normalized milk intake of male (A, C) and female offspring (B, D). Creamatocrit evaluation of PND12 milk from Veh- and LEAP2-injected mothers (E).

Supplementary Figure 5. Effects of maternal LEAP2 injections in the neurodevelopment of their offspring. Assessment of neurodevelopment through the presence of righting (A-B), cliff aversion (C-D) and negative geotaxis (E-F) reflexes and the nest seeking behaviour (G-H).

Supplementary Figure 6. Effects of maternal LEAP2 injections in the locomotor activity and auditory maturation of their offspring. Assessment of locomotion (A-B) and forepaws strength test (C-D). Maturation of auditory system (E-F).

SUPPLEMENTARY DATA

Assessment of milk intake, and creamatocrit assessment

During the lactation, milk intake has been assessed at PND6, PND11 and PND16 (Purcell *et al.*, 2011). The pups were removed from their mothers for two hours, and were placed on a warming surface at 37 °C. At the end of two hours, the body weight of each pup was measured, and the litter returned to the mother. After this, at the end of one hour, the body weight of each pup was measured (Purcell *et al.*, 2011).

Milk intake was estimated using the following formula: $Milk\ Intake = (BW_{pre} - BW_{pos})$. Relative milk intake was calculated using the following formula: $Normalised\ Milk\ Intake = (Milk\ Intake/BW_{pre})$.

At PND12, the mothers were restrained, and a blood sample of the order of microlitres was collected in a glass capillary. The milk was centrifuged to obtain creamatocrit, and the results expressed as % fat (Paul, Hallam and Reimer, 2015).

Neurodevelopmental Tests

Straightening reflex: From PND5 to PND10, straightening reflexes and cliff aversion were assessed. (Sousa *et al.*, 2020; Amaro *et al.*, 2023). The animal was placed in the supine position, with the researcher maintaining the position with a finger on its chest. The animal was given a time of up to 5 seconds to move from the supine to the prone position. The time taken was recorded. For times longer than 5 seconds, a retry was provided. Times less than 1 second were counted as 1 second.

Cliff aversion: The animal was placed on a 10x10 cm rectangular platform, elevated 10 cm from its base, with their snout and forepaw digits hanging over the edge. The time for the pup to turn away from the cliff and move its paws and snout away from the edge was counted up to 30 s.

Nest seeking behaviour: At PND8 and PND10, the maturation of the olfactory system was assessed through nest seeking behaviour. (Amaro *et al.*, 2023). The animals were placed in the centre of a rectangular box (45 x 25 cm), with bed from the nest on one side and clean bed on the other. With a maximum time of 120 seconds per trial, and in two trials, the time required for the animal to enter

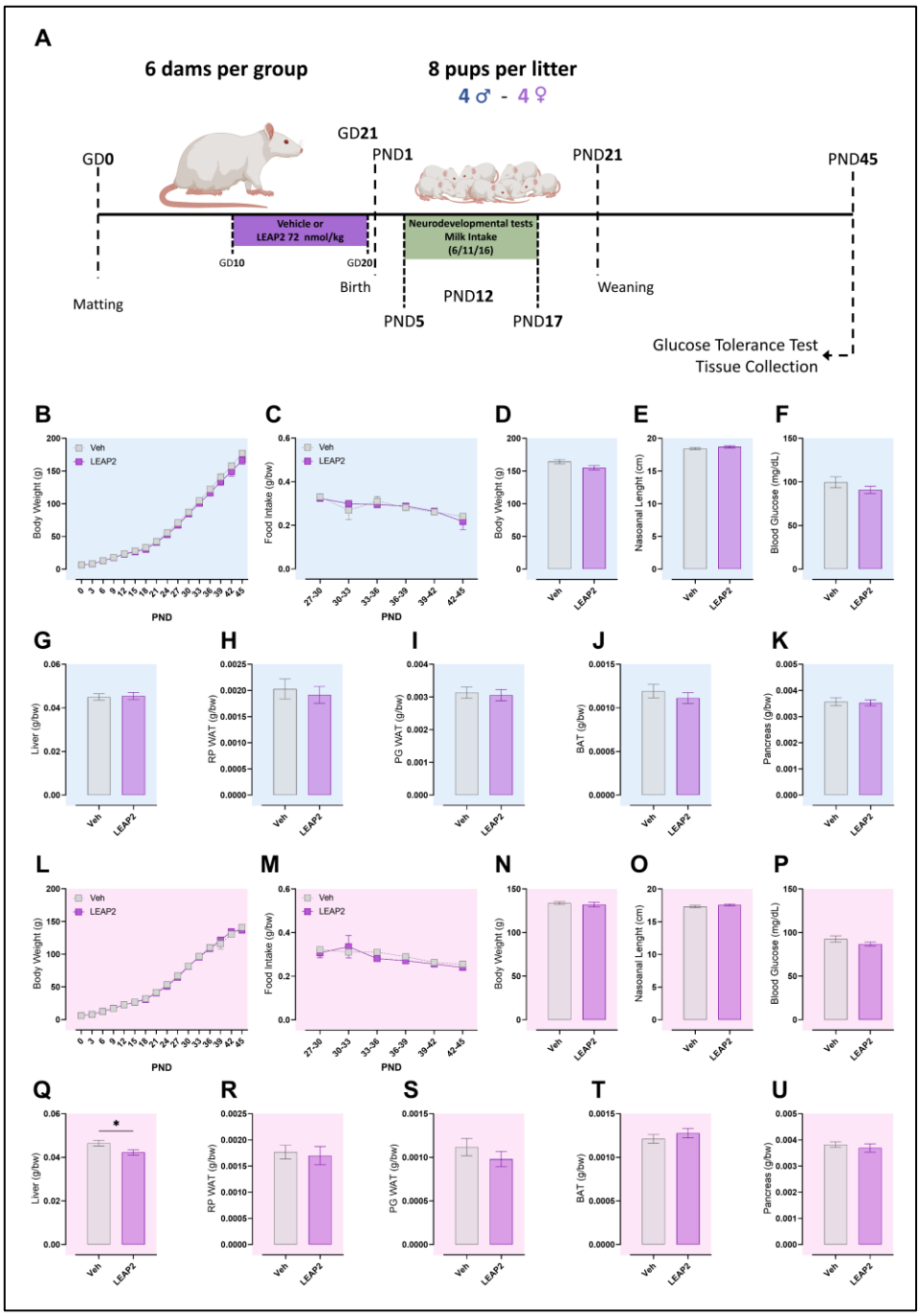
the section containing the wood from the nest was computed. For animals that choose the clean wood, a time of 120 seconds was computed, as well as for those that exceeded the time limit.

Negative geotaxis: From PND5 to PND12 the negative geotaxis reflex was assessed (Sousa *et al.*, 2020). For this, the animals were positioned on a ramp with a 35° inclination, with the head directed to the low part. The reflex was positive when the animal moved to position its head to the high part. 15 seconds were given for the animal to complete the inversion, and the time was recorded. For times longer than 15 seconds, or falling, a new attempt was granted. In the event of a further fall, or immobility, the maximum time was recorded.

Locomotion test: On alternate days, from PND5 to PND13, locomotor activity was assessed (Fox, 1965; Baharnoori, Bhardwaj and Srivastava, 2012). For this purpose, animals were placed in the centre of a 50 x 50 cm board, composed of 25 10x10 cm squares, and monitored for 120 seconds. The number of crossings of vertical and horizontal lines was computed.

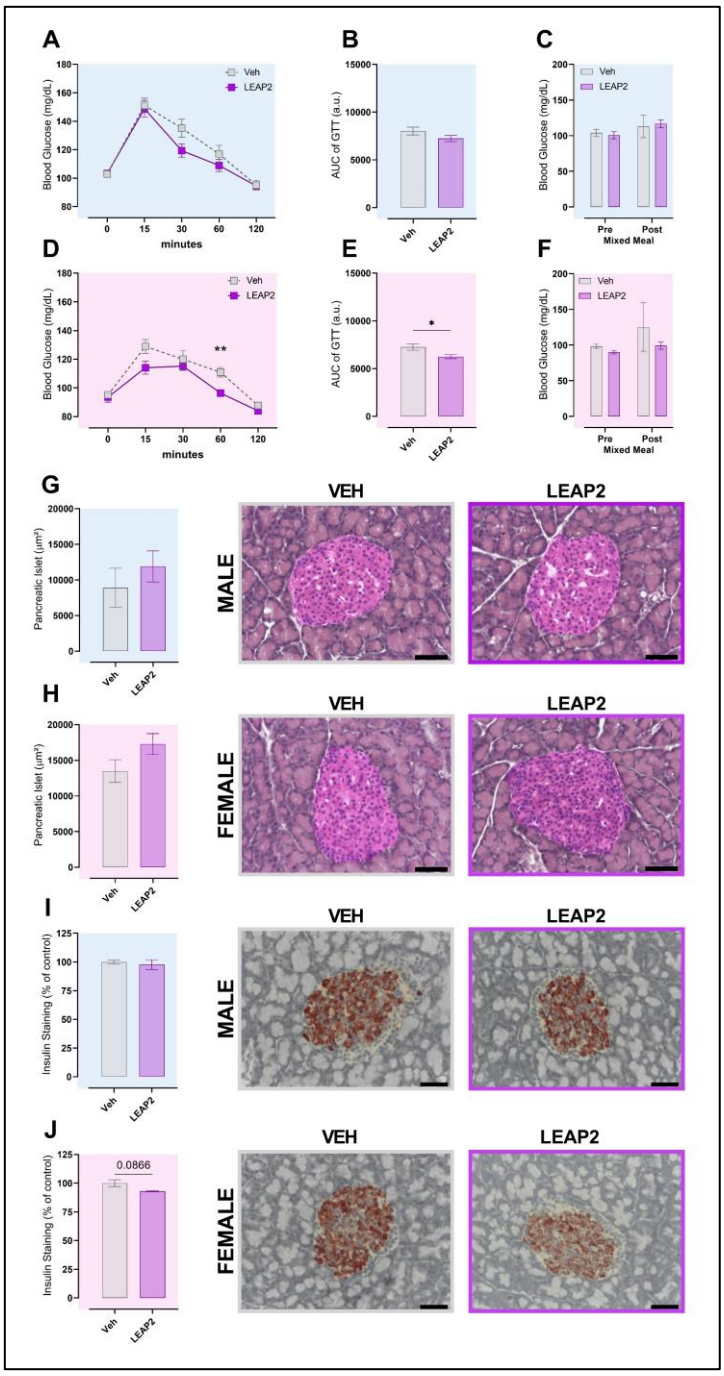
Suspension test: From PND10 to PND14, the muscle strength of the animals was assessed using the suspension test (Fox, 1965; Baharnoori, Bhardwaj and Srivastava, 2012; Amaro *et al.*, 2023). The animals had their forepaws placed in contact with a copper wire covered with non-toxic PVC (2 mm in diameter) and the experimenter recorded the maximum grabbing time.

Auditory reflex: From PND10, animals were checked daily to record the day on which the auditory reflex appeared (Fox, 1965; Baharnoori, Bhardwaj and Srivastava, 2012). The auditory reflex was positive when the animal responded with a startle to a finger snapping near its ear. The day of appearance of each event was recorded separately for each animal.



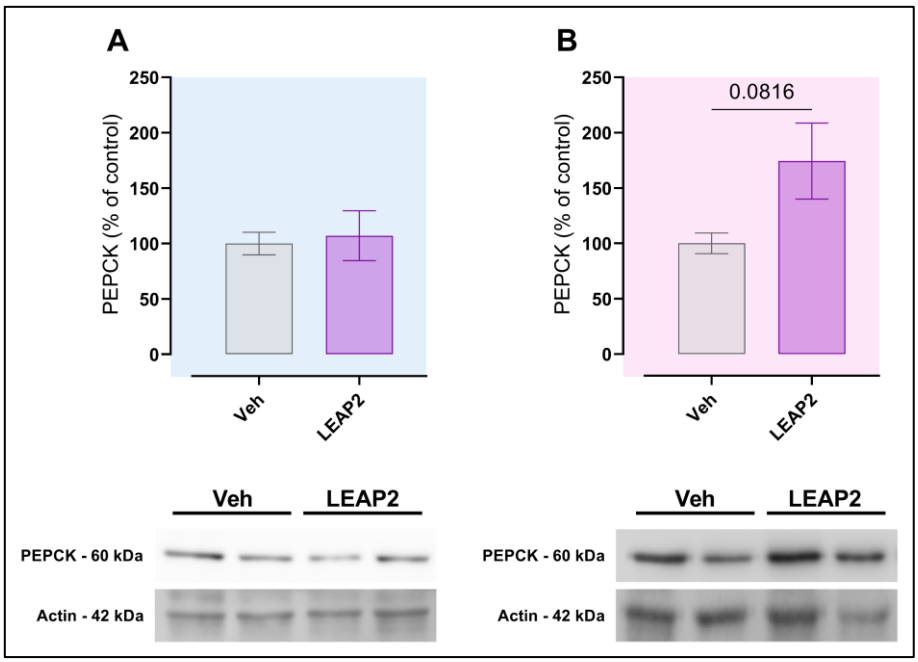
Artigo 2 - Figure 1. Effects of GHSR inhibition by the administration of LEAP2 during the pregnancy on body weight, food intake of their young offspring.

Experimental design (A). The phenotype of LEAP2 offspring was not changed by the treatment, as evidenced by the similarity between body weight gain (B, L) and food intake (C, M) throughout experimental period, and the body weight (D, N), nasoanal length (E, O), blood glucose (F, P), liver (G, Q), retroperitoneal (H, R) and perigonadal (I, S) white adipose tissues, interscapular brown adipose tissue (J, T) and pancreas (K, U) weight at the end of the experimental period in males and females, respectively. Two-way ANOVA followed by Sidak's post-hoc test was used for the analysis of time-dependent parameters. Student's t-test was used for the analysis of time-independent parameters. *p<0.05 vs Veh.



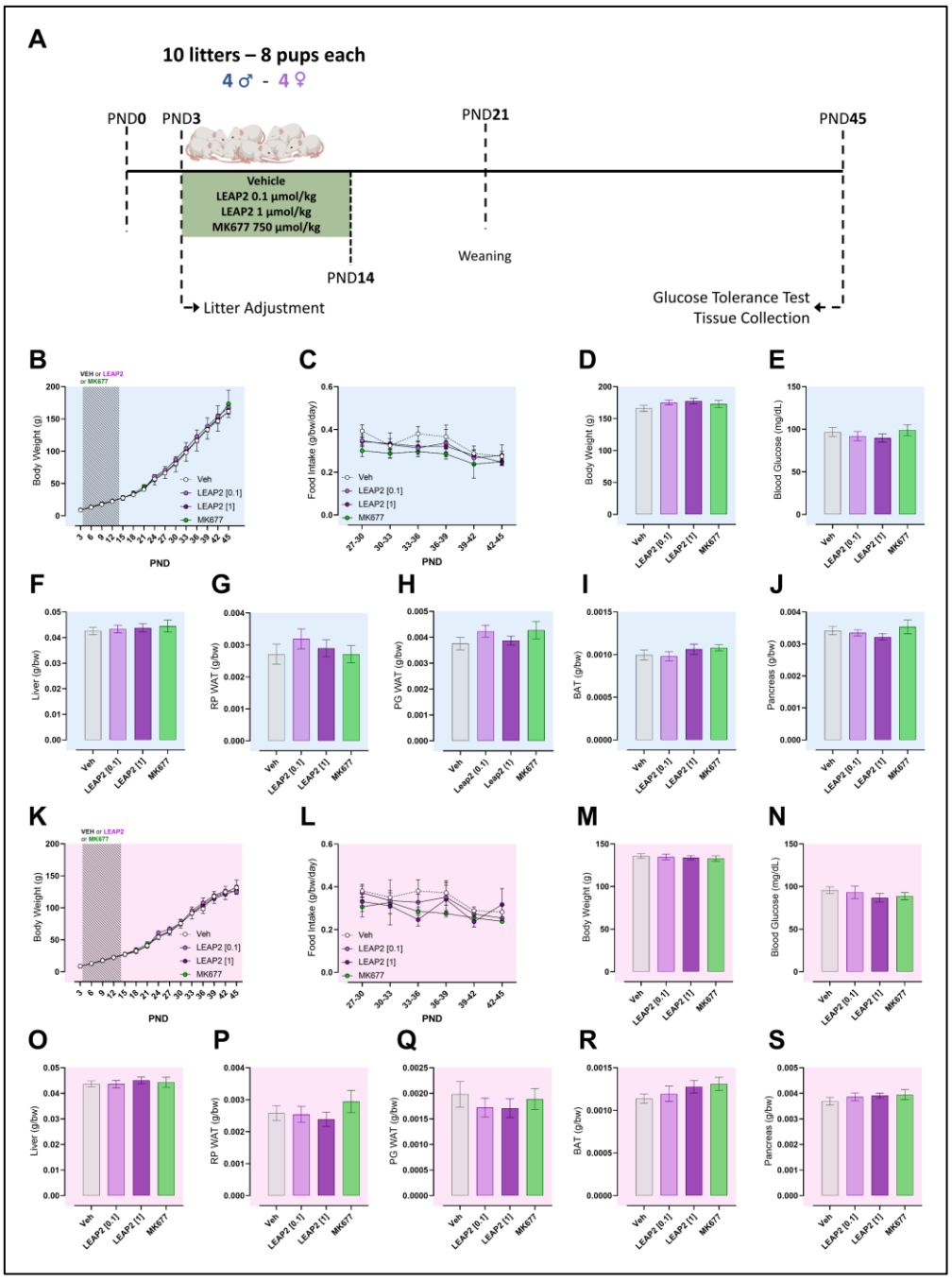
Artigo 2 - Figure 2. Effects of GHSR inhibition by the administration of LEAP2 during the pregnancy on glucose dynamics and pancreatic morphology.

During the oral glucose tolerance test, despite male LEAP2 offspring present no changes in the response (A) or AUC (B), female offspring presented increased glucose tolerance during the test (D) evidenced by the lower AUC (E). The response of a mixed meal injection was the same in both groups (C, F). No differences were observed in the pancreatic islets area (G, H) or insulin staining in the pancreatic islets (I, J). Two-way ANOVA followed by Sidak's post-hoc test was used for the analysis of time-dependent parameters. Student's t-test was used for the analysis of time-independent parameters. *p<0.05, **p<0.01 vs Veh.



Artigo 2 - Figure 3. Effects of GHSR inhibition by the administration of LEAP2 during the pregnancy on hepatic PEPCK expression.

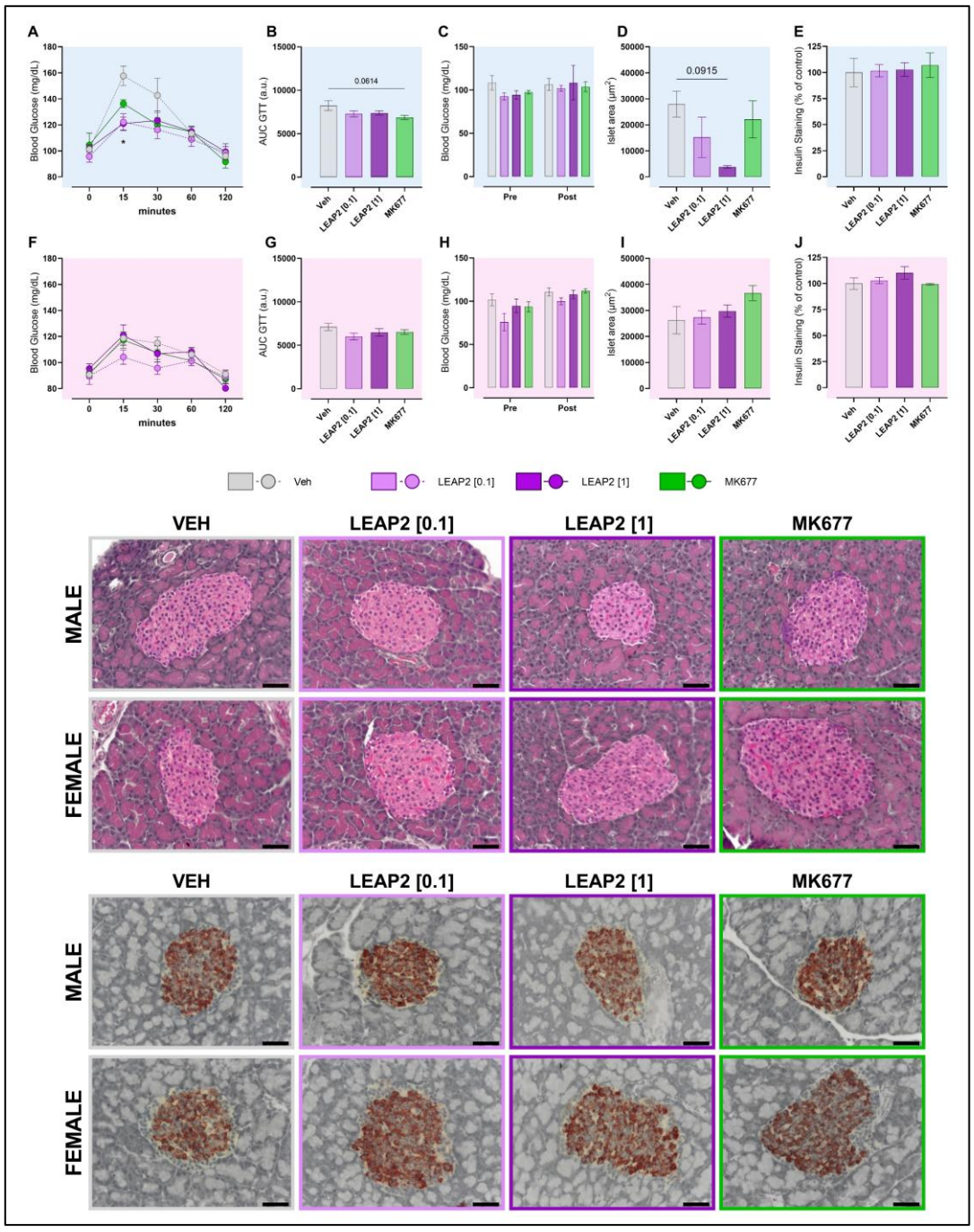
Phosphoenolpyruvate carboxykinase (PEPCK) expression in male (A) and female (B) livers from Veh or LEAP2 offsprings. Student's t-test was used. Representative immunoblots of PEPCK and Actin.



649

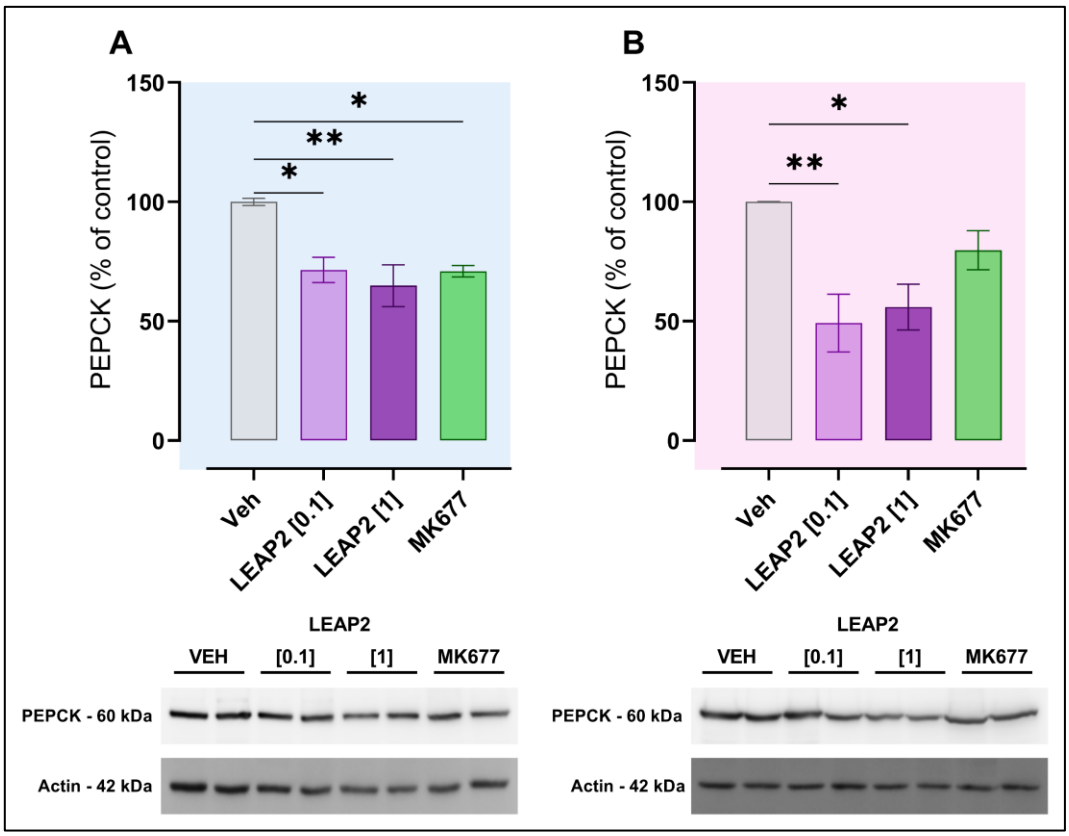
650 Artigo 2 - Figure 4. Effects of GHSR signalling modulation during lactation on body weight and food intake in young
651 animals.

652 Experimental design (A). The phenotype of the offsprings was not changed by LEAP2 or MK677 injections during
653 the lactation, as evidenced by the similarity between body weight gain (B, K) and food intake (C, L) throughout
654 experimental period, and the body weight (D, M), blood glucose (E, N), liver (F, O), retroperitoneal (G, P) and
655 perigonadal (H, Q) white adipose tissues, interscapular brown adipose tissue (I, R) and pancreas (J, S) weight at the
656 end of the experimental period in males and females, respectively. Two-way ANOVA followed by Sidak's post-hoc
657 test was used for the analysis of time-dependent parameters. One-way ANOVA followed by Tukey's post-hoc test was
658 used for the analysis of time-independent parameters.



Artigo 2 - Figure 5. Effects of GHSR signalling modulation during lactation on body weight and food intake in young animals.

During the oral glucose tolerance test, male LEAP2 offsprings ([0.1] and [1]) present increased glucose tolerance during the test, evidenced by the lower peak response to glucose overload (A), despite no changes in glucose tolerance in female offsprings (F). However, there are no changes in the AUC of the test for both sexes (B, G). The response of a mixed meal injection was the same in both groups (C, H). No differences were observed in the pancreatic islets area (D, I) or insulin staining in the pancreatic islets (E, J). Two-way ANOVA followed by Sidak's post-hoc test was used for the analysis of time-dependent parameters. One-way ANOVA followed by Tukey's post-hoc test was used for the analysis of time-independent parameters. *$p<0.05$ LEAP2 [0.1] and [1] vs Veh.



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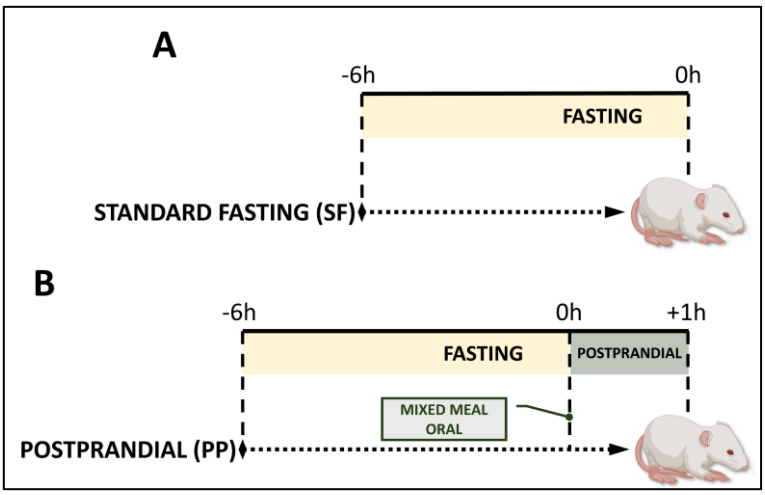
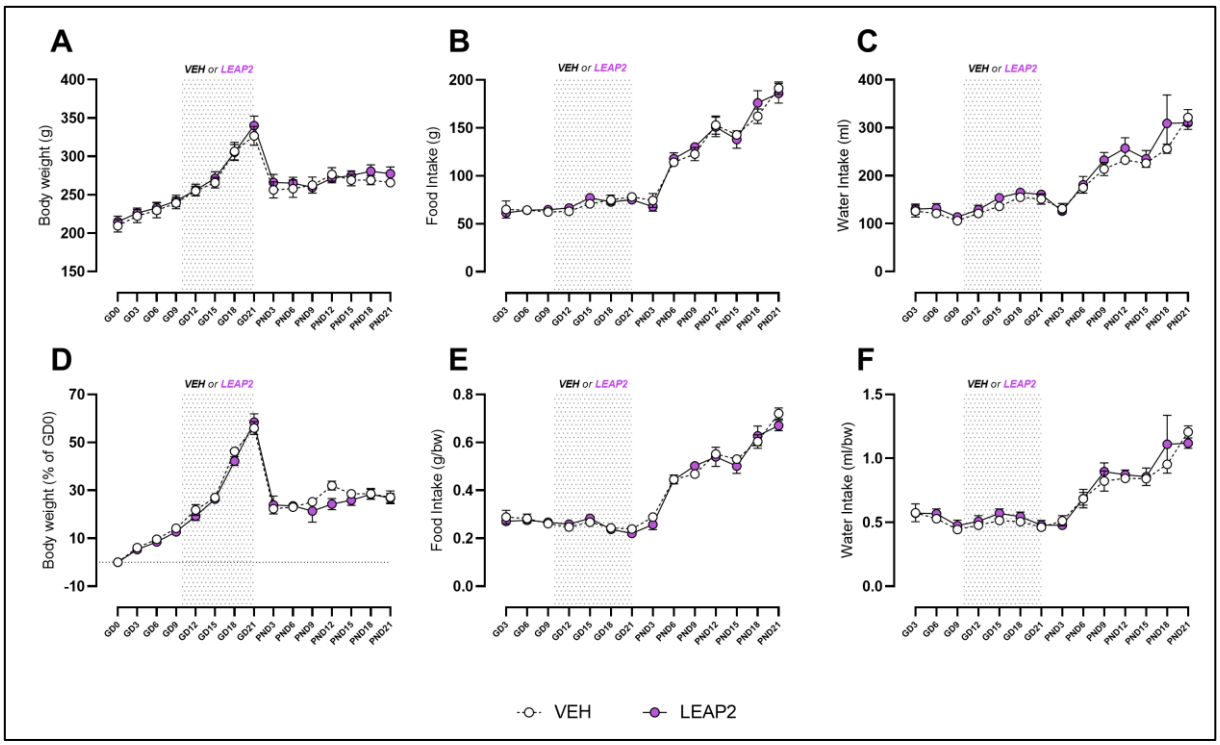
670 Artigo 2 - Figure 6. Effects of GHSR signalling modulation during lactation on glucose dynamics in young animals.

671 Phosphoenolpyruvate carboxykinase (PEPCK) expression in male (A) and female (B) livers from Veh, LEAP2 and
672 MK677 injected animals. One-way ANOVA followed by Tukey's post-hoc test was used. *$p<0.05$, **$p<0.01$.
673 Representative immunoblots of PEPCK and Actin.



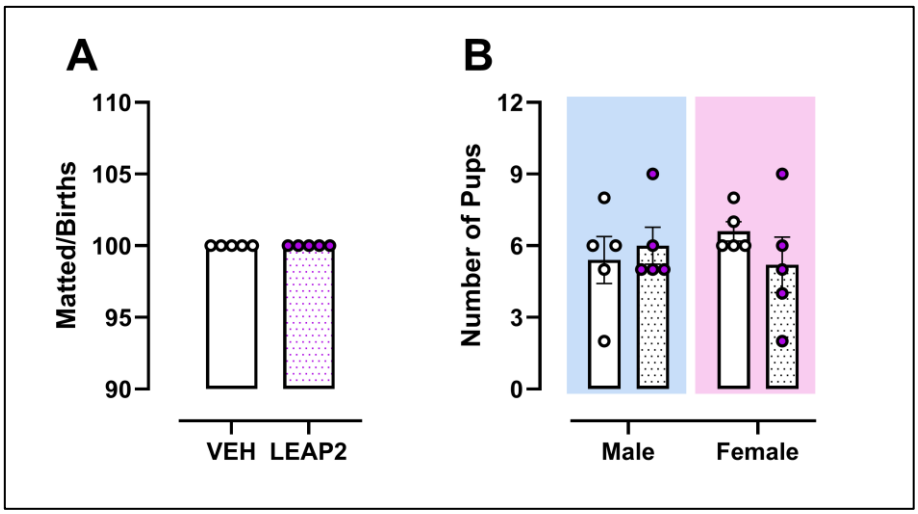
Artigo 2 - Supplementary Figure 1. Experimental design of postprandial blood glucose assessment.

Experimental design of standard fasting (A) and postprandial (B) blood collection.



Artigo 2 - Supplementary Figure 2. Effects of LEAP2 injections during pregnancy.

Effects of LEAP2 injections on raw and corrected body weight (A, D), food (B, E) and water intake (C, F).



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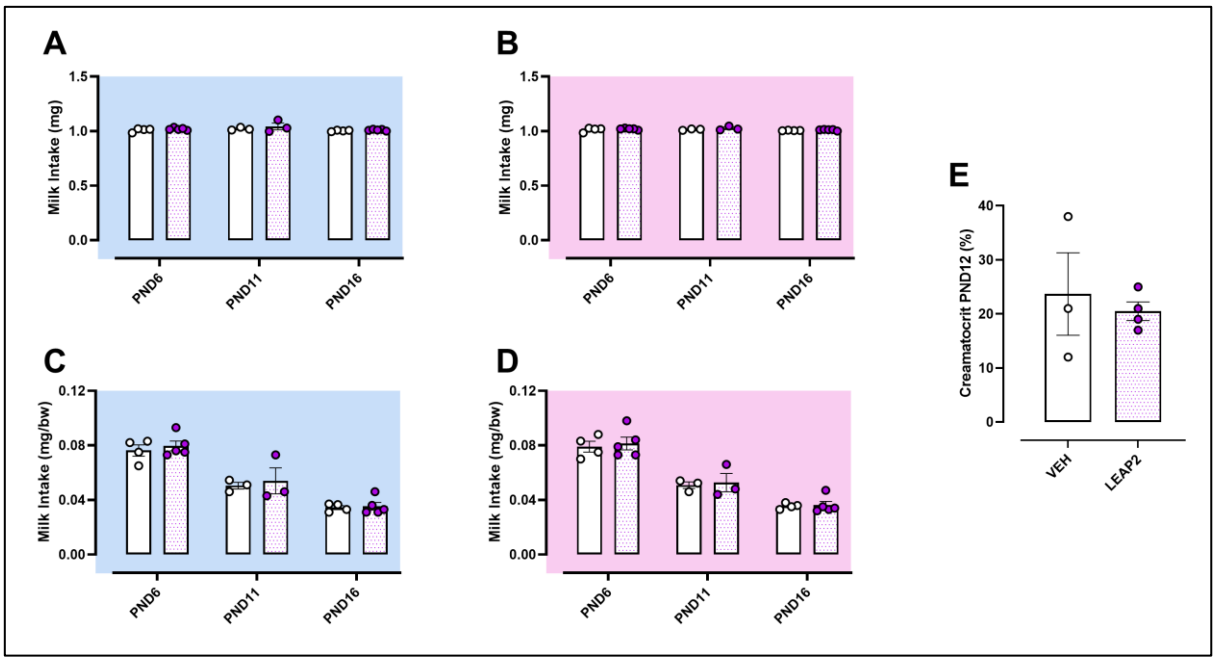
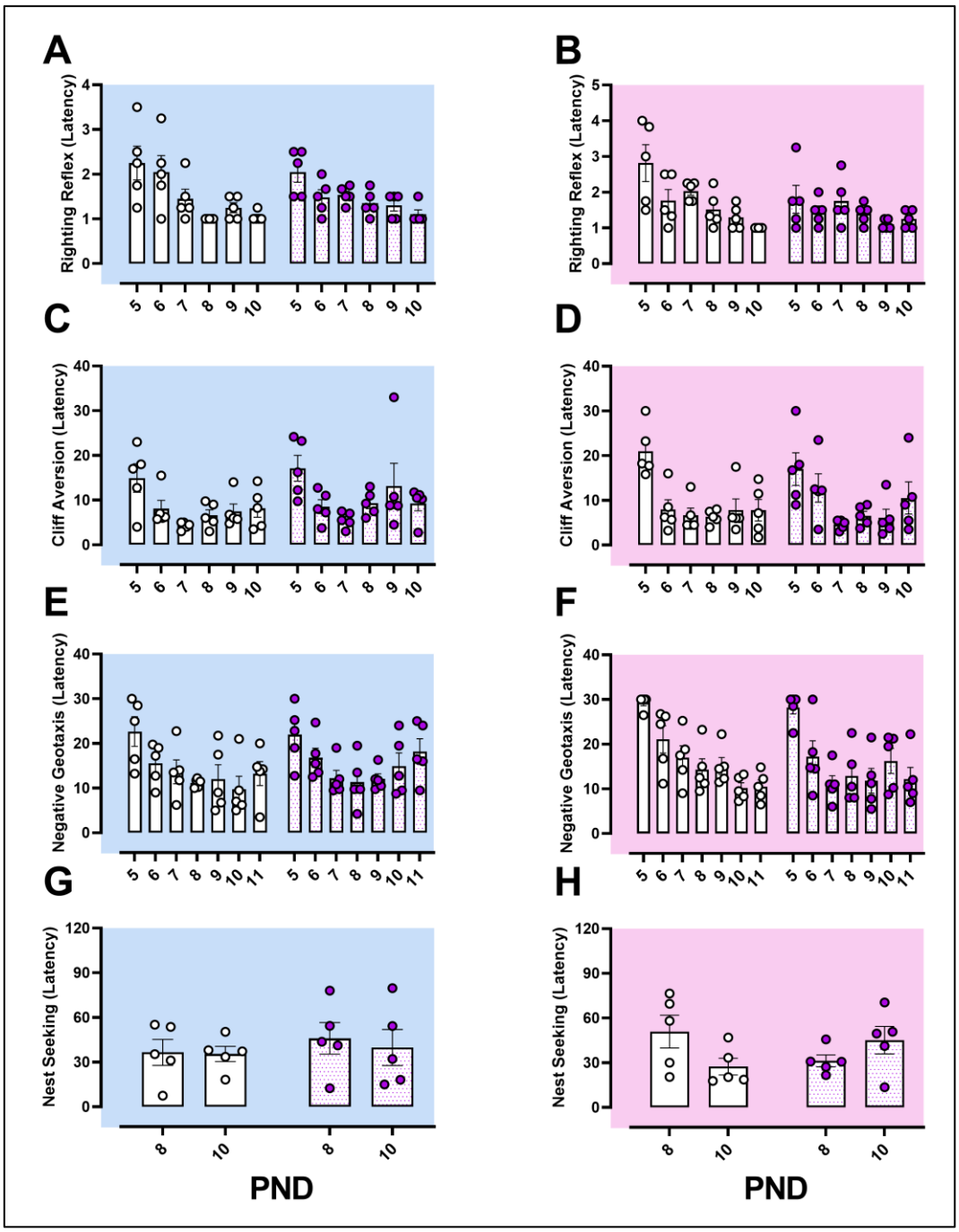
681 Artigo 2 - Supplementary Figure 3. Effects of LEAP2 injections in pregnant females on pregnancy outcome.

682 Ratio of matted rats to successful pregnancies (A) and sex of the pups (B).



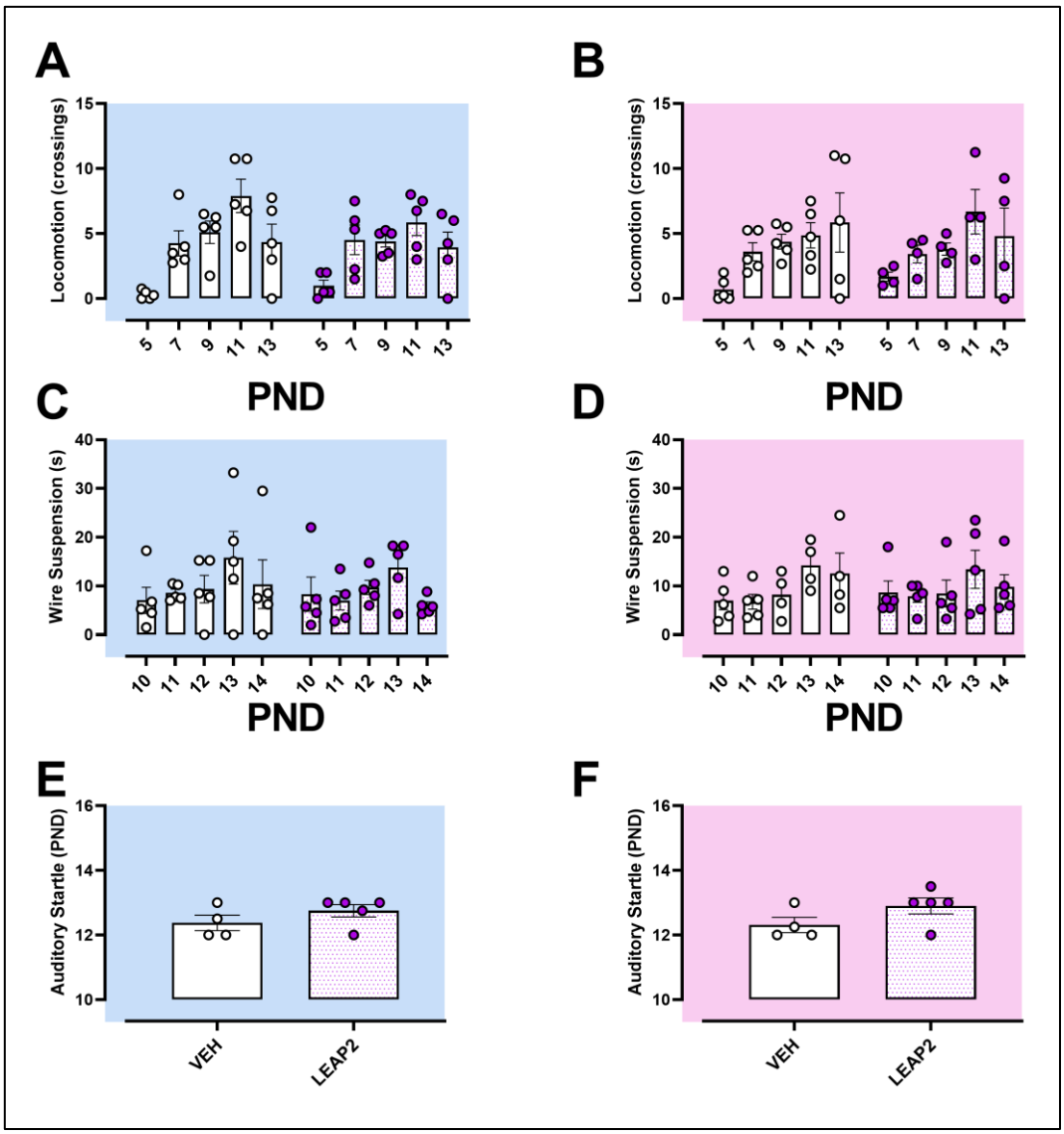
Artigo 2 - Supplementary Figure 4. Effects of maternal LEAP2 injections on the food intake of their offspring and milk composition.

Raw and normalized milk intake of male (A, C) and female offspring (B, D). Creamatocrit evaluation of PND12 milk from Veh- and LEAP2-injected mothers (E).



Artigo 2 - Supplementary Figure 5. Effects of maternal LEAP2 injections in the neurodevelopment of their offspring.

Assessment of neurodevelopment through the presence of righting (A-B), cliff aversion (C-D) and negative geotaxis (E-F) reflexes and the nest seeking behaviour (G-H).



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693 Artigo 2 - Supplementary Figure 6. Effects of maternal LEAP2 injections in the locomotor activity and auditory
694 maturation of their offspring.

695 Assessment of locomotion (A-B) and forepaws strength test (C-D). Maturation of auditory system (E-F).

6. CONCLUSÕES

Os dados do artigo 1 demonstram que a exposição precoce às glicotoxinas levou a deficiências cardíacas e vasculares. Ainda, os dados do Artigo 2 mostram que a exposição ao LEAP2 em estágios perinatais pode afetar a homeostase da glicose. Juntos, estes resultados chamam a atenção para o papel da puberdade como um período em que intervenções podem ser realizadas na tentativa de diminuir ou mitigar os efeitos, ainda que estes se manifestem de forma suave, que poderiam se agravar na fase adulta.

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ANEXOS

CERTIFICADO DE APROVAÇÃO CEUA/UFG – ARTIGO 1

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE GOIÁS
PRÓ-REITORIA DE PESQUISA E INOVAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS/CEUA

UFG

C E RT I F I C A D O

Certificamos que a proposta intitulada **"Efeitos a longo prazo da administração de metilglioxal em ratas lactantes e supernutrição durante a fase de lactação em sua prole"**, registrada com o protocolo **nº 007/21**, sob a responsabilidade de **Rodrigo Mello Gomes** e **Marcos Divino Ferreira Junior** que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Universidade Federal de Goiás (UFG), em reunião de **26/04/2021**

- Finalidade: ( ) Ensino (X) Pesquisa Científica
- Vigência da autorização (início e fim): 26/04/2021
- Espécie/linhagem/raça: Ratos, Wistar
- Nº de animais autorizados: 400 Ratos sendo pelo menos 30 fêmeas
- Peso/Idade: recém-nascidos e adultos
- Sexo: Machos e Fêmeas
- Origem (fornecedor): Biotério Central da UFG

Dra. Liliana Borges de Menezes Leite
Coordenadora da CEUA/PRPI/UFG

CERTIFICADO DE APROVAÇÃO CEUA/UFG – ARTIGO 2

CEUA
COMISSÃO DE ÉTICA EM USO DE ANIMAIS

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE GOIÁS
PRÓ-REITORIA DE PESQUISA E INOVAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-CEUA

UFG
UNIVERSIDADE FEDERAL DE GOIÁS

C E RT I F I C A D O

Certificamos que a proposta intitulada **"Contribuição da sinalização de GHSR em parâmetros metabólicos e do neurodesenvolvimento na prole de ratas gestantes alimentadas em regime ad libitum ou em restrição calórica",** registrada com o protocolo **nº 072/23**, sob a responsabilidade de **Marcos Divino Ferreira Júnior** e **Rodrigo Mello Gomes** que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto humanos), para fins de pesquisa científica (ou ensino) encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal de Goiás (UFG), em reunião de 21/08/2023.

- Finalidade: ( ) Ensino (x) Pesquisa Científica
- Vigência da autorização (início e fim): 21/08/2023 a 31/12/2027
- Espécie/linhagem/raça: *Rattus norvegicus*, linhagem Wistar
- Nº de animais autorizados: 247
- Peso/Idade: 39 com 70-90 dias e peso entre 250-400 gramas; 208 com 45 dias e peso entre 140-160 gramas
- Sexo: 117 machos; 130 fêmeas
- Instalação onde serão mantidos os animais: Biotério setorial DCiF/DFAR
- Origem (fornecedor): Centro de Produção e Ciência em Biomodelos (CPCBio)

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ELIZABETH PEREIRA MENDES
Data: 29/08/2023 13:51:43-0300
Verifique em https://validar.iti.gov.br

Dra. Elizabeth Pereira Mendes
Coordenadora da CEUA/PRPI/UFG