UNIVERSIDADE FEDERAL DO PARANÁ

BRUNA CHRIST FARIA

EFEITOS HEPÁTICOS E ÓSSEOS DA DULAGLUTIDA E DA SEMAGLUTIDA EM UM MODELO DE HEPATOTOXICIDADE AGUDA EM CAMUNDONGOS

CURITIBA

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Dissertação apresentada como requisito parcial à obtenção do título de Mestre, curso de Pós-graduação em Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná.

Orientadora: Profa. Dra. Alexandra Acco.

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **BRUNA CHRIST FARIA** intitulada: **Efeitos hepáticos e ósseos da dulaglutida e da semaglutida em um modelo de hepatotoxicidade aguda em camundongos**, sob orientação da Profa. Dra. ALEXANDRA ACCO, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestra está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 28 de Janeiro de 2022.

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NOTA EXPLICATIVA

Essa dissertação possui seus principais resultados, materiais e métodos e discussão em forma de artigo científico, de acordo com as normas do programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná. Ela é constituída, portanto, de revisão de literatura, artigo científico e considerações finais.

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RESUMO

As doenças hepáticas são uma das doenças crônicas mais comuns em todo o mundo, e a osteodistrofia uma complicação importante dos pacientes com doença hepática crônica, sendo ambas as condições de difícil tratamento. Diante desse desafio, a busca de substâncias que possam atuar como hepatoprotetoras e, também, como reparadoras do tecido ósseo, é importante. Este estudo investigou os efeitos hepáticos e ósseos dos análogos antidiabéticos do GLP-1, semaglutida e dulaglutida, no modelo agudo de hepatotoxicidade induzida por tetracloreto de carbono (CCl_4 a 1,5%). Dois protocolos foram utilizados em camundongos Swiss machos: Tratamento e Pré-tratamento com uma ou duas administrações de semaglutida (0,021 mg/Kg, i.p.) e dulaglutida (0,014 mg/Kg, i.p.), silimarina (100 mg/Kg, v.o., controle positivo) ou veículo (10 ml/kg, v.o.). No Tratamento os animais receberam primeiramente o agente hepatotóxico CCl_4 e 2 h após receberam os tratamentos, enquanto no Pré-tratamento as administrações dos fármacos foram feitas alguns dias antes da administração do CCl_4. Em ambos os protocolos os animais foram eutanasiados 48 h após o desafio com CCl_4. Os resultados indicaram que a dulaglutida tem efeitos hepatoprotetores mais evidentes que a semaglutida, reduzindo a necrose hepática, a atividade da GST e o nível plasmático de ALT, e aumentando a expressão gênica de *Ciclina D1*. A semaglutida também aumentou a expressão deste gene e melhorou a excreção biliar de colesterol, aparentemente beneficiando o sistema biliar mais do que o parênquima hepático. Ambos os análogos do GLP-1 melhoraram a microarquitetura da tíbia, aumentando o volume trabecular e reduzindo a espaçamento entre trabéculas; enquanto a dulaglutida aumentou o número de trabéculas. Estes dados evidenciaram que a dulaglutida tem potencial para ser um tratamento alternativo à hepatotoxicidade, induzida principalmente por drogas (DILI), e que a dulaglutida e semaglutida melhoraram significativamente o desenvolvimento ósseo após apenas duas administrações, demonstrando o potencial destes fármacos no tratamento de fragilidade óssea em doenças hepáticas, diabetes ou outras condições.

Palavras-chave: Hepatotoxicidade. Análogos do GLP-1. Histomorfometria óssea. Fígado. Metabolismo.

ABSTRACT

Hepatic diseases are one of the most common chronic diseases in the world, and the osteodystrophy an important complication of the patients with chronic hepatic diseases. Both conditions have challenges in their treatments. Facing this situation, the search for substances that can act as hepatoprotector and also as bone tissue repairer is important. This study investigates the hepatic and bone effects of the GLP-1 analogues, semaglutide and dulaglutide, in a model of acute hepatotoxicity induced by carbon tetrachloride (CCl_4, 1.5%). Two protocols were used in male Swiss mice: Treatment and Pretreatment, with one or two administrations of semaglutide (0.021 mg/Kg, i.p.) and dulaglutide (0.014 mg/Kg, i.p.), silymarin (100 mg/Kg, p.o., positive control) or vehicle (10 ml/kg, v.o.). In Treatment the mice received first the toxic agent CCl_4 and, 2 hours later, the treatments, while in Pretreatment the drug administrations were performed few days before the CCl_4 administration. In both protocols, the mice were euthanized 48 h after the toxic agent administration. The results indicated that dulaglutide have more evident hepatoprotective effects than semaglutide, reducing hepatic necrosis, GST activity and plasmatic levels of ALT, and increasing the *Cyclin D1* gene expression. Semaglutide also increased this gene expression and improved biliary excretion of cholesterol, apparently benefiting the biliary system more than the liver parenchyma. Both GLP-1 analogues improved the tibia microarchitecture, increasing trabeculae volume, and reducing its interspace; while dulaglutide increased the trabeculae number. These data show that dulaglutide can be an alternative treatment to hepatotoxicity, mainly induced by drugs (DILI). Both dulaglutide and semaglutide improved significantly the bone development after only two administrations, showing the potential of these drugs for the treatment of bone fragility in hepatic diseases, diabetes or other conditions.

Keywords: Hepatotoxicity. GLP-1 analogues. Bone histomorphometry. Liver. Metabolism.

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LISTA DE SIGLAS

AC – Adenilato ciclase (*adenylate cyclase*)
AKT – Serina-treonina quinase (*Serine-threonine kinase*)
ALT – Alanina aminotransferase (*alanine aminotransferase*)
ARE – Elementos de resposta antioxidante (*Antioxidant response elements*)
AST – Aspartato aminotransferase (*aspartate aminotransferase*)
ATP – Adenosina trisfosfato (*adenosine triphosphate*)
BCRP – Proteína de resistência ao câncer de mama (*breast cancer resistance protein*)
cAMP - adenosina 3',5'-monofosfato cíclico (*ciclic adenosine monophosphate*)
CAT – Catalase
CCl_4 - Tetracloreto de carbono (*carbon tetrachloride*)
cDNA – DNA complementar (*complementary DNA*)
CDNB – 1-cloro-2,4 dinitrobenzeno (*1-chloro-2,4-dinitrobenzene*)
CYP – Citocromo P-450 (*cytochrome P-450*)
DILI- Lesões hepáticas induzidas por drogas (*drug-induced liver injury*)
DNA – Ácido desoxirribonucleico (*deoxyribonucleic acid*)
DPPH - 2,2-difenil-1-picrilhidrazil (*2,2- diphenyl-1-picryhydrazyl*)
DTNB – 5,5-ditiobis(2- ácido nitrobenzóico) (*5,5'-Dithiobis(2-nitrobenzoic acid*)
FDA – Agência regulatória de alimentos e drogas dos Estados Unidos da América (*Food and drug administration*).
GLP-1 – Peptídeo 1 semelhante ao glucagon (*glucagon-like peptide 1*)
GLP-1R – Receptor de análogos do GLP-1 *(GLP-1 receptor*)
GPx – Glutationa peroxidase (Gluthatione peroxidase)
GSH - Glutationa reduzida (*reduced glutathione*)
GST – Glutationa-S-transferase (*gluthatione-S-transferase*)
IL-1b – Interleucina 1b (*interleukin-1b*)
IL-6 – Interleucina 6 (*interleukin-6*)
LDH - Lactato desidrogenase (*lactate dehydrogenase*)
LPO – Lipoperoxidação (*lipid peroxidation*)
MAPK – Proteína quinase mitógeno-ativada (*mitogen-actived protein kinase*)
MPO – Mieloperoxidase (Myeloperoxidase)
mRNA – RNA mensageiro (*messenger RNA*)
MRP – Proteína de resistência multidrogas (*multidrug resistance protein*)

NAC- N-acetilcisteína (*N-acetylcisteyne*)

NAD^+- dinucleotídeo de adenina e nicotinamida (*nicotineamide adenine dinucleotide)*

NADPH - fosfato de dinucleotídeo de adenina e nicotinamida (*nicotineamide adenine dinucleotide phosphate*)

NAFLD – Lesões gordurosas hepáticas não-alcoólicas (*non-alcooholic fatty liver disease*)

NAG – N-acetilglucosaminidase (N-acetylglucosaminidase)

NASH – Esteatohepatite não-alcoólica (*non-alcooholic steatohepatitis*)

NERF2 – Fator nuclear eritróide 2 (*nuclear erytrhoid 2-related factor*)

OATPs – Polipetídeos orgânicos transportadores de ânions (*organic anion transporting polypeptide*)

OATs – Transportadores orgânicos de ânions (*organic anion transporter*)

OCTs – Transportadores orgânicos de cátions (*organic cation transporter*)

PCR – Reação em cadeia polimerase (*Polymerase chain-reaction*)

PI3K – Fosfoinositídio 3-quinase (*Phosphoinositide 3-kinase*)

PKA – Proteína quinase A (*protein kinase A*)

RANK-L – Receptor ativador do fator nuclear Kappa B (*Receptor activator of nuclear factor kappa-B ligand*)

RNA – Acido ribonucleico (*ribonucleic acid*)

RNS – Espécies reativas de nitrogênio (*reactive nitrogen species*)

ROS – espécies reativas de oxigênio (*reactive oxygen species*)

RT-qPCR – Reação em cadeia polimerase quantitativa em tempo real (*Real time quantitative Polymerase chain-reaction*)

SINITOX – Sistema nacional de informações tóxico-farmacológicas (*National system of toxic-pharmacological informations*)

SOD – Superóxido dismutase (*superoxyde dismutase*)

TNB – 2-nitro-5-ácido mercaptobenzóico (*2-nitro-5-mercapto-benzoic acid*)

TNFα – Fator de necrose tumoral α (*Tumoral necrosis factor α*)

LISTA DE ABREVIATURAS

µg – micrograma (*microgram*)
µL – microlitro (*microliter*)
µm – micrometro (*micrometer*)
BV/TV – Volume ósseo (*bone volume*)
I.p.: Intraperitonial
Kg: Quilograma *(kilogram*)
MA.V.TV. – Volume da medula óssea (*bone marrow volume*)
mg: Miligrama (*miligram*)
mL: Mililitro (*milliter*)
mM – milimolar (*millimolar*)
nm – nanômetro (*nanometer*)
Tb.N – Número de trabéculas (*number of trabeculae*)
Tb.Sp – Separação trabecular (*separation between the trabeculae*)
Tb.Th – Espessura trabecular (*trabecular thickness*)
v.o. e p.o.: Via oral (*per os*)

SUMÁRIO

1 INTRODUÇÃO

Os medicamentos são essenciais para a manutenção da saúde. Sua administração permite prevenir, tratar ou curar doenças, desde que prescritos e recomendados para a necessidade de cada paciente (WHO, 2012). Porém a automedicação, o uso abusivo de drogas e álcool, concomitante ou não, juntamente a fatores ambientais, são responsáveis por um dos maiores problemas de saúde mundial, as lesões hepáticas por toxicidade.

No Brasil, no ano de 2017, foram reportados 20.697 casos de intoxicação hepática induzida por medicamentos, em diferentes circunstâncias (SINITOX, 2017). Destacam-se nos dados avaliados pela plataforma os acidentes individuais, com 5.051 casos; uso terapêutico, com 953 casos; e tentativa de suicídio, com 9.983 casos. Ainda, há dados de prescrição inadequada, com 19 casos; e acidente coletivo, com 17 casos, conforme tabela 1. A região Sul foi responsável por mais 55% dos casos reportados.

Tabela 1: Casos de intoxicação por medicamentos por Unidade federada, segundo circunstancias registradas, 2017.

Região/Centro \ Circunstância	Acidente Individual	Acidente Coletivo	Acidente Ambiental	Ocupacional	Uso Terapêutico	Presc.Méd. Inadequada	Erro de Administração	Auto Medicação	Abstinência	Abuso	Ingestão de Alimentos	Tentativa Suicídio	Tentativa Aborto	Violência/ Homicídio	Uso Indevido	Ignorada	Outra	Total nº	Total %
	nº	nº	nº	nº	nº	nº	nº	nº	nº	nº	nº	nº	nº	nº	nº	nº	nº		
NORTE	**176**	**0**	**0**	**0**	**65**	**2**	**85**	**27**	**0**	**1**	**0**	**35**	**0**	**0**	**0**	**1**	**2**	**394**	**1,91**
CIT/AM - Manaus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CIT/PA - Belém	176	0	0	0	65	2	85	27	0	1	0	35	0	0	0	1	2	**394**	**1,91**
NORDESTE	**186**	**0**	**0**	**0**	**185**	**0**	**14**	**23**	**1**	**4**	**0**	**236**	**4**	**0**	**3**	**13**	**1**	**670**	**3,25**
CIAT/CE - Fortaleza	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CEATOX/CE - Fortaleza	103	0	0	0	3	0	2	1	0	3	0	89	1	0	0	1	1	**204**	**0,99**
CIT/RN - Natal	9	0	0	0	0	0	0	0	0	0	0	16	0	0	0	1	0	**26**	**0,13**
CEATOX/PB - João Pessoa	22	0	0	0	1	0	0	0	0	0	0	3	0	0	0	0	0	**26**	**0,13**
CEATOX/PB - Campina Grande	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CEATOX/PI - Teresina	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CAT/PE - Recife	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CIAVE/BA - Salvador	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CIT/SE - Aracaju	52	0	0	0	181	0	12	22	1	1	0	128	3	0	3	11	0	**414**	**2,01**
SUDESTE	**1506**	**8**	**0**	**2**	**474**	**5**	**513**	**86**	**0**	**5**	**0**	**2641**	**5**	**11**	**4**	**1936**	**462**	**7658**	**37,11**
ST/MG - Belo Horizonte	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CCI/ES - Vitória	1164	0	0	0	209	3	333	2	0	4	0	2095	3	10	3	75	453	**4354**	**21,10**
CCI/RJ - Niterói	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CCI/SP - São Paulo	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CEATOX/SP - São Paulo	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CCI/SP - Campinas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1853	0	**1853**	**8,98**
CCI/SP - Ribeirão Preto	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CEATOX/SP - Botucatu	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CCI/SP - São José dos Campos	16	0	0	0	0	0	2	4	0	0	0	33	0	0	1	7	1	**64**	**0,31**
CEATOX/SP - São José do Rio Preto	194	8	0	1	145	0	69	42	0	1	0	444	2	0	0	0	2	**908**	**4,40**
CCI/SP - Taubaté	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CEATOX/SP - Presidente Prudente	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CCI/SP - Santos	132	0	0	1	120	2	109	38	0	0	0	69	0	1	0	1	6	**479**	**2,32**
HVB/SP - Butantan	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
SUL	**3020**	**7**	**0**	**3**	**212**	**11**	**742**	**246**	**3**	**34**	**0**	**6923**	**7**	**7**	**97**	**111**	**67**	**11490**	**55,68**
CCE/PR - Curitiba	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CCI/PR - Londrina	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CCI/PR - Maringá	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CIT/SC - Florianópolis	771	0	0	1	107	0	281	117	0	18	0	2664	3	1	36	28	59	**4086**	**19,80**
CIT/RS - Porto Alegre	2249	7	0	2	105	11	461	129	3	16	0	4259	4	6	61	83	8	**7404**	**35,88**
CENTRO - OESTE	**163**	**2**	**0**	**3**	**17**	**1**	**38**	**15**	**0**	**1**	**0**	**148**	**0**	**0**	**2**	**35**	**0**	**425**	**2,06**
CIT/MS - Campo Grande	163	2	0	3	17	1	38	15	0	1	0	148	0	0	2	7	0	**397**	**1,92**
CIAVE/MT - Cuiabá	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	28	0	**28**	**0,14**
CIT/GO - Goiânia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
CIT/DF - Brasília	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	**0,00**
Total	**5051**	**17**	**0**	**8**	**953**	**19**	**1392**	**397**	**4**	**45**	**0**	**9983**	**16**	**18**	**106**	**2096**	**532**	**20637**	**100**
%	**24,48**	**0,08**	**0,00**	**0,04**	**4,62**	**0,09**	**6,75**	**1,92**	**0,02**	**0,22**	**0,00**	**48,37**	**0,08**	**0,09**	**0,51**	**10,16**	**2,58**	**100**	

Fonte: MS / FIOCRUZ / SINITOX

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O paracetamol (acetaminofeno), amplamente prescrito e de venda livre no Brasil, se destaca por sua elevada hepatotoxicidade, principalmente para pessoas que fazem uso de outros medicamentos ativadores da via citocromo P450 (CYP450) e/ou de etanol. Esse medicamento pode causar intoxicação aguda ou crônica, dependendo do uso e da situação do paciente (AGRAWAL, 2020). O paracetamol é apenas um dos medicamentos que podem causar toxicidade hepática. O LiverTox, disponível para consulta na internet pela plataforma Pubmed, lista vários, desde anti-inflamatórios esferoidais e não esteroidais, antimicrobianos, sedativos e hipnóticos, a quimioterápicos e imunossupressores (LIVERTOX, 2021).

Em muitos casos, o paciente faz uso de medicamentos sabidamente tóxicos, mas o risco: benefício compensa a terapia. O uso de quimioterápicos e antimetabólicos, essenciais para a cura e sobrevivência de pacientes com câncer, são exemplos bastante conhecidos na literatura (MUD, 2021). É sabido que a retirada da substância causadora da toxicidade hepática muitas vezes soluciona o problema, porém, dependendo do dano causado ao órgão, não há tratamentos eficazes, muitas vezes culminando na necessidade de transplante (ALHADDAD, 2020).

Os exemplos aqui mencionados, que estão presentes no cotidiano de muitas pessoas, podem levar a uma condição conhecida como DILI (*drug-induced liver injury*) ou lesões hepáticas induzidas por drogas. Essa condição é transitória, porém, com a constante exposição a agentes tóxicos, pode se tornar crônica, evoluindo para uma condição chamada NAFLD (*non-alcooholic fatty liver injury*). Essa condição não é exclusiva das lesões induzidas por drogas, sendo mais comum em doenças metabólicas, como dislipidemia, obesidade, disfunções da microbiota intestinal e o *diabetes mellitus* tipo II (BENCE, 2021). A NAFLD é marcada pelo aparecimento de esteatose hepática, com hepatócitos balonados, e mal funcionamento do fígado. Essa condição pode ainda evoluir para a NASH (*non-alcooholic esteatohepatites*), onde além das condições supracitadas, inicia-se um processo de infiltrado inflamatório, levando à hepatite. Ocorrências como essas levam à fibrose e, então, à cirrose, cujo único tratamento é o transplante hepático. Nesse interim, buscar uma solução mais viável e de fácil acesso é um desafio. São poucas as alternativas para tratar uma lesão hepática induzida por drogas, e as mais utilizadas na clínica são a silimarina, para intoxicações gerais; N-acetilcisteína, para intoxicação por paracetamol; L-carnitina, para intoxicação por ácido valpróico; colestiramina, para acelerar a depuração de

drogas; ácido ursodesoxicólico, para quadros colestáticos graves e prolongados; e corticoides para evitar a progressão das lesões e em DILI imunomediada (EASL, 2019).

Considerando que a hepatotoxicidade medicamentosa pode ser grave, levando a uma variedade de lesões e falhas nas funções do fígado, para as quais ainda não foi desenvolvida uma terapia eficaz, estudos abordando fatores de risco, diagnóstico e estratégias terapêuticas para DILI são muito importantes.

2 REVISÃO DE LITERATURA

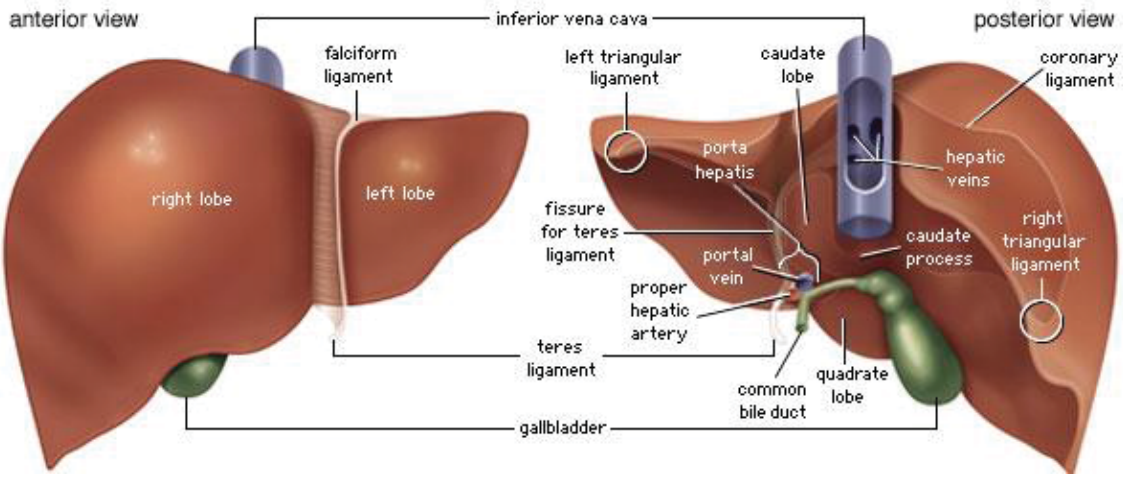
2.1 O FÍGADO E O METABOLISMO DE FÁRMACOS

O fígado é a maior glândula do corpo humano, localizado no quadrante abdominal superior direito até a linha média anterior. Morfologicamente, é dividido em quatro lobos, sendo os lobos superiores e inferiores direito e esquerdo. Os lobos direitos são substancialmente maiores que o esquerdo. Anexo a ele, encontra-se a vesícula biliar, órgão que recebe a secreção exócrina do fígado, a bile. O órgão é suprido pela veia porta-hepática e artéria hepática (KALRA; TUMA, 2021). A Figura 1 evidencia a anatomia do fígado.

As principais células que o compõem são chamadas de hepatócitos e possuem diferenças baseadas em suas funções e localizações. Na zona 1, na região periportal, os hepatócitos são mais bem supridos de sangue e oxigênio, portanto se regeneram com maior facilidade. Atuam na beta oxidação, gliconeogênese, lipólise, na formação da bile e no catabolismo de aminoácidos. A zona 2 é uma região de transição entre os tipos de hepatócitos 1 e 3. A zona 3 (perivenosa), por sua vez, é a que recebe menor quantidade de suprimento de sangue, e as células dessa região participam da detoxificação, biotransformação de drogas, liponeogênese, síntese de glicogênio, cetogênese e na formação de glutamina (KALRA; TUMA, 2021), sendo mais susceptível à necrose induzida por agentes tóxicos. Devido a estas especificidades dos hepatócitos, o fígado é responsável pela metabolização, ativação ou desativação de xenobióticos ingeridos oralmente (KALRA; TUMA, 2021).

A biotransformação de fármacos ocorre em duas fases. Na primeira fase as enzimas do complexo citocromo P450 (CYP) atuarão. Essas enzimas são classificadas através de sequências gênicas similares e ganham nomes numéricos de acordo com a família, letras para designar a subfamília e novamente um número, de acordo com a isoforma. Um exemplo dessa classificação é a CYP3A4/5, que está envolvida na biotransformação de diversas drogas (MCDONELL; DANG, 2013). Na fase I, as enzimas transformam o composto lipossolúvel em hidrossolúvel, por meio de reação de oxidação, oxirredução ou hidrólise.

Figura 1. Anatomia do fígado.

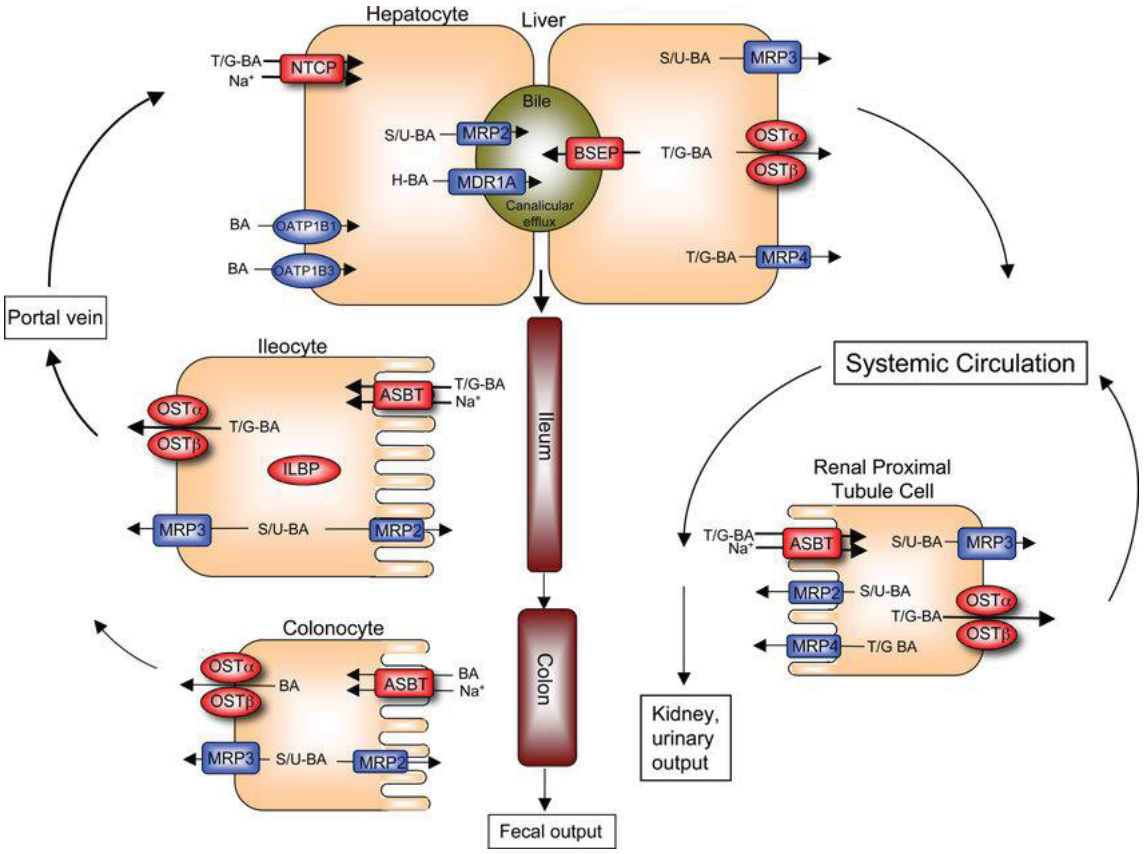


Fonte: Encyclopaedia Britannica, 2013

A fase II é conhecida como fase de conjugação, pois o produto da fase I ou a molécula original do fármaco são conjugados de forma a se tornarem ainda mais hidrossolúveis, facilitando a excreção pela bile ou por via renal (KALRA; TUMA, 2021). As enzimas envolvidas na fase II são, em sua maioria, transferases, como glucuronosiltransferases, sulfotransferases, N-acetiltransferases, glutationa-S-transferases e as metiltransferases (JANCOVA, 2010). Os produtos dessas reações podem ser um metabólito inativo ou ativo de um pró-fármaco.

É importante citar que esses complexos enzimáticos também estão presentes no intestino e nos rins. Ainda que em menor quantidade, algumas CYPs, como a CYP3A4, podem iniciar o metabolismo do fármaco antes que ele chegue à circulação. Transportadores de efluxo, como a glicoproteína-P, a MRP (proteína de resistência multidrogas) e a BRCP (proteína de resistência ao câncer de mama) também podem levar o fármaco a ser excretado antes mesmo de passar pela metabolização hepática (DEI,2019). Em contrapartida, há os transportadores de influxo, que facilitam a entrada do fármaco nas células: OATPs (polipeptídios orgânicos transportadores de ânions, OATs (transportadores orgânicos de ânions) e OCTs (transportadores orgânicos de cátions) (KATO, 2009), conforme ilustrado na Figura 2.

Figura 2: Circulação enteroepática de ácidos biliares mostrando as proteínas de transporte individuais nos hepatócitos, ileócitos (enterócitos ileais), e células do túbulo proximal renal



Fonte: Reprodução de DAWSON, 2011.

Legenda: T/G-BA: Taurina/Glicina conjugadas com ácidos biliares; NTCP: Transportador polipeptídico de taurocolato; S/U-BA: Sulfato de glicuronídeo conjugado com ácidos biliares; H-BA: hidroxilação de ácidos biliares; MRP2: proteína de resistência a multidrogas 2; MDR1A: proteína de resistência a multidroga 1A. BA: ácido biliar; OATP1B1: Polipeptídios orgânicos transportadores de ânions 1B1; OATP1B3: Polipeptídios orgânicos transportadores de ânions 1B3; BSEP: Bomba de exportação de sais biliares; MRP3: proteína de resistência a multidrogas 3; MRP4: proteína de resistência a multidrogas 4; OSTα-OSTβ: transportadores heteroméricos; ILBP: proteína ligante de lipídeos ileal; ASBT: transportador de ácido biliar sódio dependente.

Nessas reações, porém, ocorre a formação de compostos transitórios, como as espécies reativas de oxigênio (ROS). As enzimas CYP utilizam o NADPH (assim como diversas outras enzimas nos diferentes tecidos) para iniciar o processo de oxidação de substâncias. As características moleculares dos ROS provocam a peroxidação lipídica (LPO) que causa danos ao DNA, membranas celulares e tecidos. O sistema antioxidante geralmente consegue eliminar essas espécies através da atuação de enzimas como a superóxido dismutase (SOD), catalase (CAT), glutationa-S-transferase (GST) e glutationa peroxidase (GPx). No entanto, o acúmulo de ROS por diversos fatores provoca danos celulares permanentes. Estudos demonstram que

a citotoxicidade desses compostos leva à destruição de proteínas, de enzimas, de lipídios e neurotransmissores, estando envolvidos na patogênese de diferentes doenças. No fígado, o órgão mais afetado pelo estresse oxidativo, ocorrem injúrias crônicas ou agudas, destruição dos hepatócitos, necrose, esteatose hepática, entre outras lesões, podendo causar diferentes graus de hepatotoxicidade, de insuficiência hepática aguda a carcinoma (CONWAY; CAREY, 2017).

2.2 O ESTRESSE OXIDATIVO

A geração de produtos reativos de oxigênio em organismos é algo natural que ocorre em pequenas quantidades, havendo um mecanismo fisiológico que é capaz de inativar essas espécies rapidamente. O problema reside na saturação desse mecanismo antioxidante, que leva as ROS a causarem danos celulares diversos, inclusive morte celular. As mitocôndrias são as principais organelas geradoras de espécies reativas de oxigênio, pelo mecanismo de transferência de elétrons na produção de adenosina trifosfato (ATP). Os produtos gerados podem ser o peróxido de hidrogênio (H_2O_2), o radical hidroxila (OH) e o radical superóxido (O_2^-), entre outros. As enzimas CYP, envolvidas com a metabolização de drogas, também geram esses radicais livres no processo de inativação de compostos. O radical OH, diferente dos outros dois, não é eliminado por reação enzimática, sendo a mais danosa das espécies. Além das ROS, espécies reativas de nitrogênio (RNS) também contribuem com o estresse oxidativo. Assim, moléculas como a glutationa reduzida (GSH) são fundamentais em sua eliminação (IVANOV, 2017).

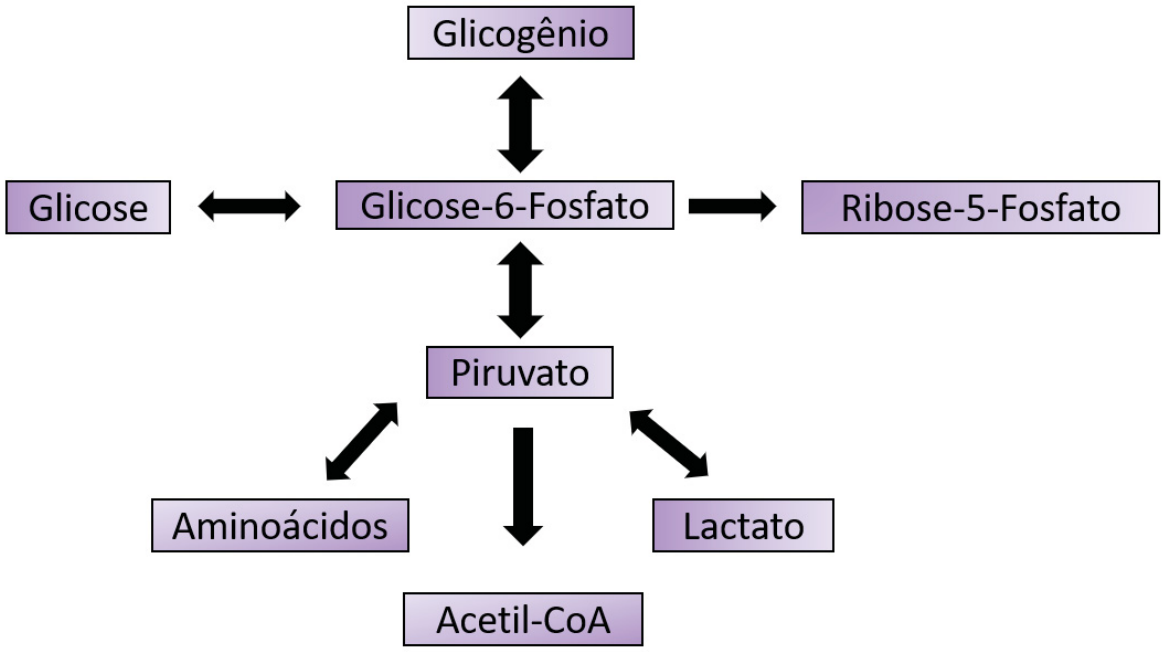
As enzimas conhecidas como de fase II atuam neutralizando algumas ROS, e são especializadas nos diferentes tipos de espécies. A superóxido dismutase (SOD) atua na neutralização do superóxido convertendo-o em H_2O_2. Enzimas como a catalase (CAT), peroxiredoxinas e glutationa peroxidases neutralizam o peróxido de hidrogênio. A glutationa peroxidase 4 (GPx4) é especialista em eliminar peróxidos lipídicos (IVANOV, 2017). A expressão dessas enzimas é controlada pelo fator Nrf2, sendo o promotor das regiões de Elementos de Resposta Antioxidante (ARE). Adicionalmente, a ciclina D1 sofre *down-regulation* em resposta ao estresse oxidativo, levando à parada do ciclo celular, bem como o *cross-talk* entre vias do NRF2 e da ciclina D1 foi demonstrado (MÁRTON et al., 2018; MORALES-GONZÁLES et al., 2017). No caso de superprodução de radicais livres, as enzimas antioxidantes não

conseguem cumprir suas funções e as células sofrem danos graves, como mutações no DNA, danificação e rompimento da membrana e morte celular. Esses efeitos podem acometer o tecido, gerando desde tumores à falência de órgãos, principalmente do fígado (IVANOV, 2017).

2.3 O METABOLISMO DA GLICOSE HEPÁTICA

O fígado possui duas funções essências no metabolismo da glicose: a gliconeogênese, quando em jejum, e estoca-la no período pós-prandial. Esses processos, quando desbalanceados, nas condições de *diabetes mellitus* tipo I e II, promovem a hiperglicemia, tanto em jejum quanto pós-prandial. A produção hepática de glicose corresponde a aproximadamente 90% da glicose endógena humana (PETERSEN, 2017). O ciclo da gliconeogênese é mostrado na figura 3.

Figura 3 – Vias da gliconeogênese.



Fonte: Adaptado de RODWELL; BENDER; BOTHAM; KENNELLY e WEILL, 2017.

Para manter os níveis homeostáticos de glicose sistêmica, os hepatócitos utilizam o glicogênio, que é um polímero de glicose, para disponibilizar às demais células a glicose que necessitam. Após a ingestão de alimentos, o fígado iniciará

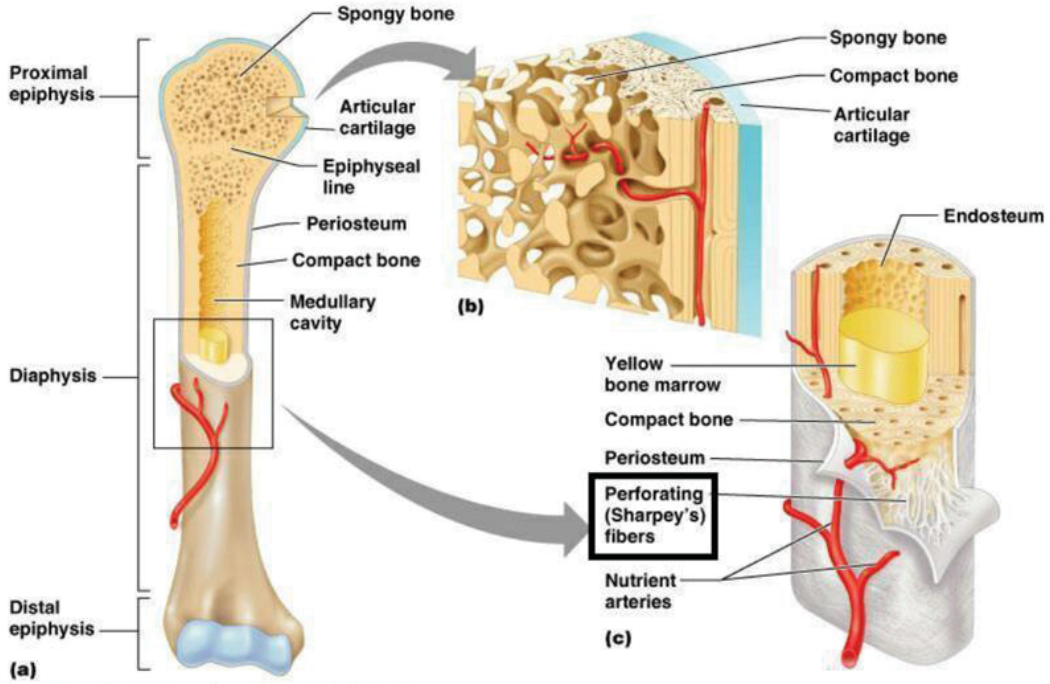
síntese de glicogênio para reposição e deixará de enviar a glicose para a circulação sistêmica (PETERSEN, 2017). Para que esse processo ocorra, tanto a glicose quanto a insulina devem estar presentes em níveis normais. Em indivíduos com NAFLD e/ou *diabetes melittu*s tipo II, ocorre supressão insulino-dependente da gliconeogênese hepática, o que demonstra resistência à insulina. Na NAFLD e sua progressão, NASH, a deposição de gordura nos hepatócitos desregula esse sistema e aumenta as chances de resistência insulínica (PETERSEN, 2017). Nestas condições clínicas, fármacos análogos do GLP-1 (peptídeo 1 semelhante ao glucagon) vêm sendo utilizados para regular o metabolismo de glicose e de lipídeos.

2.4 O TECIDO ÓSSEO

O tecido ósseo é o responsável pela estrutura do corpo humano, bem como locomoção e sustentação, assim como o alojamento e proteção da medula óssea, pela hematopoiese e pela homeostase de cálcio e outros eletrólitos. Esse tecido responde a uma variedade de estímulos, desde metabólicos a endócrinos (AMINI, 2012).

Como visto na Figura 4, o tecido possui uma estrutura compacta (*compact bone*) e outra esponjosa (*spongy bone*), ou trabecular. No estroma da medula óssea, encontram-se as células mesenquimais, que se diferenciam em tecido adiposo, osteoblastos, condrócitos, miócitos e endotélio. Durante toda a vida, o tecido passa por um processo de remodelagem, que mantém o osso integro e a homeostase. Disfunções nesse processo levam à fragilidade óssea e a riscos de fratura ou condições crônicas, como a osteoporose (FÖGER-SAMWALD, 2020). Algumas doenças metabólicas, como o *diabetes mellitus* tipo II, doenças inflamatórias gastrintestinais, como doença celíaca, doenças hepáticas, como NAFLD, e a menopausa, podem favorecer quadros de fragilidade e fraturas (FILIP, RADZKI e BIEŃKO, 2019).

Figura 4: Estrutura do tecido ósseo



Fonte: CUMMINGS, 2004

Entretanto, deve-se ressaltar que em pacientes com doenças gastrintestinais, a prevalência de doenças metabólicas ósseas não foi estudada com profundidade até o momento. Especialmente, ao considerar as doenças hepáticas crônicas, nas quais os possíveis mecanismos, além da desnutrição, incluem também distúrbios endócrinos acompanhados de alterações inflamatórias (FILIP, RADZKI e BIEŃKO, 2019). A via RANK-L (receptor ativador do fator nuclear Kappa B) é responsável pela regulação da reabsorção óssea, que pode ser indesejada em casos de doenças como osteoporose (BOYCE, 2007). O estrógeno é um regulador da remodelação óssea e da homeostase do tecido, modulando a expressão do RANK-L (STREICHER, 2017). A via Wnt é responsável pela diferenciação de células mesenquimais osteogênicas em adultos, e é um interessante alvo farmacológico, já que pode estimular a regeneração do tecido, mesmo em fraturas (HOUSCHYAR, 2019). Assim, os tratamentos farmacológicos existentes atualmente para osteoporose tentam inibir a reabsorção óssea, através da promoção da apoptose dos osteoclastos; modulação do metabolismo de cálcio; uso de anticorpos anti-RANKL; uso de agonistas do estrogênio para simular seus efeitos ósseos; e pela modulação da via Wnt (FÖGER-SAMWALD, 2020). No entanto, tem

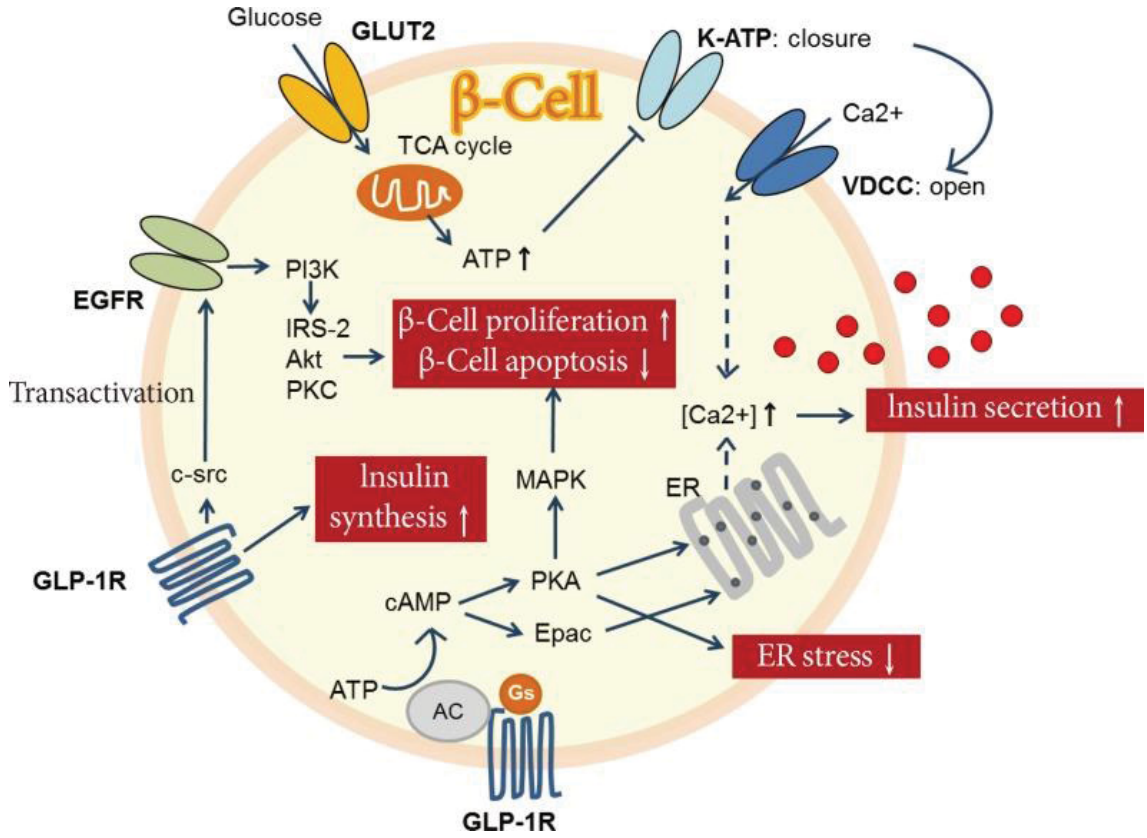
se demonstrado que alguns análogos do GLP-1 têm efeitos ósseos interessantes para o tratamento de osteopatias.

2.5 ANÁLOGOS DO PEPTÍDEO 1 SEMELHANTE AO GLUCAGON (GLP-1) E O REPOSICIONAMENTO DE FÁRMACOS.

Os análogos do GLP-1, a exemplo de liraglutida, semaglutida, dulaglutida e lixisenatida, são peptídeos sintéticos semelhantes ao glucagon, utilizados para o tratamento de condições como pré-diabetes e *diabetes mellitus* tipo II. Esses análogos se ligam ao receptor GLP-1R, acoplado à proteína G, nas células β-pancreáticas, liberando insulina apenas quando os níveis glicêmicos dos pacientes se encontram altos (CORNELL, 2020). Porém, na literatura, também há estudos sobre sua capacidade de atuar na regeneração de tecidos, como o cardíaco e o ósseo (SCHIELLERUP et al., 2019; TANDAY, FLATT e IRWIN, 2021), bem como um estudo prévio de nosso grupo de pesquisa demonstrou que a liraglutida possui ação hepatoprotetora e terapêutica em casos de intoxicação hepática (MILANI et al., 2019).

Em geral, duas vias podem ser ativadas quando análogos do GLP-1 ocupam seus receptores em células beta-pancreáticas (Figura 5). Na via principal, ocorre a ativação da proteína Gs, estímulo de adenilato ciclase (AC), formação de cAMP e consequente ativação de proteína quinase A (PKA), que permite a liberação de cálcio de estoques intracelulares, entrada de cálcio na célula e a secreção de insulina. A via secundária, ocorre com a ativação PI3K (fosfoinostídeo 3 quinase) e da via inibitória AKT (serina-treonina quinase). Durante esse processo, diversos eventos são ativados ou inibidos, como o aumento da proliferação celular, pela via MAPK e AKT, e a diminuição da apoptose, assim como regulação de vias de estresse oxidativo (ATHAUDA, 2016).

Figura 5: Mecanismo de ação dos análogos do GLP-1 quando ligados ao receptor GLP-1R.



Fonte: JUNG, 2014.

Legenda: GLUT2: Transportador de glicose 2; β Cell: célula beta-pancreática; TCA cycle: ciclo do ácido tricarboxilico; ATP: adenosina trifosfato; K-ATP: Canal de potássio sensível ao ATP; EGFR: receptor do fator de crescimento epidérmico; PI3K: fosfoinositídio 3 quinase; AKT: Serina-treonina quinase; PKC: Proteína quinase C; C-src: Tirosina proto-oncogênica quinase src; ER: Retículo endoplasmático; MAPK: Proteína quinase mitógeno-ativada; cAMP: adenosina 3',5'-monofosfato cíclico; GLP-1R: receptor do GLP-1; AC: adenilatociclase; Epac: proteínas diretamente ativadas por cAMP; VDCC: Canal de cálcio voltagem dependente.

Além dessas ações, os análogos do GLP-1 têm sido estudados por sua capacidade de aumentar a formação óssea e diminuição da reabsorção (SCHIELLERUP et al., 2019; TANDAY, FLATT e IRWIN, 2021). Em animais diabéticos, com perda de mineralização óssea, a administração desses fármacos demonstrou a recuperação do tecido (MANSUR et al., 2019). Teorias versam que a ação desses fármacos ocorre diretamente nas células mesenquimais, promovendo a ativação de genes que favorecem a formação de osteoblastos (TANDAY, FLATT e IRWIN, 2021).

O emprego destes fármacos para melhorar a remodelação óssea ou as funções hepáticas se enquadra em uma utilização *off-label*, ou seja, diferente da condição patológica para a qual foram aprovados pelas agências regulatórias.

A partir de estudos e de resultados significantes sobre um fármaco, é possível sugerir seu reposicionamento, afinal sabe-se que é seguro, pois passou por todas as etapas e testes de segurança e toxicidade anteriormente; e são conhecidos os possíveis efeitos, as vias de administração e as doses. O reposicionamento de fármacos tem sido uma prática comum, além de interessante para a população, pois o desenvolvimento de uma droga desde sua descoberta até sua aprovação pode levar em torno de dez anos. Por exemplo, muitos estudos têm buscado no reposicionamento de fármacos tratamentos para a recente pandemia de COVID-19, já que um tratamento efetivo é urgente em situações como essa (ALTAY, 2020).

Deste modo, os análogos do GLP-1 são possíveis candidatos para o reposicionamento, já que seus resultados em estudos abordando diversas situações clínicas são promissores. Recentemente a semaglutida, objeto de estudo dessa dissertação, foi aprovada como terapia contra a obesidade pelo FDA (FDA, 2021). O outro objeto desse estudo, a dulaglutida, tem-se mostrado eficaz na inibição de cascatas inflamatórias (NGUYEN, 2020). A hipótese deste trabalho é que tanto a dulaglutida quanto a semaglutida têm efeitos benéficos sobre o fígado e ossos em modelo agudo de hepatotoxicidade, e que os novos usos para esses medicamentos se enquadrariam no reposicionamento de fármacos.

2.6 OBJETIVOS

2.6.1 Objetivo geral

Analisar os efeitos terapêuticos e profiláticos da semaglutida e dulaglutida em modelo agudo in vivo de hepatotoxicidade induzida por tetracloreto de carbono (CCl_4);

Analisar os efeitos dessas drogas sobre parâmetros ósseos.

2.6.2 Objetivos específicos

Avaliar parâmetros bioquímicos plasmáticos e biliares dos animais submetidos ao desafio com agente hepatotóxico e aos tratamentos;

Avaliar os danos hepáticos através da mensuração de parâmetros histológicos, de estresse oxidativo, inflamatórios e de proliferação celular;

Avaliar os efeitos metabólicos do agente hepatotóxico e dos tratamentos;

Avaliar parâmetros ósseos através de histomorfometria da tíbia.

3 ARTIGO CIENTÍFICO

Effects of dulaglutide and semaglutide in liver and in bone microarchitecture in an acute model of hepatotoxicity in mice

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ABSTRACT

Hepatic liver diseases are among the most common chronic diseases over the world, with few available treatments. Bone diseases, such as osteoporosis, are associated with liver disease and other conditions and represent another challenge regarding treatments. This study investigated the effects of the anti-diabetic GLP-1 analogues, semaglutide and dulaglutide, in the acute CCl_4-hepatotoxicity model, and over the bone microarchitecture. Two protocols were used in Swiss male mice: Treatment and Pretreatment with one or two doses of semaglutide (0.021 mg/Kg, i.p.) and dulaglutide (0.014 mg/Kg, i.p.), silymarin (100 mg/Kg, v.o., positive control) or vehicle (10 ml/kg, s.c.). The mice were euthanasiated 48 h after the challenge with 1.5% CCl_4. The results indicated that dulaglutide has greater hepatoprotective effects than semaglutide, reducing the liver necrosis and glutathione-S-transferase activity, and the plasmatic alanine aminotransferase level. However, both drugs induced the gene expression of *Cyclin D1*, improving the proliferative phase of liver regeneration. Semaglutide improved the biliary excretion of cholesterol, benefiting more the biliary system than the hepatic parenchyma. Both GLP-1 analogues improved the tibia microarchitecture, increasing the trabecular volume and reducing the trabecular thickness. Also, dulaglutide increased the number of trabeculae. These data evidenced that dulaglutide has potential to be an alternative treatment to hepatotoxicity, mainly induced by drugs, and that dulaglutide and semaglutide improved significantly the bone development after two administrations, demonstrating the potential value of these GLP-1 analogues in the treatment of fragility fractures in liver diseases, diabetes or other conditions.

3.1 INTRODUCTION

The glucagon-like peptide-1 receptor agonists, or GLP-1 analogues, are largely used in therapeutics because of their effects in *diabetes mellitus* type II or in pre-diabetic patients. Liraglutide, semaglutide, dulaglutide and lixisenatide are examples of these hypoglycemic drugs that only respond to high levels of plasma glycemia, which reduce collateral effects. Recent studies show that these drugs have other beneficial effects, as weight loss, reduction of cardiovascular diseases risk and hepatic protection (NAUCK, 2021; MILANI et al., 2019). This effect is important concerning different aspects of the liver: this organ is constantly exposed to compounds that can induce hepatotoxicity, such as drugs and alcohol; the hepatic steatosis is the most common chronic disease in the world, with no specific pharmacological treatment; and free prescription drugs, like acetaminophen, and their misuse are the responsible for most cases of drug induced liver injury (DILI) in the world (KE, 2020). Hepatic injuries, like non-alcoholic fatty liver diseases (NAFLD) and its progression to non-alcoholic steatohepatitis (NASH) are commonly related to DILI. Although the NAFLD is reversible, it can lead to irreversible damage provoked by NASH, fibrosis and cirrhosis (CUYKX et al., 2018).

Hepatic drug biotransformation can induce the formation of reactive chemical metabolites that may bind to nucleic acids, proteins and lipids, thus leading to DNA damage, loss of protein function, and lipid peroxidation (SHEHU, MA and VENKATARAMANAN, 2017). Among the risk factors, changes in hepatic drug metabolism and antioxidant defense system are associated with the incidence of DILI (JEONG et al., 2020; SOTANIEMI et al., 1997), by generating reactive oxygen species (ROS). Normally the cell redox system can deal with small quantities of these radicals, different from the exposure to high levels of toxic agents, or chronic exposure. In these cases, the cells are exposed to oxidative stress and tend to die. To reduce the damages, acute liver injuries can be treated, for example, with N-acetylcysteine (NAC), silymarin, L-carnitine, ursodeoxycholic acid and nowadays with corticosteroids (ANDRADE et al., 2019; GARCIA-CORTES et al., 2020). However, in many cases there is no improvement of lesions and DILI is still an orphan disease from a therapeutic standpoint (ANDRADE et al., 2019). Considering this scenario, and the absence of effective therapies for those lesions, the search for new treatments is necessary. The liraglutide was previously studied by our research group (MILANI,

2019) and showed benefits in hepatic injury induced by carbon tetrachloride (CCl_4) in mice, a model of DILI. This model is very described in literature, and can cause different levels of hepatic injury, with very similar results of intoxication by drugs and other compounds (ERNST, 2020) However, the hepatoprotective effects of the modern GLP-1 analogues, such as semaglutide and dulaglutide, in DILI are not known. These drugs have a long half-life (~4 - 7 days), allowing the application of a single weekly dose, in contrast to the liraglutide, which has a half-life of ~12 h. Furthermore, another feature of semaglutide is its role in bone recovery, which can be a relevant aspect considering that NAFLD and NASH can cause decrease of bone mass, by lowering level of vitamin D, which increases the risk of fracture, growth hormone decreases and chronic inflammatory processes, that can lead to osteoporosis (FILIP, 2018).

Considering that the discovery of new drugs and their commercialization can take 10 to 15 years of research, and that thousand compounds are analyzed while only one can become a marketable and safe drug (HANQING XUE, 2018), the repositioning of drugs can reduce costs, time, and offer some safety to users. This study was based in this concept and focused on the potential off-label use of the GLP-1 analogues dulaglutide and semaglutide to prevent hepatic and osseous tissues changes in an acute liver injury model.

3.2 MATERIAL AND METHODS

3.2.1 Reagents

The GLP-1 analogues used were commercially available: semaglutide, Ozempic® (Novo Nordisk; Copenhagen, Denmark) and dulaglutide, Trulicity® (Eli Lilly; Indiana, USA). The silymarin were purchased from Pharma Nostra (Rio de Janeiro, Brazil). The carbon tetrachloride (CCl_4) was obtained from Dinâmica (Indaiatuba, Brazil). For the lactate and glycogen tests the Bioclin® Kits assays were used (Belo Horizonte, Minas Gerais). Amyloglucosidase, NAD^+, NADH, Bradford reagent, reduced glutathione, glutathione reductase, DTNB (5,5′-Dithiobis(2-nitrobenzoic acid), DPPH (2,2 diphenyl-1- picrylhydrazyl) and CDNB (1-chloro-2,4-dinitrobenzene) were purchased from Sigma-Aldrich (St. Louis, USA); pyrogallol from VETEC® (Duque de Caxias, Brazil); and methanol from NEON® (Suzano, Brazil). TriZol and primers were obtained from Invitrogen-ThermoFisher (Waltham, USA). High-Capacity cDNA

Reverse Transcription Kit and SYBR Green PCR Master Mix were purchased from Applied Biosystems-ThermoFisher (Waltham, USA).

3.2.2 Animals

Three-months-old Male Swiss mice (*Mus musculus*) from Federal University of Paraná animal facility were used in the experiments. The animals were maintained in 12 hours night/day cycle, under controlled temperature (24°C ± 2), and with *ad libitum* water and food. Along the experiment, the mice were disposed in groups of 5 or 7 per cage with environmental enrichment, aiming the animal welfare. The protocols followed the international rules for laboratory animal use in research and were approved by the local Animal Experimentation Ethics Committee (CEUA, certificate # 1344). The mice were used in two protocols of experiment, namely Treatment and Pretreatment.

3.2.3 Experimental protocols

3.2.3.1 Treatment

In this protocol, at the first day, the hepatotoxicity was induced in the mice with 1.5% CCl_4 in corn oil (0.25 mL i.p.), except for the basal group, which received corn oil (vehicle) at the same volume via i.p.. Two hours later the animals received the treatments: the groups CCl_4 and basal were treated with saline solution (0.25 mL i.p); the positive control group received silymarin (100 mg/Kg, 0.1 ml, orally); and the groups of GLP-1 analogues received semaglutide (0.021 mg/Kg, i.p.) or dulaglutide (0.014 mg/Kg, i.p.), diluted in saline solution. The doses of GLP-1 analogues were calculated by interspecific allometry (PACHALY and BRITO, 2001) based on the lowest dosage indicated for humans, and in a pilot study (data not shown) using high and low doses of the drugs. The dose of silymarin was a higher and non-toxic dose for mice based in previous study (STOLF et al., 2018). In the second day, the glycemia was measured at 9 a.m., under fed state, using glucose strips and respective glucose monitor (AccuChek® Guide), in a blood drop collected by a tale's puncture. At the third day, the animals were anesthetized with 100 mg/Kg of ketamine and 10 mg/Kg of xylazine for biological material collection. Through the laparotomy, blood samples were collected from the abdominal cava vein with heparinized syringes; liver, and other organs were withdrawn, weighted and properly stored for further analysis; and the bile

was collected by direct puncture of gallbladder. The experimental design of Treatment (*n* = 5-10 mice/ group) is presented in Figure 1A.

3.2.3.2 Pretreatment

The complete pretreatment protocol was conducted during 10 days, as presented in Figure 1B. The animals (*n* = 5-10 mice/ group) were treated with one (1x) or two administrations (2x) of semaglutide (0.021 mg/Kg, i.p.) or dulaglutide (0.014 mg/Kg in a volume of0.25 mL i.p). Animals receiving two administrations were treated in the first and sixth days of the study protocol, while those submitted to one-administration scheme were treated in the sixth day only. The basal and CCl_4 groups were treated with saline (0.22 mL, i.p.) in the first and sixth days, while the silymarin group (100 mg/kg, 0.1 mL, v.o.) received the drug daily for 7 days. In the eighth day, all the groups, except for the basal, were challenged with the toxic agent 1.5% CCl_4 in corn oil (0.22 mL, i.p.). The basal group received the same volume of corn oil. The mice's glycemia was measured in fed state at 9 a.m., with glucose strips at the second and seventh day. In the tenth day the animals were anesthetized and the biological material collected as mentioned in the Pretreatment protocol.

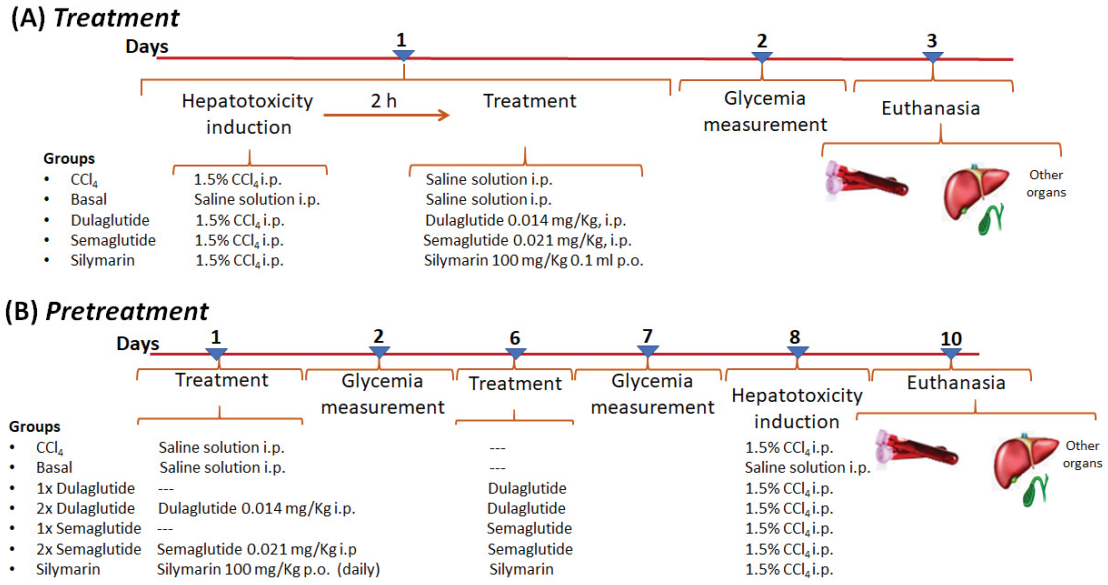


Figure 1. Experimental design and timeline of the Treatment **(A)** and Pretreatment **(B)** protocols.

3.2.4 Hepatic histological evaluation

Liver fragments were harvested and fixed in formalin 10%. Formalin-fixed tissues were processed according to histological routine techniques (dehydration in alcohol, clearing in xylene, and embedding in paraffin). Thin sections of each sample (5 µM) were stained with hematoxylin and eosin and histological slides were examined by light microscopy (blind study). The examination of histological injury included findings of necrosis, vacuolization, ballooning and inflammation, classified as described by Milani et al. (2019), pursuant to the intensity of the lesion, ranging from absent (grade 0) to severe (grade 3).

3.2.5 Plasmatic and biliary biochemistry analysis

The blood was centrifuged at 3,400×gfor 10 min to obtain the plasma for the measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The bile samples were diluted in saline solution adjusting the volume to 100 µL for the analysis of cholesterol and total bilirubin. All these parameters were measured using commercial kits in an automatic analyzer (Mindray BS-200, Shenzhen, China).

3.2.6 Hepatic metabolism biomarkers

To analyze the metabolic function of the liver some parameters were evaluated. Glycogen and lactate rates were performed as biomarkers of glucose metabolism, and total cytochrome P450 (CYP) activity as indicator of hepatic biotransformation capacity.

The hepatic contents of glycogen were measured by the Kepler and Decker method (1974). Briefly, liver samples were homogenized in perchloric acid, still frozen. The samples were centrifuged in 10,000×g for 10 minutes to measure the free glucose in 505 nm. For the measurement of the total glucose, 100 µL of the homogenate were used, added of 980 µL 0.2 M sodium acetate buffer (pH 4.8), 60 µL of 1M potassium bicarbonate and 20 µL of amyloglucosidase. The samples were stirred in a water bath (37ºC) for 2 hours. The reaction was stopped using 500 µL of 0.6 N perchloric acid and centrifugated at 6,000×g for 10 minutes. The absorbance was read in 505 nm in a microplate reader (BioTek Synergy HT, Highland Park, VT, USA). The lactate assay was performed using a commercial kit, through the conversion of lactate to pyruvate by the reaction with lactic dehydrogenase (LDH), and reduction of NAD^+ to NADH. The reading was performed in 90 wells microplate at 340 nm using a multi-mode microplate

reader. To measure the total CYP levels the liver homogenates were prepared with 10 mg wet tissue/mL in CYP buffer (50 mM Tris-HCL pH 7.4; 150 mM KCl; 10 mM $MgCl_2$) and samples were suspended in CO by 1 min, followed by a absorbance reading at 400 to 600 nm. Then 6 µl of sodium dithionite were added to the samples and the spectrum was obtained in 450 to 490 nm according to Matsubara et al. (1976). The CYP levels were calculated with the molar extinction coefficient (104 $mM^{-1} \cdot cm^{-1}$) and expressed in nmol CYP/mg tissue.

3.2.7 Antioxidant evaluation in vivo and in vitro

3.2.7.1 Hepatic oxidative stress biomarkers

Liver samples (0.3 g) were homogenized in potassium phosphate buffer (pH 6.5, 1:10) and centrifuged at 10,000×g at 4°C for 20 min. The homogenate was used to measure the levels of reduced glutathione (GSH) and lipid peroxidation (LPO), while the supernatant was used to measure superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) activities and protein levels. All the measurements were performed in 90 wells microplate.

GSH levels were determinate by the reaction of glutathione with DTNB, resulting in 2-nitro-5-mercapto-benzoic acid (TNB), which reading was taken in 412 nm (SEDLAK and LINDSAY, 1968). The activity of SOD was measured though the inhibition of the oxidation of pyrogallol, where 50% of inhibition (IC_{50}) were equivalent to one unit (U) of SOD (AEBI, 1984). The CAT activity was measured by the consumption of H_2O_2 in the reaction and reading was taken at 0, 15, 30 45 and 60 sec at 240 nm (AEBI, 1984). The determination of GST activity was by the conjugation of the GSH with the CDNB reagent, with reading at 340 nm (KEEN, HABIG and JAKOBY, 1976). The LPO analysis was performed by the measurement of the hydroperoxides, following the iron oxidation by the orange xylenol (FOX method), with reading at 550 nm (JIANG et al., 1991). Finally, the determination of proteins was performed in 1:10 homogenized samples, by the Bradford method (1976) at 595 nm.

3.2.8 In vitro antioxidant capacity evaluation of GLP-1 analogues

In complement to the in vivo analyses of the hepatic redox system, it was performed an assay to investigate the antioxidant capacity *per se* of the GLP-1

analogues. The reactivity of the range of concentrations from 1 to 300 μg/mL of semaglutide and dulaglutide with the free radical 2,2 diphenyl-1-picrylhydrazyl (DPPH) was determined (CHEN et al., 2004). The ascorbic acid (50 μg/mL) was used as the positive control and distilled water as the negative control.

3.2.9 Hepatic gene expression

Total RNA was extracted from the liver tissues using the TRIzol reagent according to the manufacturer's instructions. Two µg of RNA were reverse-transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions. Gene expression was measured by RT-qPCR using SYBR Green PCR Master Mix (Applied Biosystems). Two genes were assessed: *Cyclin D1* and Nuclear Factor Erythroid 2 Like 2 (*Nfe2l2* or *Nrf2*). Both the gene expression levels were normalized to the Ribosomal Protein Lateral Stalk Subunit P0 (*Rplp0*). Relative expression values were obtained using the $2^{-\Delta\Delta Ct}$ method, and normalized to the control value for percent fold changes (LIVAK and SCHMITTGEN, 2001). The primer gene sequences are listed in Supplementary Table S1.

3.2.10 Inflammatory parameters evaluation

Samples of liver tissue were homogenized in 0.1% Triton X-100 saline to determine the enzymatic activity of myeloperoxidase (MPO) and N-acetylglucosaminidase (NAG), indicating neutrophil and macrophage (mononuclear cell) migration, respectively. The homogenates were centrifuged at 9,000×g at 4ºC for 10 min, and the supernatants were used to determine MPO and NAG activity. The reading of MPO absorbance was performed at 620 nm as described by Bradley et al. (1982). The NAG activity was measured at 405 nm as the hydrolysis of p-nitrophenyl-N-acetyl-β-d-glucosamine (substrate) in N-acetyl- β-d-glucosamine, which releases p-nitrophenyl, according to the method of Sánchez & Moreno (1999). The measurements were performed in a microplate reader (BioTek Synergy HT, Highland Park, VT, USA).

3.2.11 Histomorphometric analysis of tibias

The tibias from mice submitted to the pretreatment with two doses of semaglutide or dulaglutide and CCl_4 were collected for analysis. The bones were left in 70% ethanol for 48 hours to fixation, followed by 7 days in 100% ethanol to dehydration. Then, the samples were soaked in 3 different solutions of methylmethacrylate for 7 days. The solutions were: I, 25% of dibutylphthalate/75% of methylmethacrylate; II, 25% of dibutylphthalate/75% of methylmethacrylate + 1 g of benzoyl peroxide [1%]; and III, 25% of dibutylphthalate/75% of methylmethacrylate + 2 g of benzoyl peroxide [2%]). The samples were put in a solution III (37ºC, 24 h) till total polymerization of the methylmethacrylate. The histomorphometric analyses were performed by the same investigator using a microscope (Nikon Labophot II, Tokyo, Japan) equipped with a high-resolution UV color video camera (Olympus DP71, Center Valley, PA, USA). The following parameters were analyzed: bone volume [BV/TV, %], trabecular thickness [Tb.Th, μm], number of trabeculae [Tb.N, N/mm], separation between the trabeculae [Tb.Sp, μm] and bone marrow volume [Ma.V.TV, %].

3.2.12 Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). The comparisons were performed with analysis of variances (ANOVA) followed by the *post hoc* test of Bonferroni. For the comparison of two groups the Student *t* test was applied. Significances were considered when $p<0.05$. All data, figures and tables were analyzed and created using Graphpad Prism® version 9 software (San Diego, USA).

3.3 RESULTS

3.3.1 Hepatic macroscopy and microscopy evaluation

The livers were weighted and expressed in percentage of the body mass. In both protocols the administration of CCl_4 induced a significant increase of the liver mass, and both treatments and pretreatments did not change this condition (Fig. 2A, B). In accordance, CCl_4 group was able to promote the death of hepatocytes by necrosis, with a centrilobular distribution pattern (Fig. 2D). Necrosis was predominant in CCl_4

and semaglutide groups (grade 2), followed by dulaglutide and silymarin groups (grade 1). The degenerative change of hepatocellular ballooning and the inflammatory changes were similar in all groups (grade 1), excluding the basal group (grade 0). The Figure 2 C-G demonstrates the hepatic histological condition of liver in the groups from the treatment protocol.

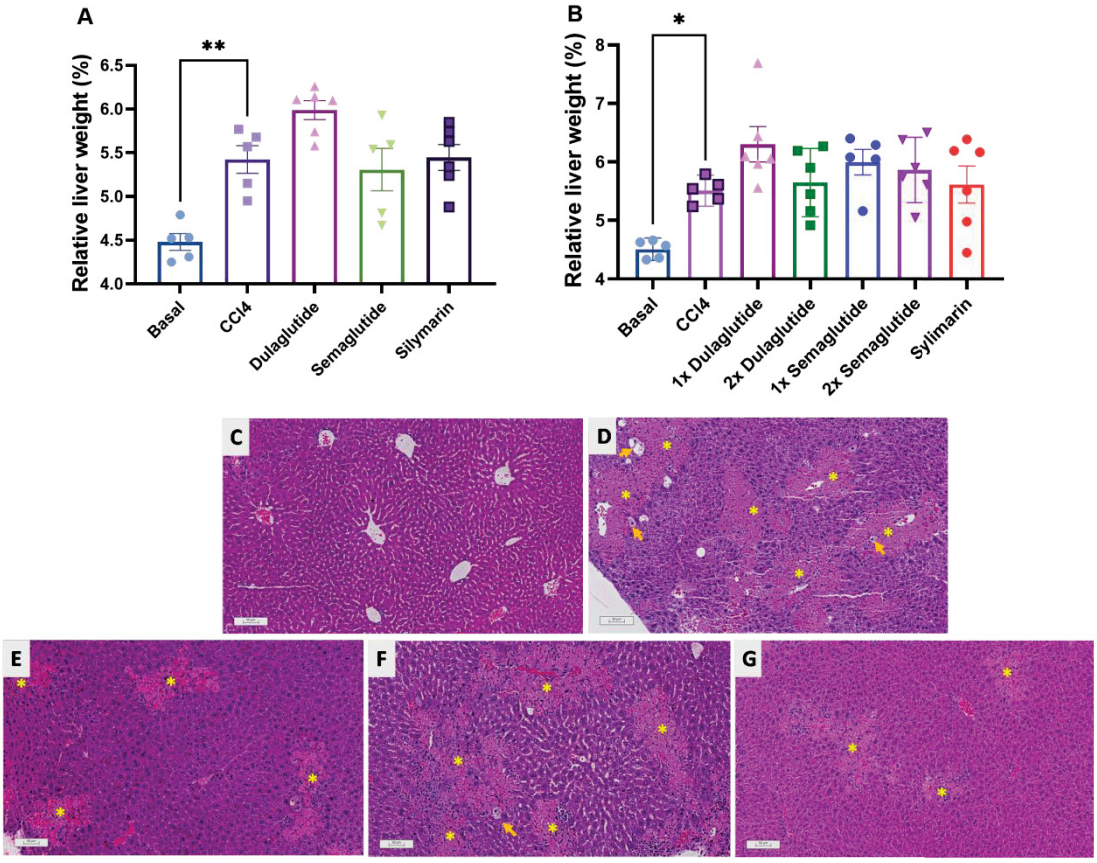


Figure 2. Hepatic relative weight and liver histology. **(A-B)** shows liver relative weight of mice from treatment and pretreatment protocol, respecively. **(C-G)** are representative histological images of **(C)** basal, **(D)** CCl_4, **(E)** dulaglutide, **(F)** semaglutide and (**G)** silymarin groups from the treatment protocol. The results in A and B are expressed as the mean ± SEM and were analyzed by one-way ANOVA followed by Bonferroni's test. *p<0.05; **p<0.01; → hepatocellular ballooning; * centrilobular necrosis; scale bar = 50 µm.

3.3.2 Plasmatic and biliary biochemistry

The plasmatic transaminases, ALT and AST, increased enormously in the groups that received CCl_4 in comparison with the Basal group, in both treatment (Fig. 3A) and pretreatment (Fig. 3B) protocols. However, the late treatment with dulaglutide reduced significantly the ALT activity in ~52%, while semaglutide and silymarin reduced the ALT activity in ~28% (Fig. 3A). In contrast, the pretreatment with the drugs did not protect

the liver against the elevation of ALT and AST induced by CCl_4 (Fig. 3C, D). The glycemia was reduced by the CCl_4 administration, but it was normalized by the treatment with dulaglutide and silymarin. (Fig. 3E). Instead, in the pretreatment only 2 doses of semaglutide reduced 16% the glycemia in relation to CCl_4 group, while silymarin increased the glycemia in 15% (Fig. 3F).

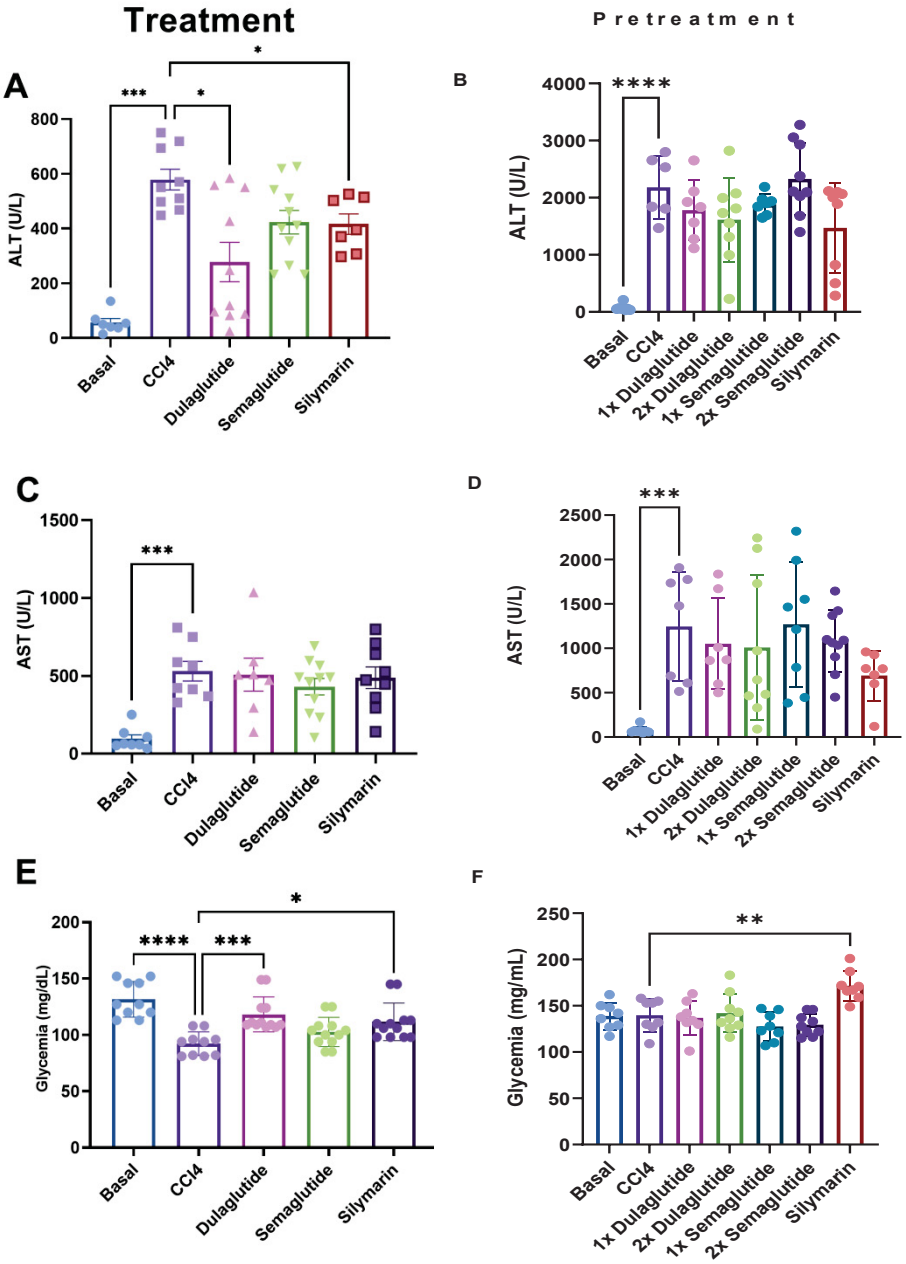


Figure 3. Plasmatic parameters in mice challenged with CCl_4 under different treatments. **(A-B)** show the ALT levels, **(C-D)** evidence the AST levels, and **(E-F)** show the glycemia in treatment and pretreatment protocols, respectively. The results are expressed as the mean ± SEM and were analyzed by one-way ANOVA followed by Bonferroni's test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Enough amounts of bile samples were collected only in the treatment protocol. In these mice the CCl_4 administration tended to reduce the secretion of cholesterol into the bile, while semaglutide and silymarin improved this secretion (Fig. 4A). The biliary bilirubin secretion was not modified by the toxicant CCl_4 or any treatment (Fig. 4B).

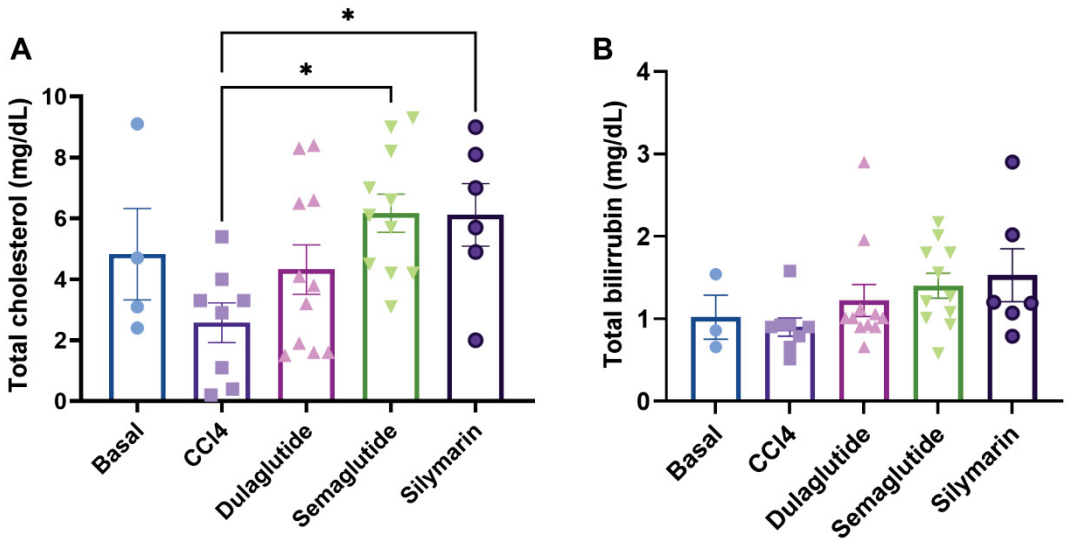


Figure 4. Biliary secretion of total cholesterol **(A)** and total bilirubin **(B)** in mice submitted to treatment protocol. The results are expressed as the mean ± SEM and were analyzed by one-way ANOVA followed by Bonferroni's test. *p<0.05.

3.3.3 Hepatic glycogen, lactate and total CYP levels

Considering that the GLP-1 analogues and CCl_4 can interfere in the glucose metabolism, the hepatic contents of glycogen and lactate was measured. It was not found statistical differences among the groups in both protocols of treatments, as shown in Table 1. Curiously, the lowest hepatic total CYP level was obtained in semaglutide groups, in both treatment and pretreatment protocols (Table 1).

Table 1. Hepatic contents of lactate, glycogen and total CYP in mice challenged with CCl_4.

	Lactate (mg/dL)	Glycogen (mmol/g liver)	Total CYP (nmol/mg protein)
Treatment groups			
Basal	1.60 ± 0.13	4.85 ± 0.28	4.38 ± 0.26
CCl_4	1.52 ± 0.14	4.74 ± 0.30	4.37 ± 0.34
Dulaglutide	1.51 ± 0.26	4.78 ± 0.30	4.09 ± 0.18
Semaglutide	1.40 ± 0.22	4.81 ± 0.20	3.59 ± 0.17
Silymarin	2.12 ± 0.25	4.61 ± 0.12	3.96 ± 0.23
Pretreatment groups			
Basal	1.67 ± 0.20	4.20 ± 0.28	4.03 ± 0.39
CCl_4	1.74 ± 0.18	4.86 ± 0.25	3.41 ± 0.22
1x Dulaglutide	1.56 ± 0.41	6.03 ± 0.23	3.78 ± 0.39
2x Dulaglutide	1.50 ± 0.15	5.33 ± 0.44	4.12 ± 0.29
1x Semaglutide	1.70 ± 0.50	4.92 ± 0.27	3.00 ± 0.19
2x Semaglutide	0.87 ± 0.09	5.09 ± 0.39	3.16 ± 0.26
Silymarin	0.73 ± 0.08	4.30 ± 0.26	3.46 ± 0.33

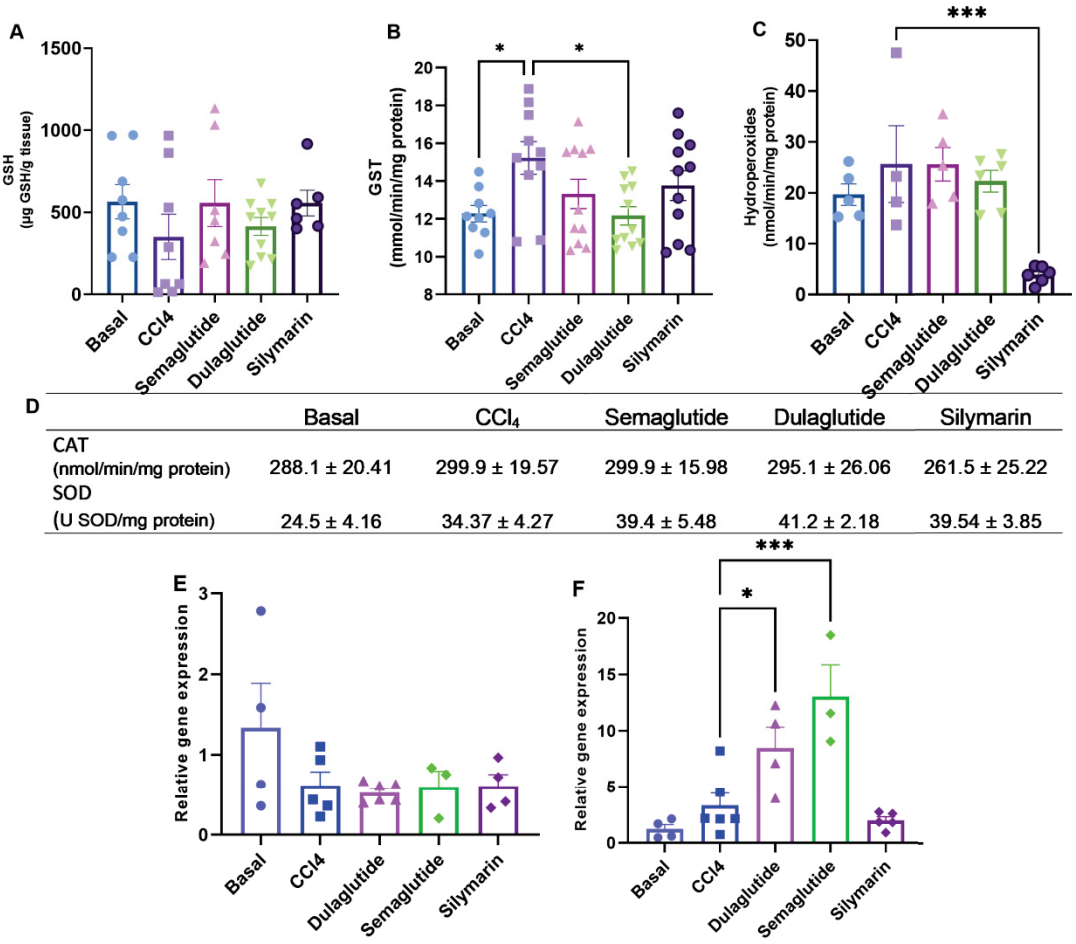
The results were analyzed using one-way ANOVA and Bonferroni's test, expressed by the mean ± SEM.

3.3.4 Oxidative stress, gene expression and inflammatory parameters

In the treatment protocol the level of hepatic GSH was reduced ~37% in the CCl_4 group in comparison with the basal, despite the absence of statistical significance. The treatment with semaglutide, dulaglutide and silymarin increased the GSH level similarly to the basal, but also without statistical difference (Fig. 5A). On the other hand, the GST activity was significantly increased by the toxicant CCl_4, and reduced to the basal level by dulaglutide treatment (Fig. 5B). The LPO level was slightly increased by CCl_4, but significantly reduced only by the silymarin treatment (Fig. 5C). The other biomarkers of oxidative stress were not different among groups in the treatment (Fig. 5D) and pretreatment protocols (Table 2). In accordance, semaglutide and dulaglutide did not modify the gene expression of *Nrf2* (Fig. 5E); as well these drugs did not show

antioxidant activity in vitro against the DPPH radical, different of the positive control of this assay, the ascorbic acid (Fig. 6). However, dulaglutide and semaglutide increased 2.2-folder and 4-folder the hepatic gene expression of *Cyclin D1*, respectively, while silymarin did not promote alteration (Fig. 5F).

Regarding the liver inflammation, both inflammatory biomarkers, the enzymatic activity of MPO and NAG, did not demonstrate differences among the groups (Table 3).



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	Basal	CCl_4	Semaglutide	Dulaglutide	Silymarin
CAT (nmol/min/mg protein)	288.1 ± 20.41	299.9 ± 19.57	299.9 ± 15.98	295.1 ± 26.06	261.5 ± 25.22
SOD (U SOD/mg protein)	24.5 ± 4.16	34.37 ± 4.27	39.4 ± 5.48	41.2 ± 2.18	39.54 ± 3.85

Figure 5. Hepatic oxidative stress parameters and gene expression of mice submitted to treatment protocol. **(A)** GSH levels**, (B)** GST activity and **(C)** LPO values. Table **(D)** shows CAT and SOD activity. **(E)** *Nrf2* and **(F)** *Cyclyn D1* gene expression. The results are expressed as the mean ± SEM and were analyzed by one-way ANOVA followed by Bonferroni's test. **p*<0.05; ****p*<0.001.

Table 2. Hepatic oxidative stress parameters in mice pretreated with GLP-1 analogues and challenged with CCl_4.

Pretreatment groups	GSH (μg/g tissue)	GST (mmol/min/mg protein)	LPO Hydroperoxides (nmol/mg protein)	CAT (nmol/min/mg protein)	SOD (U SOD/mg protein)
Basal	535.9 ± 79.60	13.2 ± 0.64	22.53 ± 1.28	309.5 ± 20.89	28.10 ± 8,351
CCl_4	485.7 ± 26.70	14.83 ± 1.13	29.98 ± 4.17	313.6 ± 39.52	35.77 ± 9.65
1x Dulaglutide	554.0 ± 93.74	14.37 ± 1.60	27.00 ± 2.98	334.0 ± 8.64	33.19 ± 4.18
2x Dulaglutide	612.5 ± 35.62	15.06 ± 1.31	30.40 ± 2.07	329.6 ± 25.50	44.15 ± 2.01
1x Semaglutide	565.3 ± 74.42	14.98 ± 1.56	20.81 ± 2.03	271.0 ± 9.33	47.37 ± 4.16
2x Semaglutide	398.9 ± 37.90	14.58 ± 0.69	25.11 ± 2.58	264.3 ± 69.42	53.57 ± 10.02
Silymarin	634.6 ± 102.3	15.43 ± 1.65	23.43 ± 3.39	266.7 ± 34.77	55.87 ± 10.34

The data were analyzed using descriptive statistics of one-way ANOVA and Bonferroni's test, expressed by the mean ± SEM.

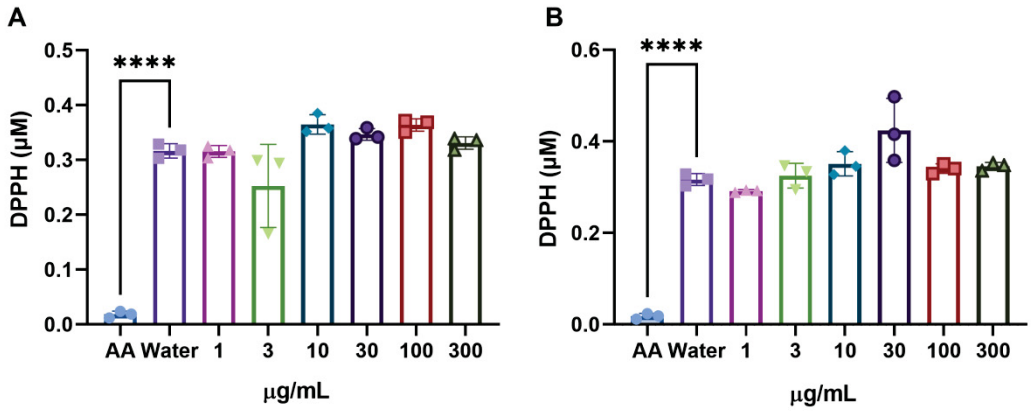

Figure 6. In vitro antioxidant capacity of dulaglutide **(A)** and semaglutide **(B)** against the DPPH radical, performed in triplicate.Ascorbic acid (AA, 50 μg/mL) was the positive control and water the negative control. The results are expressed as mean ± SEM and were analyzed using one-way ANOVA and Bonferroni's test. ****p<0.0001.

Table 3. Contents of the enzymes NAG and MPO in liver of mice challenged with CCl_4.

	NAG (μmol/min/g tissue)	MPO (μmol min^{-1}/g hepatic tissue)
Treatment groups		
Basal	6.00 ± 0.46	199.7 ± 0.46
CCl_4	5.48 ± 0.38	190.4 ± 0.38
Dulaglutide	6.74 ± 0.37	180.8 ± 0.37
Semaglutide	5.20 ± 0.25	197.0 ± 0.25
Silymarin	4.67 ± 0.30	185.2 ± 0.30
Pretreatment groups		
Basal	4.84 ± 0.17	182.5 ± 0.17
CCl_4	4.52 ± 0.47	204.3 ± 0.47
1x Dulaglutide	5.19 ± 1.12	207.6 ± 1.12
2x Dulaglutide	4.52 ± 0.61	188.7 ± 0.61
1x Semaglutide	4.08 ± 0.56	209.4 ± 0.56
2x Semaglutide	5.01 ± 0.38	199.8 ± 0.38
Silymarin	4.77 ± 0.48	170.2 ± 0.48

The results were analyzed using descriptive statistics of one-way ANOVA and Bonferroni's test, expressed by the mean ± SEM.

3.3.5 Bone histomorphometry

The tibias of mice submitted to the pretreatment protocol were analyzed and showed that two doses of semaglutide or dulaglutide improved the bone microarchitecture. Both treatments increased the BV.TV, and reduced the Tb.Sp and Ma.V.TV, while dulaglutide also increased the TV.N, as evidenced in the Figure 7.

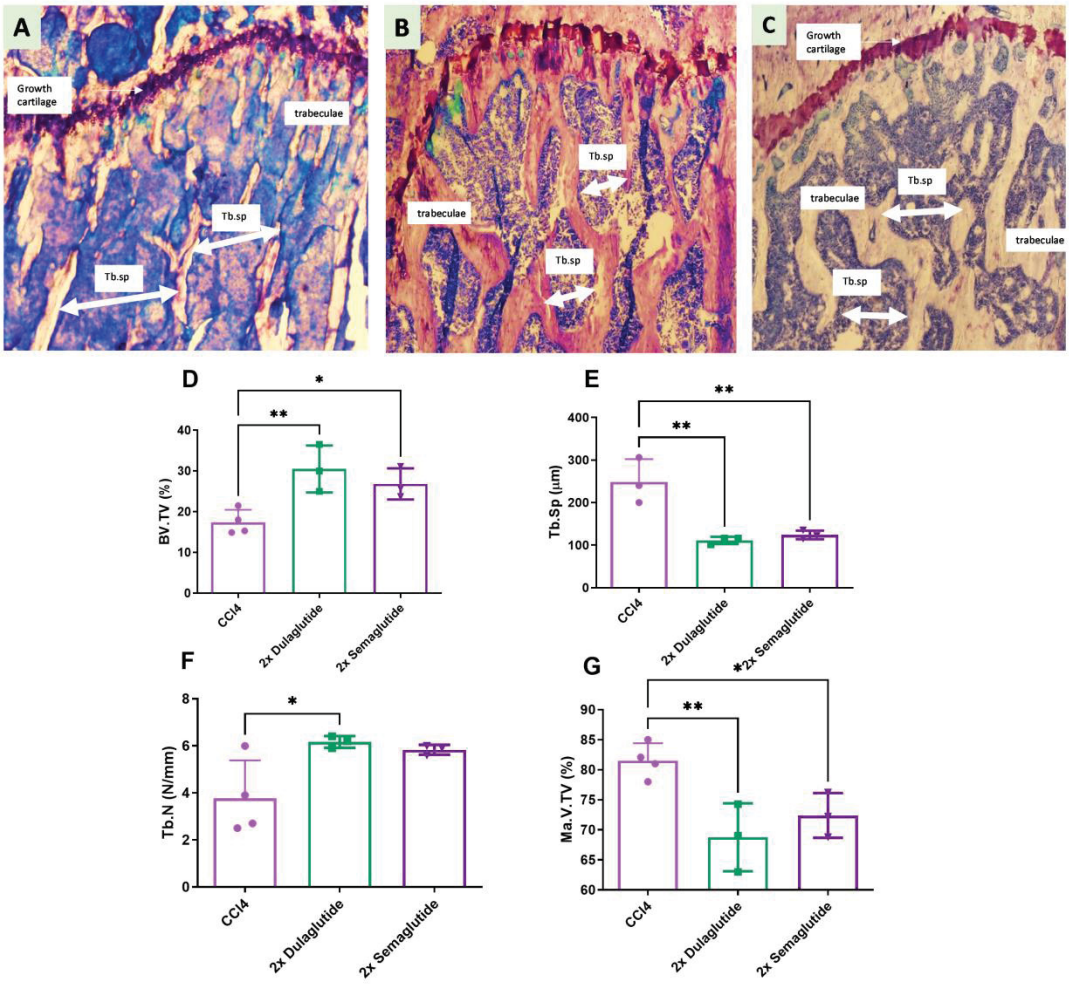

Figure 7. Trabecular bone measurements in mice submitted to pretreatment protocol. Representative histomorphometry of tibia from **(A)** CCl_4, **(B)** 2x Semaglutide and **(C)** 2x Dulaglutide groups (magnitute 4x), and the biomarkers of bone development: **(D)** bone volume (BV/TV), **(E)** trabecular separation (Tb.Sp), **(F)** number of trabeculae (Tb.N), and **(G)** bone marrow volume (Ma.V.TV). The results of D-G are expressed as mean ± SEM and were analyzed using one-way ANOVA and Bonferroni's test. **p*<0.05, ***p*<0.01.

3.3.6 Systemic toxicity evaluation

The kidneys, spleens and abdominal fat pad were collected and weighted, as macroscopic evaluation of a possible systemic toxicity. No significant differences were found among the groups for both experimental protocols regarding the organs and body weight, as showed in Supplementary Table S2.

3.4 DISCUSSION

The present work demonstrates that dulaglutide has more significant hepatoprotective effects than semaglutide upon the CCl_4-induced hepatotoxicity model, in both tested protocols, namely treatment and pretreatment. However, both dulaglutide and semaglutide showed beneficial effects on bone development.

The features of CCl_4-induced hepatotoxicity were striking as previously described (MILANI et al., 2019; JEONG et al., 2020): increased liver weight, ALT and AST plasmatic levels, reduced glycemia, increased hepatic lipoperoxidation, and centrilobular necrosis. These lesions cause cells death by several pathways, like necrosis and apoptosis, leading to fibrosis, cirrhosis, steatohepatitis and liver failure, which is very similar to DILI lesion (PIOTROWSKA-KEMPISTY, 2020). The treatments with GLP-1 analogues were able to improve some characteristics, but not equally. Dulaglutide improved the liver necrosis and the ALT and glycemia levels, while semaglutide recovered the ability to excrete biliary cholesterol. Those parameters were also improved by the positive control silymarin. This compound is known for its hepatoprotective effects and is capable of preventing liver cirrhosis or fibrosis that are induced by CCl_4 in rats (MOURELLE et al., 1989; CLICHICI et al., 2015; STOLF et al., 2018). In some aspect the GLP-1 analogues behaved like the positive control, but it is worth to mention that the dosage of silymarin (100 mg/Kg, v.o.) was higher than the dose of the GLP-1 analogues (semaglutide 0.021 mg/Kg i.p., dulaglutide 0.014 mg/Kg i.p.), the administration was also through different routes, and that the action mechanisms of these drugs are different. Apart from these effects the three drugs did not induce alteration in other organs, at least macroscopically in kidneys and spleen and in body wheight, evidencing low systemic adverse effects.

The reduction of glycemia was induced by CCl_4 in treatment protocol, while the treatment with the GLP-1 analogues did not change this parameter in treatment and pretreatment, except for the 2x Semaglutide group that presented significant reduction of glucose levels. This effect is probably a summation of actions from the hepatotoxin CCl_4 and the two doses of semaglutide, and not an isolated effect of semaglutide. The reason is even if GLP-1 analogues are hypoglicemiants in diabetes condition, they maintain the glucose levels near to normal in non-diabetic. They act only in response for high levels of glucose, targeting GLP-1 receptor that inhibits glucagon, slows gastric

emptiness and stimulate pancreatic β-cells to release insulin (DRUCKER, 2018). Despite these variations on glycemia in both protocols, the hepatic glycogen and lactate, which is a precursor of gluconeogenesis, did not vary by the challenge with CCl_4 or the treatments. These results differ from those obtained by Milani et al. (2019), in which CCl_4 reduced the hepatic glycogen and increased the liver lactate levels, while liraglutide improved both parameters to basal levels. The origin for these differences could be the amount of CCl_4, since in the present study the hepatotoxic agent was used at 1.5%, against 2% and 5% previously applied (MILANI et al., 2019). Different doses of CCl_4 can induce unlike metabolic changes and lesions in the liver. Conversely, still there is no reports about the interference of semaglutide and dulaglutide in hepatic glycogen and gluconeogenesis, what requires more investigation in future pre-clinical and clinical studies.

The toxicity of CCl_4 is related with its metabolism, attributable to trichloromethyl radicals (•CCl3), trichloromethylperoxy radical (CCl3OO•) and phosgene, reactive metabolites that are formed by hepatic metabolism via cytochrome P450 enzymes, mainly CYP2E1 (RECKNAGEL et al.,1989; MILANI et al., 2019; JEONG et al., 2020). Herein, the isolated activity of CYP2E1 was not measured, but no difference in the hepatic total CYP levels was found comparing the treated animals with the CCl_4 group. This result is not a surprise since the alteration of one or more CYP enzymes can be surpassed by the activity of another CYP (STIPP and ACCO, 2021). Furthermore, hepatic metabolism is not the main pathway for the elimination of GLP-1 analogues. Liraglutide, dulaglutide and semaglutide are endogenously metabolized into their component amino acids by general protein catabolism pathways, and no specific organ is presumed to be major route of elimination for these drugs (GANGOPADHYAY and SINGH, 2017; LILLY, 2021; NOVO NORDISK, 2018). Surprisingly, the groups that received semaglutide in the pretreatment and treatment protocols exhibited the lowest activity of total CYP among the groups. This can be a consequence of hepatic interaction between CCl_4 and semaglutide. However, this hypothesis needs investigation because as far as we know there is no report of drug interactions with semaglutide at biotransformation level.

Another enzyme involved in drug metabolism, specifically related to phase II of biotransformation, is the GST, which was increased by CCl_4 and reduced by dulaglutide in the treatment protocol, but not in the pretreatment. This enzyme is also part of the glutathione system, relevant for the redox homeostasis, which is impaired

by CCl_4 administration, generating hepatic oxidative stress (JEONG et al., 2020; CHAVES et al., 2020; MILANI et al., 2019). The pretreatment protocol did not induce alterations in oxidative stress parameters, but some changes were observed in livers from the treatment protocol: increased of GST activity and LPO level, and GSH level reduction (not statistically significant) in the CCl_4 group. These mild changes were not as expressive as previously reported in experiments that applied higher concentrations of CCl_4 (CHAVES et al., 2020; MILANI et al., 2019). Even more, the age influences the severity of hepatic lesions. CCl_4 markedly reduced the hepatic GSH levels in the weaning mice, but not in adult mice (JEONG et al., 2020), as used in our experiment. Despite the reduction on GST activity induced by dulaglutide in the treatment protocol, the analogues of GLP-1 did not modify other oxidative stress parameters (GSH, LPO, CAT and SOD) and the gene expression of *Nrf2.* Nrf2 is a transcription factor that regulates the expression of genes involved in oxidative stress response and drug detoxification (HE, RU and WEN, 2020). Together, these data indicate that theseGLP-1 analogues have little impact in the hepatic redox system, at least in the CCl_4-induced acute toxicity. This result is compatible with the absence of the in vitro antioxidant activity of semaglutide and dulaglutide, as evidenced by the DPPH test.

Interestingly, both semaglutide and dulaglutide induced the hepatic gene expression of *Cyclin D1*, a key regulator of the cell cycle. This result was also demonstrated with the GLP-1 and the analogue exendin-4 in the pancreatic beta-cell line INS-1 (FRIEDRICHSEN et al., 2006; KIM et al., 2006). Cyclin D1 is part of the cell pathways involved in liver regeneration, as demonstrated in the liver cells lineage Huh7 (GUPTA et al., 2019) and in partial hepatectomy model (ZHU et al., 2021). This effect of both GLP-1 analogues implicates in a potential mechanism underlying hepatocytes proliferation to recovery from the injury induced by the CCl_4.

The GLP-1 analogues did not influence the hepatic inflammation, since no differences were found in inflammatory cells on liver histology and in MPO and NAG activities. These enzymes are biomarkers of the neutrophil and macrophage (mononuclear cell) migration, respectively. Based on these results we thought not necessary to investigate other inflammatory parameters, as cytokine levels or inflammatory genes expression. However, anti-inflammatory effect of dulaglutide in injured muscle was previously reported, with reduction of mRNA levels of TNF-α, IL-1b, and IL-6 (NGUYEN, CHOI and JUN, 2020). Liraglutide also decreased the inflammation and initiation of fibrosis in the liver of methionine-choline deficient (MCD)

dietary model after four weeks of treatment (SOMM et al., 2021), different of the acute protocols herein performed.

Some authors have reported that GLP-1 may directly affect bone cells and regulate bone turnover, increasing osseous formation and decreasing the reabsorption (SCHIELLERUP et al., 2019; TANDAY, FLATT and IRWIN, 2021). The hepatotoxicity can decrease levels of important factors of bone formation, as vitamin D and growth hormone, as well as increase inflammatory factors, such as TNF-α, osteoprotegerin, osteopontin, osteocalcin, leptin and adiponectin, that can lead to osteoporosis (FILIP, 2018). A theory suggests that GLP-1 acts on bone marrow stromal cells, with transcription of genes to promote osteoblast differentiation and inhibiting adipocyte differentiation to ultimately favor bone formation (TANDAY, FLATT and IRWIN, 2021). Our results corroborate these data, since semaglutide and dulaglutide improved the bone microarchitecture of mice. Several parameters of bone development were found improved on tibia histomorphometry analysis, including the increase in trabecular volume and number, and reduction of trabecular space and bone marrow volume. Interestingly, these parameters ameliorate in few days and only with 2 administrations of dulaglutide and semaglutide. The present data add further information about the osseous effects of semaglutide and dulaglutide, beyond the exenetide literature (MANSUR et al., 2019).

This article extends our prior study on GLP-1 analogues (MILANI et al., 2019) and their collective results demonstrate that despite having the same action mechanism, these drugs may display different effects on hepatoprotection and metabolism. These differences can occur due pleiotropic pharmacodynamics effects or pharmacokinetics reason. Liraglutide is the true GLP-1 agonist with 9-12 h half-life (indicated once a day in diabetic patients), dulaglutide is associated with a Fc fragment of human antibodies increasing the half-life to 4.7 days (administered once weekly), and semaglutide is strongly linked to albumin, increasing the half-life to 7 days (administered once weekly) (MIHAI et al., 2015; LILLY, 2021; NOVA NORDISKI, 2018). From the present data we cannot affirm the exact reason for the different results found among the tested drugs. Further studies approaching pharmacokinetics and cell molecular pathways are still necessary.

In conclusion, a single dose of dulaglutide exerts beneficial liver effects when administered just after the hepatotoxic agent CCl_4, improving plasmatic transaminases and glycemia, the hepatic GST activity, and regulating *Cyclin D* expression. Thus, it

has potential to be an alternative treatment to DILI. Additionally, dulaglutide and semaglutide improved significantly the bone development after only two doses, demonstrating the potential value of these GLP-1 analogues in the treatment of fragility fractures in liver diseases or other conditions. These suggested uses of dulaglutide and semaglutide are in accordance with the drug repositioning concept.

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SUPPLEMENTARY MATERIAL

Table 4(S1). Sequence of primers used for quantitative real time PCR.

Gene	**Forward and reverse primers** (5'→3') (*Mus musculus*)
Cyclin D1	F: AGAAGTGCGAAGAGGAG R: GGATAGAGTTGTCAGTGTAGAT
Nrf2	F: GTGGATCCGCCAGCTACTCCCA R: TGGGGATATCCAGGGCAAGCGA
Rplp0	F: CGACCTGGAAGTCCAACTAC R: ACTTGCTGCATCTGCTTG

Table 5(S2). Kidney,spleen and body weight from mice submitted to treatment and pretreatment protocols.

	Kidney (%)	Spleen (%)	Body Weight Gain (g)
Treatment groups			
Basal	1.48 ± 0.07	0.38 ± 0.01	3,.10 ± 0,23
CCl_4	1.49 ± 0.13	0.38 ± 0.01	3.19 ± 0.30
Dulaglutide	1.37 ± 0.05	0.36 ± 0.02	3.61 ± 0.70
Semaglutide	1.48 ± 0.03	0.35 ± 0.01	3.70 ± 0.56
Silymarin	1.33 ± 0.06	0.35 ± 0.01	3.60 ± 0.52
Pretreatment groups			
Basal	0.65 ± 0.03	0.37 ± 0.03	1.09 ± 0.46
CCl_4	0.65 ± 0.02	0.34 ± 0.03	2.50 ± 1.10
1x Dulaglutide	0.64 ± 0.02	0.41 ± 0.03	3,78 ± 0.84
2x Dulaglutide	0.70 ± 0.04	0.37 ± 0.01	3.77 ± 0.69
1x Semaglutide	0.64 ± 0.03	0.37 ± 0.03	3.49 ± 1.26
2x Semaglutide	0.67 ± 0.02	0.41 ± 0.03	2.87 ± 0.76
Silymarin	0.70 ± 0.01	0.42 ± 0.02	1.02 ± 0.29

The data represent the organ weight relatively to the total body weight, and the body weight gain represents the (final weight – initial weight). The results are expressed as mean ± SEM and were analyzed using descriptive statistics of one-way ANOVA and Bonferroni's test.

4 CONSIDERAÇÕES FINAIS

Os resultados obtidos a partir desse estudo demonstram que os análogos do GLP-1estudados têm ações benéficas diferentes e pontuais, sendo os efeitos mais expressivos observados no tratamento ao invés do pré-tratamento.

A dulaglutida mostrou capacidade em reduzir significativamente os níveis de ALT e de GST e o grau de necrose hepática, enquanto a semaglutida elevou a excreção biliar de colesterol. Ambas as drogas aumentaram a expressão gênica de *Ciclina D1*, indicando possível regeneração hepática após a agressão com o CCl_4. Entretanto, nenhuma das drogas apresentou efeito antioxidante e antiinflamatório expressivo diante dos biomarcadores avaliados.

Ambas as drogas mostraram resultados significativos na análise histomorfométrica de tíbia, aumentando o volume ósseo, diminuindo a separação trabecular, aumentando o número de trabéculas e diminuindo o volume da medula óssea, em relação ao grupo CCl_4. Ainda, os fármacos testados não mostraram efeitos sistêmicos relevantes sobre os órgãos macroscopicamente avaliados, demonstrando certo grau de segurança.

Considerando os benefícios demonstrados pela dulaglutida, existe a possibilidade de utilizá-la como terapia coadjuvante para tratamento de intoxicações hepáticas. Assim como, pelos resultados histomorfométricos, que demonstraram alterações importantes no tecido ósseo mesmo em experimentos agudos, ambas as drogas podem contribuir para o tratamento de fraturas ou lesões ósseas não crônicas.

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