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JHONY WILLAMS GUSMÃO DO NASCIMENTO

TRATAMENTO COM LIRAGLUTIDA *IN VITRO* E *IN VIVO*: EFEITOS CELULARES, NA MOTILIDADE GASTROINTESTINAL E NA RESPOSTA INFLAMATÓRIA DE TECIDO EPITELIAL INTESTINAL DE RATOS

Maceió-AL
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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia – RENORBIO, Ponto Focal Alagoas, Universidade Federal de Alagoas, como requisito parcial para a obtenção do Título de Doutor em Biotecnologia, Área de Concentração: Biotecnologia em Saúde.

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"*Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes".*

(Marthin Luther King)

RESUMO

A obesidade é uma doença crônica, caracterizada por um estado de inflamação de baixo grau, que afeta a permeabilidade e a motilidade gastrointestinal, sendo a ação das citocinas nesse cenário pouco relatadas. A Liraglutida é um análogo do peptídeo semelhante ao glucagon -1 (GLP-1) que induz a perda de peso por mecanismos diversos que envolvem o trato gastrintestinal. O objetivo desse estudo é avaliar os efeitos da Liraglutida em células epiteliais intestinais *in vitro* e em parâmetros metabólicos, morfo-funcionais e inflamatórios no trato gastrintestinal de ratos obesos. **Métodos:** No ensaio *in vitro*, as células IEC-6 foram tratadas com concentrações de 0,25 a 100 μM de Liraglutida e avaliadas quanto à viabilidade celular, morte celular por apoptose e necrose, análise morfológica, reorganização do citoesqueleto de actina e ensaio de migração celular para cicatrização de feridas. No ensaio *in vivo*, ratos Wistar obesos foram distribuídos aleatoriamente para receber solução de salina, 400 ou 1.200 μg de Liraglutida/kg/dia via subcutânea por 30 dias consecutivos, uma vez um dia. Ganho de peso, eficiência alimentar, consumo calórico, motilidade gástrica, adiposidade, parâmetros histomorfométricos, murinométricos, bioquímicos e foram avaliados antes e após o tratamento. **Resultados:** Não houve alteração na viabilidade das células tratadas com Liraglutida nas concentrações de 0,25, 0,5 e 1 μM; além disso, o tratamento com a droga diminuiu a taxa de apoptose das células IEC-6 em relação ao controle. As células tratadas mostraram um citoesqueleto de actina modificado, com fibras de estresse proeminentes e diminuição da migração celular. Os efeitos da Liraglutida nos animais tratados foram dose-dependente. A dose de 1200 μg/dia/kg proporcionou menor ganho de peso, menor eficiência alimentar e menor consumo calórico, com lentificação do esvaziamento gástrico e menor amplitude das contrações gástricas. Os efeitos gastrintestinais foram acompanhados por reduções na espessura da camada muscular e na profundidade das criptas. A Liraglutida reduziu os depósitos de tecido adiposo retroperitoneal e visceral, diminuiu os níveis de TNF-α e aumentou os níveis de TGF-β1. Houve redução no colesterol total, triglicerídeos e transaminases hepáticas. **Conclusão:** A Liraglutida afetou diretamente as células intestinais, diminuindo a taxa de apoptose, a disposição do citoesqueleto de actina, reduzindo a migração celular. Em ratos, reduziu o acúmulo de gordura, melhorou os parâmetros metabólicos e minimizou a expressão da sinalização inflamatória no trato gastrointestinal.

PALAVRAS-CHAVE: Inflamação, Liraglutida, Migração Celular, Trato Gastrointestinal, Viabilidade Celular.

ABSTRACT

Obesity is a chronic disease, characterized by a state of low-grade inflammation, which affects gastrointestinal permeability and motility, and the action of cytokines in this scenario is little reported. Liraglutide is an analogue of glucagon-like peptide-1 (GLP-1) that induces weight loss by different mechanisms involving the gastrointestinal tract. The objective of this study is to evaluate the effects of Liraglutide on intestinal epithelial cells *in vitro* and on metabolic, morpho-functional and inflammatory parameters in the gastrointestinal tract of obese rats. **Methods**: In the *in vitro* assay, IEC-6 cells were treated with concentrations of 0.25 to 100 μM of Liraglutide and evaluated for cell viability, cell death by apoptosis and necrosis, morphological analysis, actin cytoskeletal reorganization and assay of cell migration for wound healing. In the *in vivo* assay, obese Wistar rats were randomly assigned to receive saline solution, 400 or 1200 μg Liraglutide/kg/day subcutaneously for 30 consecutive days, once a day. Weight gain, feed efficiency, caloric intake, gastric motility, adiposity, histomorphometric, murinometric, and biochemical parameters were evaluated before and after treatment. **Results:** There was no change in the viability of cells treated with Liraglutide at concentrations of 0.25, 0.5 and 1 μM; moreover, drug treatment decreased the rate of apoptosis of IEC-6 cells relative to control. Treated cells showed a modified actin cytoskeleton, with prominent stress fibers and decreased cell migration. The effects of Liraglutide in treated animals were dose dependent. The dose of 1200 μg/day/kg provided lower weight gain, lower feed efficiency and lower caloric intake, with slower gastric emptying and lower amplitude of gastric contractions. Gastrointestinal effects were accompanied by reductions in muscle layer thickness and crypt depth. Liraglutide reduced retroperitoneal and visceral adipose tissue deposits, decreased TNF-α levels, and increased TGF-β1 levels. There was a reduction in total cholesterol, triglycerides and liver transaminases. **Conclusion:** Liraglutide directly affected intestinal cells, decreasing the rate of apoptosis, the disposition of the actin cytoskeleton, reducing cell migration. In rats, it reduced fat accumulation, improved metabolic parameters, and minimized the expression of inflammatory signaling in the gastrointestinal tract.

KEYWORDS: Inflammation, Liraglutide, Cell Migration, Gastrointestinal Tract, Cell Viability.

SUMÁRIO

1. INTRODUÇÃO

A obesidade é uma doença crônica, complexa e multifatorial desencadeada por mecanismos que prejudicam o controle homeostático da ingestão e gasto energético resultando em acúmulo excessivo de gordura (ELLULU *et al.*, 2017, DISPIRITO *et al*., 2015; JÉQUIER & TAPPY, 1999).

Definida pela Organização Mundial de Saúde (OMS) como o acúmulo anormal ou excessivo de gordura [índice de massa corporal (IMC) ≥ 30 kg/m²] (OMS, 2020), tem sido caracterizada por disfunções metabólicas, imunológicas, estresse oxidativo, alterações mitocondriais e inflamação crônica de baixo grau (KARCZEWSKI *et al*., 2018; KAWAI; AUTIERI; SCALIA, 2021).

O gatilho da obesidade não é totalmente compreendido; entretanto, as evidências sugerem que esteja relacionado ao desequilíbrio homeostático desencadeado por um estado hiperanabólico nos adipócitos, tendo o tecido adiposo (TA) um papel importante neste processo (MCARDLE *et al*., 2013; KARCZEWSKI *et al*., 2018).

O TA possui funções que vão além da regulação da homeostase energética no organismo, pois participa da comunicação com o sistema nervoso central e com o trato gastrointestinal (TGI), sendo este um fator chave na resposta inflamatória (MCCARDLE *et al*., 2013; SPERETTA; LEITE; DUARTE, 2014). Estudos apontam que a hipertrofia dos adipócitos promove a infiltração de macrófagos e aumento da inflamação através da intensificação de citocinas pró-inflamatórias, como Fator de Necrose Tumoral-alfa (TNF-**α**) (ALVAREZ-LEITE; SOARES; TEIXEIRA, 2016; SPERETTA; LEITE; DUARTE, 2014), e das adipocinas, a exemplo do fator de crescimento de transformação Beta (TGF-β), identificado como promotor de redução do potencial adipogênico (SILVA *et al.*, 2017).

Ademais, a obesidade é caracterizada por um estado de inflamação de baixo grau agudo que leva ao comprometimento da absorção de nutrientes, pois afeta a permeabilidade e a motilidade gastrointestinal (BONA *et al.*, 2022; EMERENZIANI *et al*., 2019). A manutenção da integridade e homeostase intestinal depende do efeito de barreira do epitélio, que limita a translocação de antígenos luminais e promove uma regulação imunológica intestinal (TRONCONE et al. 2018). A barreira intestinal não é uma estrutura estática, mas é regulada por vários estímulos fisiológicos ou relacionados à medicamentos e doenças (SALVO ROMERO *et al.*, 2015). Um desequilíbrio na estrutura da barreira intestinal pode resultar em uma reação imune incontrolável no

microambiente intestinal ou permitir o crescimento desenfreado da microbiota, que leva a várias doenças, incluindo distúrbios inflamatórios intestinais e distúrbios metabólicos como diabetes e obesidade (PETERSON; ARTIS, 2014; CHELAKKOT; GHIM; RYU, 2018). Além disso, estudos apontam o papel da sinalização epitelial e citocinas na proteção epitelial contra lesões, cicatrização de feridas e tumorigênese (KAGNOFF, 2014).

Na mucosa gastrointestinal são sintetizados mais de 30 peptídeos, incluindo hormônios, secretados de acordo com os nutrientes ingeridos, que regulam os processos digestivos (MASELLI; CAMILLERI, 2021; MIRON; DUMITRASCU, 2019). Dentre os hormônios gastrintestinais mais importantes, destaque para o peptídeo similar ao glucagon 1 (GLP-1). Este hormônio regula a liberação de insulina pelo pâncreas endócrino, regula a motilidade gástrica e a secreção ácida (MASELLI; CAMILLERI, 2021). É possível que alterações na regulação da liberação desses hormônios possam afetar a homeostase energética e contribuir para a obesidade.

A Liraglutida é um análogo de GLP-1 de longa duração disponibilizada, inicialmente, para o tratamento do diabetes tipo 2. Este fármaco possui 97% de homologia sequencial ao GLP-1 humano, liga e ativa o receptor de GLP-1, potencializando a secreção de insulina dependente de glicose pelas células ß- pancreáticas (HASANZAD *et al*. 2020). Além do controle glicêmico, a Liraglutida induz a perda de peso por mecanismos diversos, incluindo atraso no esvaziamento gástrico, redução da motilidade, aumento da saciedade, aumento do gasto de energia em repouso, além de efeitos diretos sobre os centros de apetite no cérebro (ARD, 2021; BAGGIO; DRUCKER, 2007; HALAWI *et al*., 2017; WEBSTER *et al.,* 2023).

Adicionalmente às funções conhecidas na motilidade do TGI e na secreção de insulina, sugere-se que o GLP-1 atue como um fator protetor da integridade da barreira intestinal, pois exerce efeito positivo na secreção de muco, diminuindo a inflamação e protegendo a mucosa intestinal (YUSTA *et al.*, 2015). Esta ação protetora reforça os achados de estudos que não relacionam a perda de peso como sendo efeito adverso dos análogos do GLP 1 (LEAN *et al*., 2014 BURCELIN; GOURDY, 2017), ainda que efeitos citotóxicos dependentes da concentração destes análogos tenham sido reportados (MAOR *et al.,* 2021), como por exemplo inibição do crescimento de células da linhagem IEC-6 concentração dependente (TAKIZAWA *et al.,* 2022).

Neste contexto, um modelo experimental de obesidade é interessante para avaliar o efeito da Liraglutida em parâmetros metabólicos, inflamatórios e morfo-funcionais do

TGI. A aplicação de glutamato monossódico (MSG) em camundongos recém-nascidos causa lesões em várias regiões do cérebro, incluindo o núcleo arqueado do hipotálamo. Assim, na fase adulta, estes animais desenvolvem obesidade, induzida por alterações neuroendócrinas, não por hiperfagia (CAMPOS *et al.*, 2008; MACHADO *et al*., 2021)

Diversas técnicas tem sido propostas para avaliar os aspectos funcionais do TGI, contribuindo para compreender a fisiologia, o impacto de doenças ou efeitos de fármacos neste sistema (CAMILERI & LINDEN., 2016). Ademais, o monitoramento não invasivo e em tempo real da motilidade do TGI é idealmente interessante, sendo a técnica de Biosusceptometria de Corrente Alternada (BAC) uma alternativa viável, de baixo custo e validada para estudos em modelos animais (AMERICO *et al.*, 2010; QUINI *et al*., 2012).

A obesidade é frequentemente associada às alterações metabólicas; porém a relação da obesidade e de seu tratamento com o epitélio e a motilidade gastrintestinal é conflitante, sendo a ação das citocinas nesse cenário pouco relatadas. Este trabalho tem por objetivos buscar subsídios, a partir de ensaios *in vitro* e *in vivo* em modelos experimentais, para avaliar o efeito da Liraglutida em parâmetros morfológicos, metabólicos, inflamatórios e funcionais.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Avaliar os efeitos da Liraglutida em células epiteliais intestinais *in vitro* e em parâmetros metabólicos, morfo-funcionais e inflamatórios no trato gastrintestinal de ratos obesos.

2.2. OBJETIVOS ESPECÍFICOS

Ensaios *in vitro:*

- Analisar a viabilidade das células de cripta intestinais da linhagem (IEC-6) após a tratamento com Liraglutida nas concentrações de 0,25 a 100 μM;
- Quantificar apoptose e necrose nas células IEC-6;
- Analisar a morfologia e migração celular.

Ensaios *in vivo:*

- Avaliar o ganho de peso, a eficiência alimentar e o consumo calórico após o tratamento com Liraglutida nas doses de 400 e 1200 μg/kg/dia;
- Avaliar a contratilidade, o esvaziamento gástrico e a morfometria do tecido gástrico e intestinal;
- Quantificar os parâmetros murinométricos e bioquímicos;
- Determinar concentração de Fator de Necrose Tumoral- alfa (TNF- α) e Fator de Crescimento e Transformação – beta (TGF-β) no tecido duodenal;

3. REVISÃO DE LITERALTURA

3.1. O EFEITO INCRETINA E O HORMÔNIO GLP 1

Os hormônios incretinas são hormônios intestinais que amplificam a secreção de insulina em resposta à ingestão de refeições (HOLST, 2013). A glicose oral leva a um maior estímulo da secreção de insulina do que uma infusão intravenosa de glicose, mesmo quando um mesmo perfil de concentração plasmático de glicose ("isoglicemia") é alcançado. Esse fenômeno é chamado de efeito incretina e é atribuído ao fato de que a glicose oral leva à liberação dos hormônios incretinas: o peptídeo inibidor gástrico (GIP) e peptídeo-1 semelhante ao glucagon (GLP-1), de células enteroendócrinas especializadas no intestino (acopladas à absorção de glicose), enquanto a glicose intravenosa não produz este efeito. (NAUCK&MEIER, 2018). Aproximadamente 50–70% da secreção total de insulina após glicose oral, pode ser atribuída à ação das incretinas (SFAIROPOULOS *et al.,* 2018).

O GLP-1 é um peptídeo de 30 aminoácidos que exerce seus efeitos pela ligação ao receptor GLP-1 (GLP-1R), um receptor acoplado à proteína G de 463 aminoácidos, que está presente em abundância nas células alfa e beta pancreáticas, intestino, hipotálamo, células endoteliais, neurônios, pulmão, coração, rim, vasos sanguíneos e sistema nervoso periférico, sugerindo que o GLP-1 pode ter funções adicionais além da redução da glicose (RAJEEV; WILDING, 2016; LEE; JUN, 2016).

O principal estímulo para a secreção de GLP-1 é a presença de alimentos no lúmen intestinal, especialmente ricos em gordura e carboidratos. (IEPSEN; TOREKOV; HOLST, 2014). O GLP-1 é secretado pelas células endócrinas no epitélio do intestino delgado que expressam o gene do proglucagon, as chamadas células L (HOLST, 2013) e é clivado do proglucagon, expresso no intestino, pâncreas e cérebro. O processamento de proglucagon nas células L intestinais resulta na formação de glucagon, GLP-1 e GLP-2 (um fator de crescimento intestinal). A molécula GLP-1 torna-se uma molécula ativa através do efeito da proconvertase 1, e é inativada pela clivagem de dois aminoácidos em seu terminal N pela enzima, dipeptidil peptidase 4 (DPP-4) (MASELLI; CAMILLERI, 2021).

O GLP-1 é mais eficaz que o GIP no aumento da secreção de insulina e também inibe a secreção de glucagon. O GLP-1 aumenta a saciedade central e retarda o esvaziamento gástrico resultando em perda de peso (JELSING ,2012; TOMLINSON *et al.,* 2016), além de impedir a entrada rápida de glicose na circulação, fator importante

para o controle de excursões glicêmicas pós-prandiais. O papel do GLP-1 na indução da saciedade ocorre através da ativação central do GLP-1R no cérebro, bem como mecanismos periféricos (diminuição da motilidade gastrointestinal dose dependente). O potencial terapêutico desses efeitos faz com que esses agentes sejam eficazes no tratamento do diabetes tipo 2 (DM2) e obesidade. Além desses efeitos bem caracterizados, o GLP-1 também diminui níveis de triglicerídeos prandiais e ácidos graxos livres (RAJEEV; WILDING, 2016; LEE; JUN, 2016).

3.2. LIRAGLUTIDA - AGONISTA DO GLP-1

O GLP-1 endógeno tem um meia-vida curta (menos de 2 min), pois é rapidamente desativado após sua secreção pela enzima dipeptidil peptidase 4 (DPP-4) (SFAIROPOULOS *et al.,* 2018). Uma vez na corrente sanguínea, o GLP-1 é rapidamente degradado pela DPP-4, que também está presente no fígado (KRIEGER *et al.,* 2020). Agonistas do receptor GLP-1 sintéticos foram então desenvolvidos e são mais resistentes à degradação da DPP-4, prolongando a duração da atividade semelhante ao GLP-1 (EDWARDS *et al.,* 2012).

A Liraglutida é 97% homóloga ao GLP-1 humano. A adição de uma cadeia de ácido graxo na sua estrutura é responsável pela a ligação à albumina, o que resulta em uma meia-vida estendida, além de e prevenir a degradação pela DPP-4, permitindo seu uso como dose única diária (GROSSMAN *et al.,* 2009; LIN *et al.,* 2020). A Liraglutida atinge as concentrações plasmáticas máximas em 9 a 12 horas após administração subcutânea única. Mostra extensa ligação (> 98%) a proteínas plasmáticas e sua meia-vida média de eliminação é de, aproximadamente, 13 horas. Essas propriedades farmacocinéticas tornam a Liraglutida adequada para administração subcutânea uma vez ao dia (LIN *et al.,* 2020).

3.3. LIRAGLUTIDA NO TRATAMENTO DA OBESIDADE

A Liraglutida foi aprovada pela Federal Drug Administration (FDA), pela European Medicines Agency (EMA) e pela Agência Nacional de Vigilância Sanitária (ANVISA) para o tratamento de obesidade, em pacientes adultos obesos sem DM2, como adjuvante à dieta hipocalórica e prática de atividade física (TOMLINSON *et al.,* 2016; Brasil, 2023). É amplamente utilizada no Brasil e, segundo dados da Associação da Indústria Farmacêutica de Pesquisa, uma entidade setorial, sem fins lucrativos, alcançou em 2021

o 4º lugar no ranking nacional em faturamento de vendas em farmácias (INTERFARMA, 2022).

A recomendação da Liraglutida para o tratamento da obesidade teve como embasamento ensaios clínicos fase III, sobretudo os ensaios "*Liraglutide Effect and Action in Diabetes (LEAD)*" (GARBER et al., 2009; MARRE et al., 2009; NAUCK et al., 2009; ZINMAN et al., 2009; RUSSELL-JONES et al.,2009) e *"Liraglutide Satiety and Clinical Adiposity – Liraglutide Evidence in individuals with and without diabetes (SCALE)"* (WADDEN *et al.*, 2013; BLACKMAN *el al.*, 2014; PI-SUNYER *el al.*, 2015; DAVIES *et al.*, 2015). No ensaio LEAD, foram evidenciadas reduções no peso corporal entre 1,0 e 3,4 kg no tratamento com doses únicas diárias de 1,2 mg ou 1,8 mg (LIN *et al.*, 2020). Já no ensaio SCALE, foram investigadas a eficácia e segurança da Liraglutida como agente redutor de peso em homens e mulheres adultos com sobrepeso ou com obesidade. Os resultados mostraram que a administração subcutânea da Liraglutida na dose única diária de 3,0 mg resultou em perda de peso substancial ao longo de um período de 56 semanas, comparado com placebo (WADDEN *et al.*, 2013; BLACKMAN *el al.*, 2014; PI-SUNYER *el al.*, 2015; DAVIES *et al.*, 2015; BURCELIN; GOURDY, 2017; SFAIROPOULOS *et al;* 2018).

Em humanos, a dose inicial de Liraglutida é de 0,6 mg por via subcutânea diariamente com aumento da dose em 0,6 mg semanalmente, conforme tolerada, até atingir a dose máxima de 3,0 mg. O medicamento deve ser descontinuado se a perda de peso ≥4% não for alcançada após 16 semanas de dose de manutenção. Os eventos adversos mais comuns relatados são náuseas, vômitos, diarréia, hipoglicemia e constipação (SAUNDERS et al., 2016).

Em modelos experimentais de obesidade utilizando-se roedores, a administração da Liraglutida também resultou em perda de peso significativa, uma vez que foi demonstrado que a ativação das vias anorexígenas centrais contribui para a redução da ingestão energética e melhora dos parâmetros metabólicos relacionados à obesidade (RAUN *et al.*, 2007; LADENHEIM, 2015). Não há consenso referente à dosagem de Liraglutida para estudos em roedores, as quais foram escolhidas tendo como referência o peso do animal e variam de 400 μg/kg/dia ou 1200 μg/kg/dia (KNUDSEN, 2010a; ZHAO *et al.*, 2018).

Além da perda de peso, a Liraglutida promove a redução do tecido adiposo, conforme demonstrado em estudos com humanos e roedores (JENDLER *et al*., 2009; OLIVEIRA *et al*., 2022; LYU *et al*., 2022).

3.4. OBESIDADE E O TRATO GASTROINTESTINAL

O estado de inflamação de baixo grau crônico evidenciado na obesidade compromete a absorção de nutrientes, consequente ao comprometimento da permeabilidade e das funções motoras do TGI (MIRON; DUMITRASCU, 2019; BONA *et al*., 2022; EMERENZIANI *et al*., 2019).

Dentre as funções que desempenha, o TGI é responsável pelos processos digestivos, especialmente a absorção de nutrientes. Os enterócitos são células fundamentais no revestimento epitelial e são adaptados para exercer funções digestivas, metabólicas e de manutenção da integridade física da barreira. Eles também desempenham um papel no desenvolvimento de atividade imunológica, pois expressam receptores envolvidos em a resposta imune inata, atuam como células apresentadoras de antígenos e liberam várias citocinas e quimiocinas. (CHEROUTRE *et al*., 2011; SALVO ROMERO *et al.,* 2015).

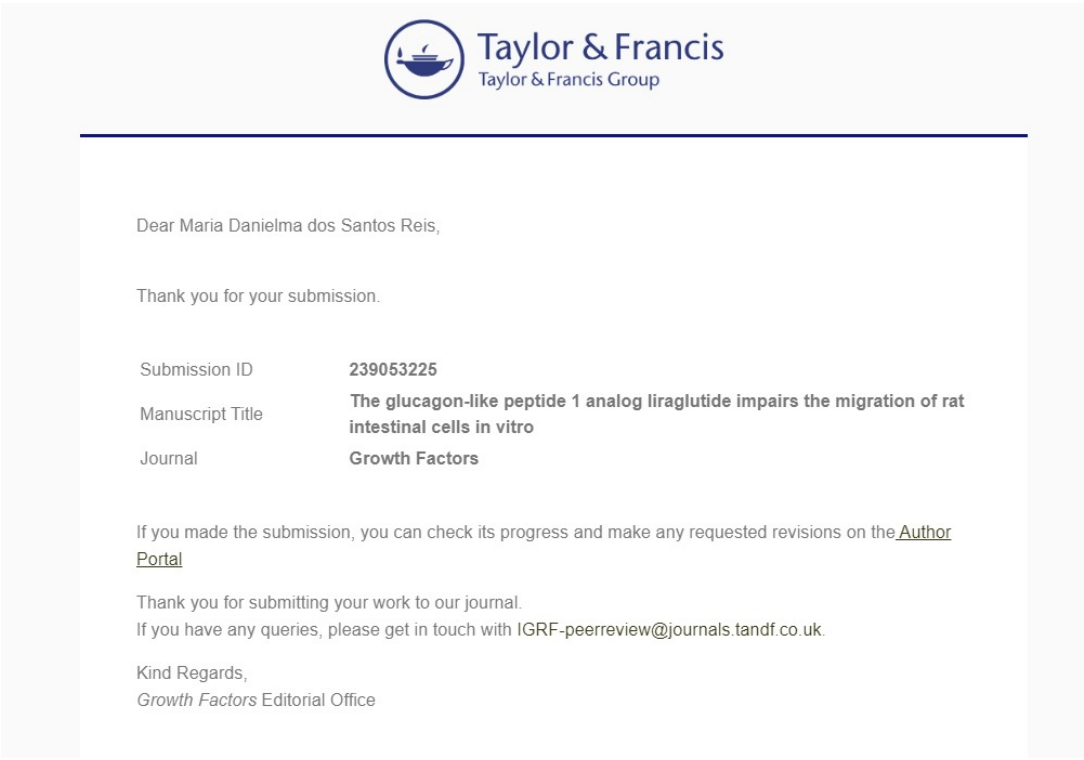
Nos estudos *in vitro,* as células da linhagem epitelial intestinal (IEC-6) são amplamente empregadas. As IEC-6, desenvolvidas e caracterizadas por Quaroni e col. (1979), são derivadas de células não diferenciadas da cripta do intestino delgado de *Rattus norvegicus*. Esta linhagem é caracterizada pela presença da borda em escova, que consiste em numerosas microvilosidades que se projetam da superfície de cada célula e aumentam a área absortiva, além da presença de pseudópodes, que se estendem pela superfície celular gerando contatos intercelulares (QUARONI et al., 1979). A linhagem IEC-6 é usada como modelo padrão-ouro em modelos *in vitro* para avaliar migração celular (MCCOMACK et al., 1992).

Sugere-se que a perda de peso associada aos agonistas do receptor de GLP-1 ocorra através de múltiplos mecanismos, incluindo atraso no esvaziamento gástrico e diminuição da motilidade (BAGGIO; DRUCKER, 2007; HALAWI *et al*., 2017, WEBSTER *et al.,* 2023). A inibição da motilidade do TGI ocorre, a princípio, pela redução de contrações fásicas, com lentificação do esvaziamento gástrico e relaxamento da musculatura do estômago proximal (MASELLI ; CAMILLERI,2021).

Neste contexto, a Biosusceptometria de Corrente Alternada (BAC) desponta como um método alternativo utilizado na avaliação da contractilidade e do trânsito gastrintestinal. Trata-se de uma técnica biomagnética, não invasiva e validada para o monitoramento em tempo real da motilidade do TGI em modelos animais (AMÉRICO et

al., 2010; QUINI et al., 2012; MARQUES et al., 2013; DALL'AGNOL et al., 2017). Os sensores BAC são constituídos por pares de bobinas de indução, sendo cada par composto por uma bobina de excitação (externa) e uma bobina de detecção (interna). Um sinal, com frequência pré definida de 10 kHz, é gerado por meio de amplificadores sensíveis à fase *Lock-in* para a bobina de excitação. O campo magnético gerado excita o material susceptível, sendo a resposta detectada como um sinal analógico contínuo, digitalizado e armazenado para análise posterior. A intensidade do sinal detectado depende da distância do sensor e da quantidade de material magnético. A ferrita em pó (Fe_2MnO_4; $80 \leq ø \leq 53\mu m$) é o principal material magnético utilizado como traçador ou marcador para as medidas com os sensores BAC. Nos ensaios em modelos animais, a ferrita é misturada com a ração comercial e peletizada, a fim de se obter uma ração magneticamente marcada. A ração marcada é ingerida pelos animais, sendo continuamente monitorada com o sensor BAC. Os movimentos gastrintestinais modulam o sinal e permitem o registro da atividade contráctil; já o deslocamento da ração ao longo dos segmentos do TGI, proporciona o monitoramento do esvaziamento gástrico e do trânsito intestinal. A BAC é uma ferramenta segura e efetiva para avaliar a motilidade do TGI, em diferentes condições, sem a necessidade de preparo prévio ou eutanásia de animais.

4. CAPÍTULO 1



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The glucagon-like peptide 1 analog liraglutide impairs the migration of rat intestinal cells *in vitro*

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Running title: Liraglutide impairs migration of intestinal cells.

ABSTRACT

Liraglutide is important in weight control and glucose regulation. However, research has shown adverse effects in patients treated with the drug, with the gastrointestinal tract being one of the affected systems. This study aimed to evaluate the *in vitro* effects of liraglutide on intestinal epithelial cells of rats (IEC-6). The cells were treated with different liraglutide concentrations and evaluated for cell viability, cell death by apoptosis and necrosis, morphology analysis, actin cytoskeleton reorganization and wound-healing cell migration assay. There was no change in the viability of cells treated with liraglutide at concentrations of 0.25, 0.5, and 1 μM; moreover, treatment with the drug decreased the rate of apoptosis of IEC-6 cells relative to the control. Also, the treated cells showed a modified actin cytoskeleton, with prominent stress fibers. Regarding cell migration, there was a decrease in the percentage of closure of the cell-free area over 24 h, relative to the untreated cells. In conclusion, the results of this study showed that liraglutide directly affects the intestinal cells by decreasing the rate of apoptosis, interfering with the disposition of the actin cytoskeleton, and reducing cell migration.

Key words: Glucagon-like peptide 1 analogs; liraglutide, cell viability; cell migration

1. INTRODUCTION

Obesity is a chronic disease that presents clinical complications associated with multiple metabolic disorders. Although poorly understood, the pathophysiology of obesity is related to hormonal, genetic, lifestyle, and dietary issues (O'neil et al. 2018).

Food intake triggers several physiological responses in the digestive system, including the release of gastrointestinal hormones from enteroendocrine cells that are involved in appetite regulation (Miron; Dumitrascu, 2019), the main ones being the incretins, glucose-dependent insulinotropic polypeptide and glucagon-like peptide 1 (GLP-1). The expression of GLP-1 receptors (GLP-1R) occurs in neurons of the myenteric plexus (Körner et al. 2007; Ladenheim, 2015; Yusuke et al. 2022) and, through them, GLP-1 acts on the physiological regulation of the gastrointestinal tract (Nauck et al. 1997). The secretion of GLP-1 provides adequate release of insulin by the endocrine pancreas, in addition to delaying the entry of chyme into the intestine through the decrease of gastric motility and acid secretion (Maselli; Camilleri, 2021). Therefore, factors that interfere with the regulation of the release of these hormones affect energy homeostasis and contribute to obesity.

In this context, drugs that target GLP-1 activation pathways are promising for treating obesity (Knudsen; Lau, 2019). Liraglutide is a long-acting GLP-1 analog that was initially made available for treating type 2 diabetes. However, due to its ability to induce weight loss, liraglutide has become the drug of choice for treating obesity, associated with diet and exercise (Ladenheim, 2015; O'neil et al. 2018). This drug has 97% sequence homology with human GLP-1 and binds to and activates GLP-1R, thus potentiating glucose-dependent insulin secretion by β-pancreatic cells. Studies in animal models have shown that liraglutide increases satiety, stimulates insulin secretion, slows gastric emptying, and inhibits duodenal motility (Hasanzad et al. 2020).

In addition to the known actions of GLP-1 on gastrointestinal motility and insulin secretion, it acts as a protective factor of intestinal barrier integrity as it has a positive effect on mucus secretion, decreasing inflammation, and protecting the intestinal mucosa (Yusta et. al. 2015). However, concentration-dependent cytotoxic effects of these analogs have been reported (Maor el al., 2021), such as inhibition of cell growth (Takizawa et al. 2022), in addition to gastrointestinal adverse effects as nausea, vomiting, diarrhea, and constipation (Saunders et al. 2016).

This study aimed to investigate the relationship between the concentration and the effects of liraglutide in intestinal epithelial cells.

2. METHOD

2.1. Cell culture

The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) with high glucose concentration (4.5 g/L) and supplemented with 10% fetal bovine serum (FBS), 4 mM of L-glutamine, 0.1 U/mL of human insulin (Invitrogen), and 0.02 ml of penicillin/streptomycin solution. When necessary, the passage of cells was performed using 0.25% trypsin-EDTA solution. They were incubated at 37°C with 5% CO_2 until being used in the experimental procedures.

2.2. Cell viability

The cells were seeded at a concentration of 10^4 cells/well in a 96-well plate. The treatment was performed on the following day using liraglutide hydrochloride (Victoza®, Novo Nordisk) at concentrations of 0.25, 0.5, 1, 25, 50, and 100 μM, diluted in DMEM medium with 2% SBF for 24 h. After the treatment, the cells were incubated with thiazolyl blue tetrazolium bromide (MTT; 5 mg/mL) for 3 h. After this period, the medium was removed and dimethylsulfoxide (DMSO) was added for cell lysis and release of formazan crystals. MTT absorbance was measured by spectrophotometry (Polaris^2 Celer Biotecnologia S.A) at a wavelength of 540 nm.

2.3. Quantification of apoptosis and necrosis

Cell apoptosis was determined by examining cell morphology and DNA degradation using acridine orange (AL, 1 mg/mL) and propidium iodide (PI, 1 mg/mL) under a fluorescence microscope. The cells were seeded at a concentration of 10^4 cells/well in 24-well plates containing round coverslips and treated with liraglutide at concentrations of 0.25, 0.5, and 1 μM diluted in DMEM 2% SBF for 24 h.

For labeling, AL and PI were diluted in 1X PBS. After treatment, the cells were washed with PBS and incubated for 2 min with the solution containing the dyes. After this period, the coverslips were mounted on glass slides and visualized under a Nikon Eclipse 50i fluorescence microscope. AL labeling was visualized using a 528-nm filter.

To visualize the cells labeled with PI, a 461-nm filter was used. The

analysis of cell apoptosis was based on cell staining and morphology (RIBBLE et al., 2005), divided into three groups: viable cells: with the nucleus stained in green; cells in apoptosis: stained green, with changes in their membrane; cells in necrosis: stained orange or reddish (Figures 1A, B and C, respectively). The cells were counted in five random fields of each well using the ImageJ software. The frequency of viable, apoptotic, and necrotic cells was calculated according to the equation:

$$\% = \frac{A}{T} \times 100$$

Where: A = number of viable /apoptotic /necrotic cells in the field; T = total number of cells per field.

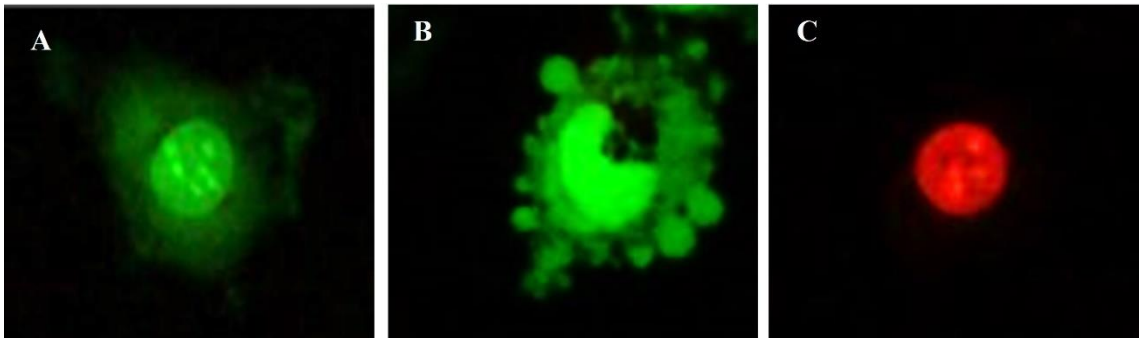

Fig. 1. Morphological aspects of IEC-6 after acridine orange (green) and propidium iodide (red) staining.

2.4. Morphological analysis

For the morphological analysis and evaluation of the F-actin cytoskeleton, IEC-6 cells were seeded at a concentration of 5×10^3 cells/well in Labtek-type 8-well plates. After 24 h, the cells were treated with liraglutide diluted in DMEM 2% FBS at concentrations of 0.25, 0.5, and 1 μM for a period of 24 h. After this period, the cells were washed with 1X PBS and fixed with 4% paraformaldehyde for 10 min. After fixation, the cells were washed again with 1X PBS and permeabilized with Triton X-100 at 0.5%. After washing with 1X PBS, the cells were labeled for F-actin with phalloidin conjugated with fluorescein isothiocyanate (FITC) for a period of 1 h in the dark. In the final step, after another PBS wash, the slides were mounted with glycerol at a ratio of 1:3 in PBS

for analysis under the Nikon Eclipse 50i fluorescence microscope. Labeling analysis was performed qualitatively, observing the morphological changes and the arrangement of the F-actin filaments.

2.5. Cell migration

The scratch wound-healing assay was used to quantify the percentage of migration of IEC-6 cells. The cells were seeded at a concentration of 8×10^4 cells/well in a 24-well plate. On the day of treatment, a cell-free area was made on the monolayer of cells using a sterile tip. Then, the cells were washed with PBS to remove cellular debris and treated with liraglutide hydrochloride at concentrations of 0.25, 0.5, and 1 μM diluted in DMEM 2% FBS. Cell migration was followed using an inverted light microscope (T1-SM Nikon) until the closure of the cell-free area, with photomicrographs taken at 0, 6, 12, and 24 h. After 24 h, the cells were fixed with 4% paraformaldehyde for 10 min, washed with PBS 1X, and stained with crystal violet (2 mg/mL) for 5 min. The cells were visualized under an inverted microscope and photomicrographs were taken using the 20X objective. The quantification of the area of cell migration was performed using the ImageJ software, in which the area (in μm^2/pixel) that remained open after the treatment period was manually delimited. The calculation of the percentage of closure of the cell-free area was performed according to Yue et al. 2010, using the following equation:

$$\% \text{ of Closure} = At0 - Ath\ At0 \times 100$$

Where: At0 is the scratched area at time zero and Ath is the scratched area at the time of analysis.

2.6. Statistical analysis

Statistical analysis was performed using the GraphPad Prism software, version 7.00 (GraphPad Prism Software, Inc.). The data obtained were evaluated by ANOVA followed by Tukey's post-test, with a significance level set at $p < 0.05$ and were expressed as mean ± standard error of the mean (SEM).

3. RESULTS

3.1 Effect of liraglutide on intestinal cell viability

It was found that after 24 h of treatment with liraglutide at concentrations of 25, 50, and 100 μM there was a significant reduction of approximately 25% and 30% in cell viability compared to the group of untreated cells (control). In contrast, concentrations of 0.25, 0.5, and 1 μM did not significantly alter cell viability (Figure 2).

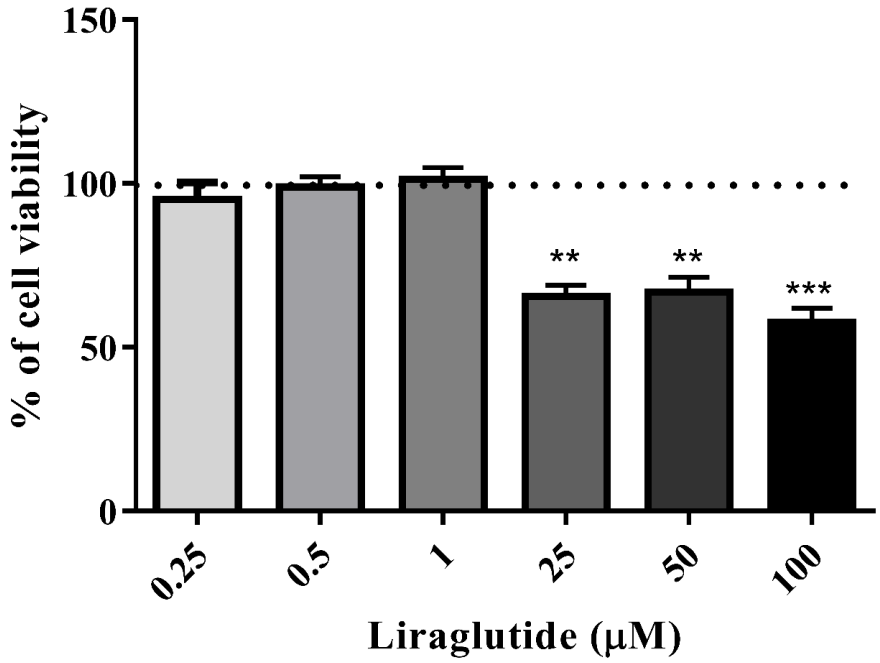

Fig 2. The percentage of viability of IEC-6 cells after treatment with liraglutide at concentrations of 0.25, 0.5, 1, 25, 50, and 100 μM for 24 h. The viability of the untreated cells was considered 100%. **$p < 0.01$ and ***$p < 0.0001$ relative to the control.

3.2 Evaluation of apoptosis and cellular necrosis

It was observed that treatment with liraglutide significantly decreased the percentage of apoptotic cells (2.5% at 0.25 μM; 1.62% at 0.5 μM; and 1.66% at 1 μM) relative to the control (9%) (Figures 3 and 4). However, there was no difference in the percentage of viable and necrotic cells (Figure 4).

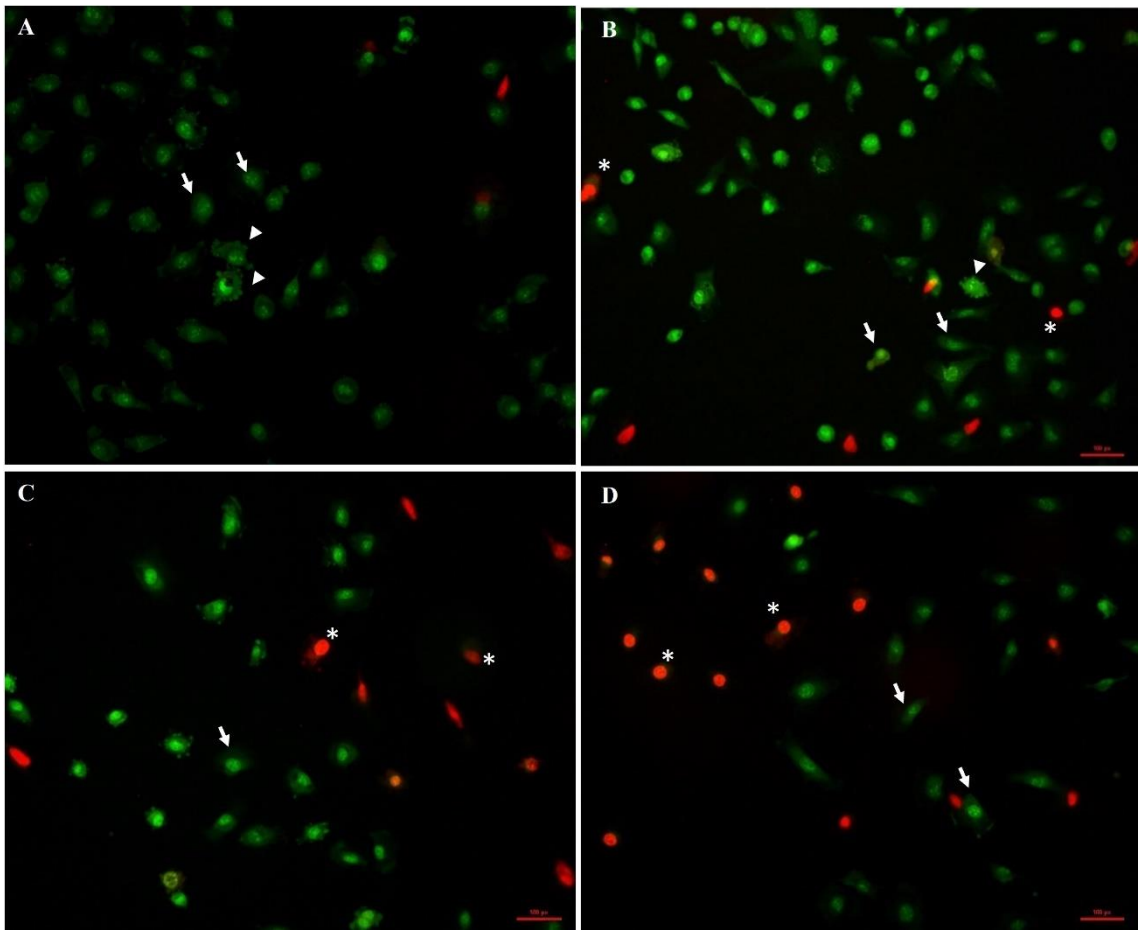

Fig. 3. The effect of liraglutide on apoptosis and necrosis of rat intestinal epithelial cells after 24 h. The photomicrographs shown in (A) cells of the control group, (B) cells treated with liraglutide at a concentration of 0.25 μM, (C) cells treated with liraglutide at a concentration of 0.5 μM, and (D) cells treated with liraglutide at a concentration of 1 μM after staining with acridine orange (green) and propidium iodide (red). The arrowheads indicate cells in apoptosis, with blistering on the membrane; the asterisks indicate cells in necrosis, and the white arrows indicate viable cells. The magnification of 200X.

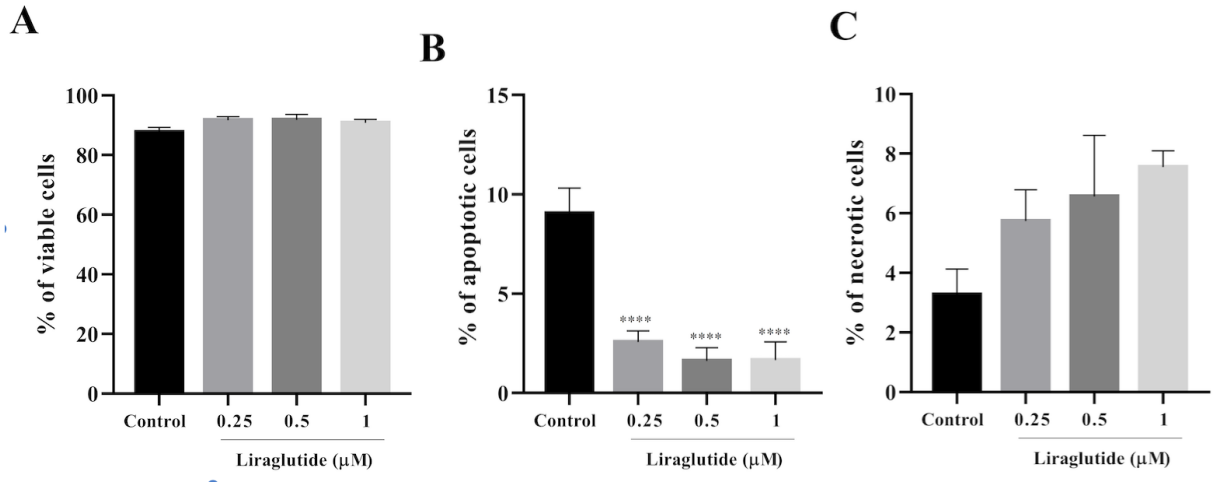

Fig. 4. Percentage of viable (A), apoptotic (B), and necrotic (C) cells after treatment with liraglutide at concentrations of 0.25, 0.5, and 1.0 μM. The bars indicate mean ± SEM. ****$p < 0.0001$ relative to the control.

3.3 Effect of liraglutide on the morphology and actin cytoskeleton of IEC-6 cells

Figure 5 shows the morphological changes exhibited by IEC-6 cells after treatment with liraglutide. The untreated IEC-6 cells exhibited typical epithelial morphology, i.e., a monolayer of cells with polygonal shape (Figure 5A). After treatment with the drug at concentrations of 0.25 μM (Figure 5B), 0.5 μM (Figure 5C), and 1.0 μM (Figure 5D), the cells showed reduced size and less cell-to-cell contact, suggesting an effect on cell junctions. Regarding the organization of the actin cytoskeleton in IEC-6 cells, in the untreated cells there were F-actin filaments, forming fibers in the cell periphery, mainly in the regions of cell–cell junctions, as shown by the increased fluorescence intensity at the site (Figure 5A). The cells treated with liraglutide at concentrations of 0.25 (Figure 5B), 0.5 (Figure 5C), and 1 μM (Figure 5D) had prominent stress fibers, with distinct rearrangements, which were concentrated both inside and in the periphery of cells, in perpendicular bundles.

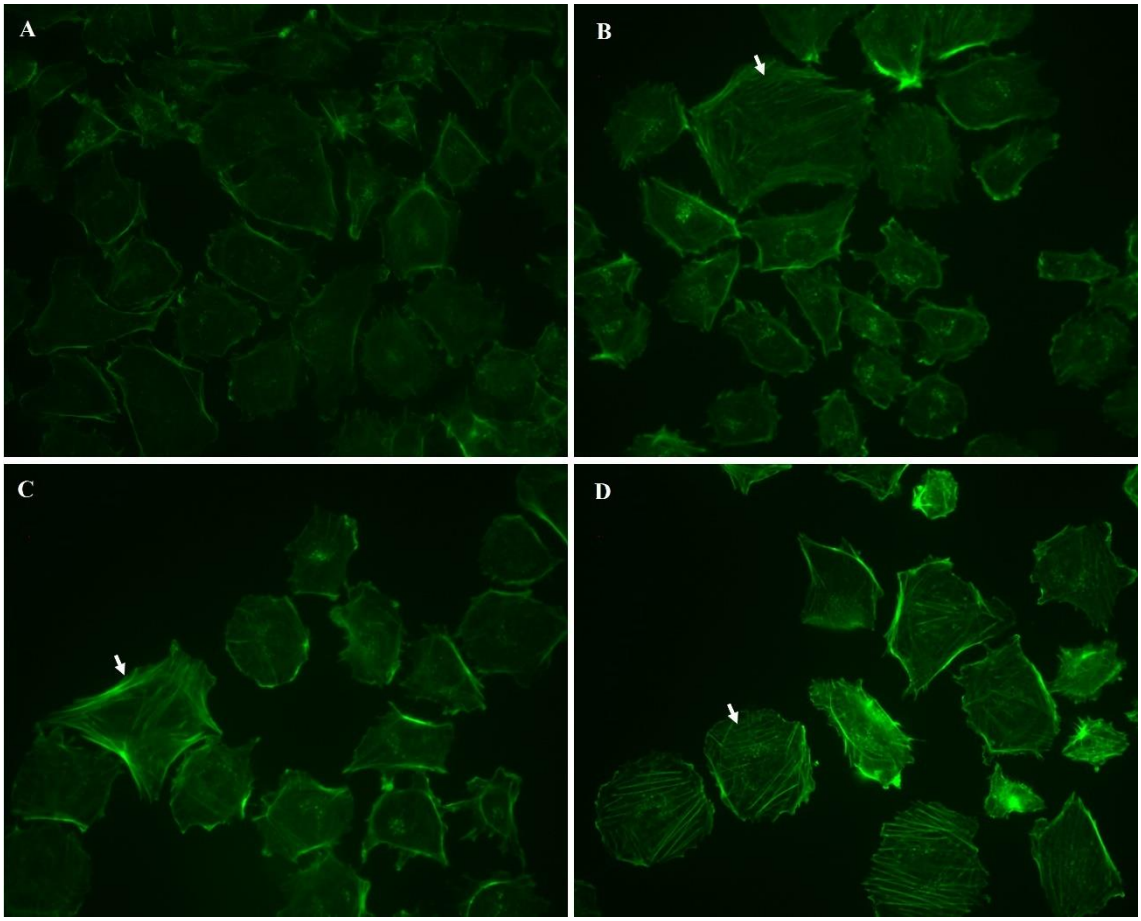

Fig. 5. The effect of liraglutide on the morphology and F-actin cytoskeleton in the IEC-6 cells. The photomicrographs shown in (A) cells of the control group, (B) cells treated with liraglutide at 0.25 μM, (C) cells treated with liraglutide at 0.5 μM, and (D) cells treated with liraglutide at 1 μM after labeling of actin filaments with phalloidin-FITC (green). The white arrows indicate the stress fibers. The magnification of 400X.

3.4 Evaluation of cell migration during treatment with liraglutide

Figure 6 illustrates the effect of different concentrations of liraglutide on cell migration before (t = 0 h), 6 and 12 h after treatment. The cells treated with liraglutide showed a lower closure rate than the control. Figure 7 shows the cells of the control group occupying the entire area injured after 24 h of treatment, whereas cell-free areas are still observed in the treated groups. There was a significant difference in the percentage of closure between the untreated and treated cells at 1 μM of liraglutide at 6 h, and 0.25, 0.5 and 1 μM over 12 h and 24 h of treatment, respectively (Figure 8).

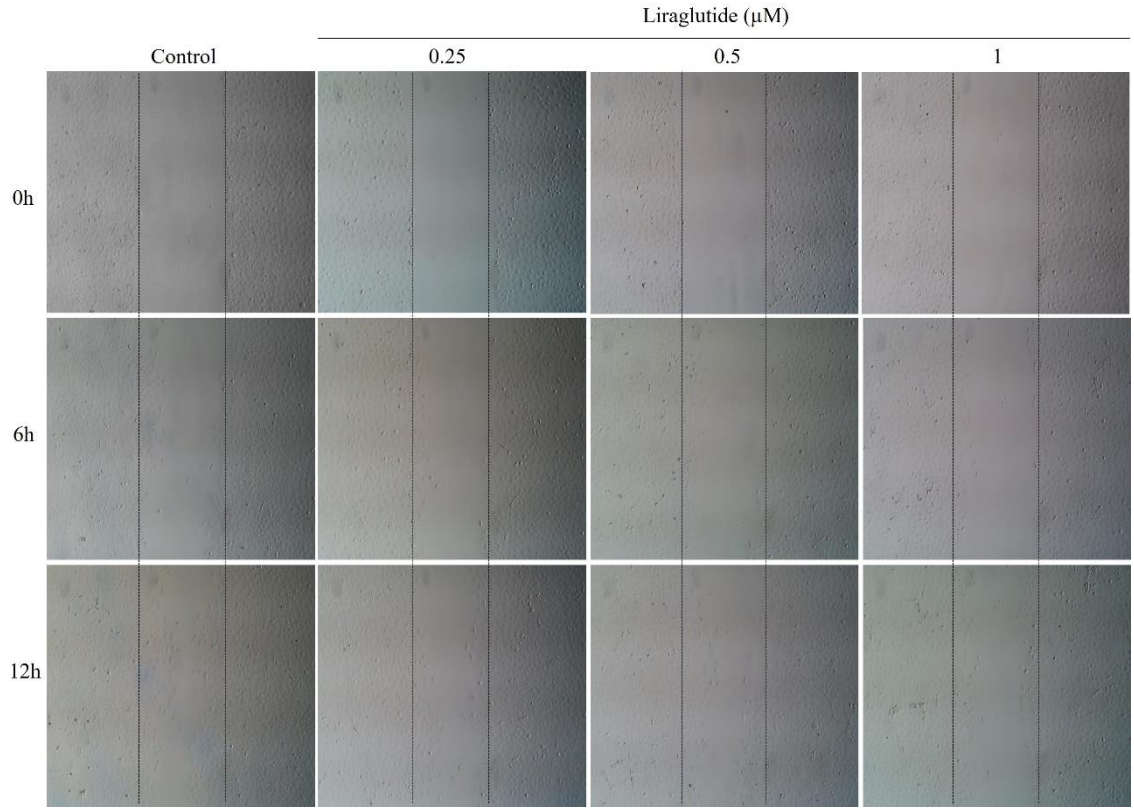

Fig. 6. The effect of liraglutide treatment at concentrations of 0.25, 0.5, and 1 μM on IEC-6 cell migration. Cell migration was followed under the inverted light microscope until the closure of the cell-free area at intervals of 0, 6, and 12 h after treatment. Dotted lines delimit de cell-free area in the 0h. The magnification of 40X.

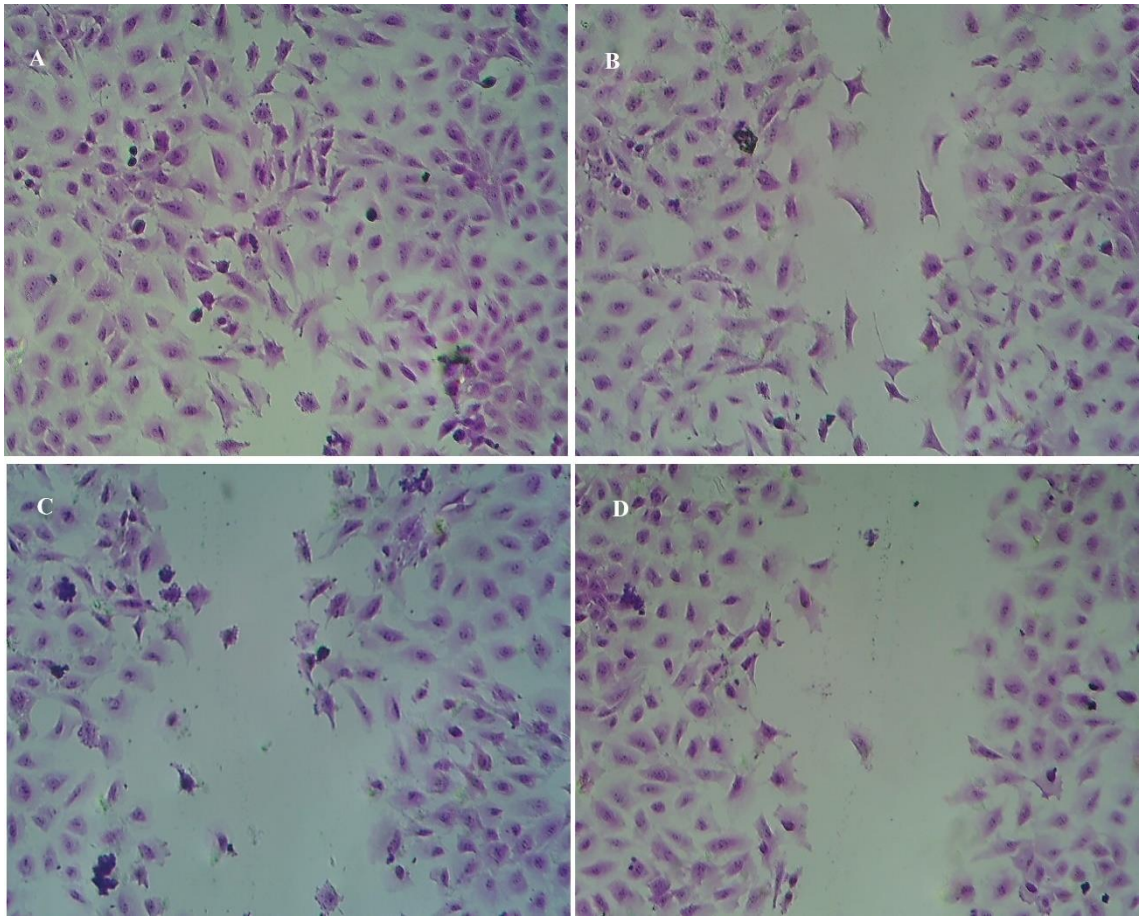

Fig. 7. The effect of liraglutide on the migration of IEC-6 cells after 24 h of treatment.-In (A) cells of the control group, (B) cells treated with liraglutide at a concentration of 0.25 μM, (C) cells treated with liraglutide at a concentration of 0.5 μM, and (D) cells treated with liraglutide at a concentration of 1 μM. Staining with 2% crystal violet. The magnification of 200X.

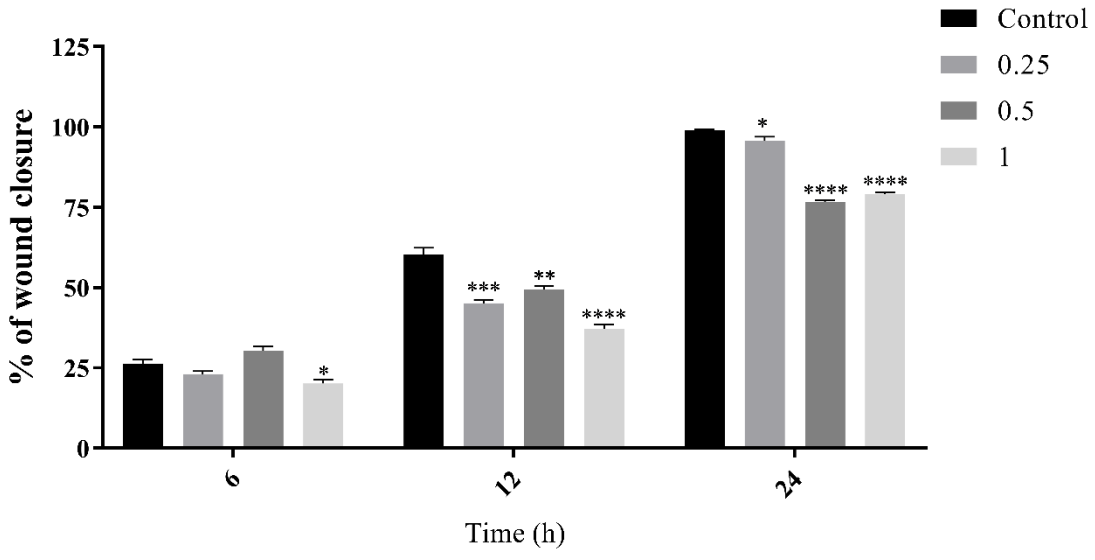

Fig. 8. The percentage of migration of IEC-6 cells treated with liraglutide. The graph shows the percentage of the closure of the cell-free area over time. The data are expressed as mean ± SEM. *p < 0.05, ** and ***p < 0.01, **** p<0.0001 compared to the control.

4. DISCUSSION

This study showed that treatment with liraglutide affected the viability, arrangement of the F-actin cytoskeleton, and migration of IEC-6 intestinal epithelial cells. The higher concentrations were responsible for the reduction of approximately 25% and 30% in cell viability, while concentrations of 0.25, 0.5, and 1 μM did not significantly alter cell viability. Similarly, a study by Yusuke et al. (2022) showed that IEC-6 cell growth was slightly concentration-dependently inhibited by liraglutide (Yusuke et al. 2022). This finding may be related to the appearance of more pronounced gastrointestinal effects at the beginning of treatment and when, subsequently, the weekly dose is changed for treating obesity (Pi-Sunyer et al. 2015).

Moreover, it was observed that treatment with liraglutide reduced the number of apoptotic cells. This finding is in line with previous studies on the role of GLP-1 in cellular apoptosis. Hui et al. (2003) found that GLP-1 inhibited apoptosis in mouse insulinoma (MIN6) cells through a signaling pathway dependent on cyclic adenosine monophosphate (cAMP) and phosphoinositide 3-kinase (PI3K). Additionally, research with freshly isolated human pancreatic islets showed that treatment with GLP-1 reduced the number of apoptotic cells through the downregulation of active caspase-3 and the upregulation of the anti-apoptotic protein BCL-2 (Farilla et al. 2003).

Similarly, a study by Challa et al. (2012) showed a decrease in apoptosis in pre-adipocytes of the 3T3-L1 strain treated with GLP-1 and liraglutide at a concentration of 0.01 μM. The authors showed that the protective effect of the substances was a result of the activation of signal-regulated extracellular kinase (ERK), protein kinase C (PKC), and serine/threonine kinase (AKT) signaling pathways, which are important in the suppression of apoptosis. According to Quoyer et al. (2010), the inhibition of apoptosis in pancreatic β cells by GLP-1 is mediated by β-arrestin 1, causing the activation of the ERK1/2 pathway. That study demonstrated that the activation of this pathway leads to the phosphorylation of the homologous Bcl-xL/Bcl-2-associated death promoter (BAD), thereby inactivating it.

Similar results have been reported in the literature, e.g., the study by Yao et al. (2021) showed the protective capacity of liraglutide against apoptosis of nucleus pulposus cells, causing decreased expression of pro-apoptosis molecules, such as BCL2-associated protein X, cell death (BAX), and caspase-3 regulator and increased BCL2 protein. It was found that exenatide inhibited the apoptosis of baby hamster kidney fibroblasts through the same antiapoptotic mechanism, i.e., by reducing the synthesis of caspase-3, caspase-8, and caspase-9 (Li et al. 2003). Thus, it is possible to enunciate the potential cellular mechanisms by which GLP-1 and liraglutide act to inhibit the apoptosis of IEC-6 cells.

Studies have shown that the permeability of intercellular connections can be altered because of contact with toxic inputs and pathological agents (Yuhan et al. 1997; Fasano; Uzzau, 1997; Philpott et al. 1998; Holmgren et al. 2003). This may occur due to a change in the arrangement of the F-actin filaments that make up the enterocyte membrane, causing pores to open at the adherent junctions and leading to increased intestinal absorption (Yuhan et al. 1997; Philpott et al. 1998). Therefore, the next step of the study was to analyze the cytoskeleton of F-actin in IEC-6 cells after treatment with liraglutide. It was observed that the treated cells showed cytoskeletal reorganization and prominent stress fibers compared with the untreated cells. Similar results were reported by Zhao et al. (2019), who showed that treatment with exenatide, a GLP-1 analog, led to increased stress fibers and morphological changes in SH-SY5Y human neuroblastoma cell lines and in rat pheochromocytoma-derived PC12 cells.

The remodeling of the cytoskeleton can be triggered by the phosphorylation of the enzyme cofilin, which is responsible for the state of polymerization and depolymerization of actin fibers. Based on this, it was found that cells treated with exenatide showed an increase in the expression of phosphorylated cofilin (inactivation), which may be an indicative of actin polymerization (Zhao et al. 2019). This reorganization of the cytoskeleton may be associated with a decrease in the migratory capacity of the cells, as will be discussed below.

As presented in the results, liraglutide significantly decreased the ability of intestinal epithelial cells of rats to migrate during the 24-h treatment. Similarly, treatment with exenatide significantly reduced the migration of SH-SY5Y cells through the inactivation of cofilin (Zhao et al. 2019). In this context, the reorganization of actin fibers may be an important factor for cell motility. The ribosomal protein S6 kinase β-1 (p70 S6K) is important for the organization of actin fibers and regulation of cell migration. According to Berven et al. (2004), p70 S6K can be found in the actin arc, where the

activators of cell motility are, and in the stress fibers that inhibit the migration process. Factors such as rapamycin may inhibit the migration of fibroblasts and epithelial cells through the inhibition of p70 S6K (Chandrasekher et al. 2001). Thus, it is possible to suggest that the decrease in cell migration induced by GLP-1 analogs is mediated by the inactivation of proteins related to the reorganization of the actin cytoskeleton.

In summary, the findings of this study show that liraglutide directly affects the intestinal epithelial cells, thereby influencing cell death processes and the arrangement of the F-actin cytoskeleton. Importantly, this drug has a negative effect on the migration of intestinal epithelial cells. However, future studies are needed to relate these actions and their mechanisms to gastrointestinal clinical outcomes.

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Declaration of Interest statement

The authors declare that they have no conflict of interest.

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5. CAPÍTULO 2

Submission Confirmation

Thank you for your submission

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Liraglutide modulates morpho-functional and inflammatory gastrointestinal responses in rats

Running Title: Liraglutide effects in obese rats model.

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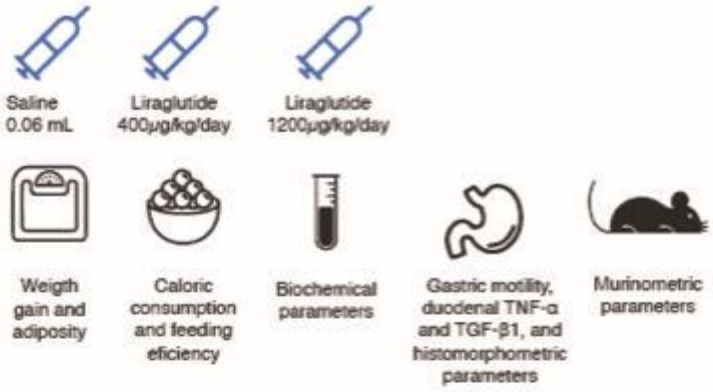
ABSTRACT

Background: Obesity impairs homeostatic control of energy and is associated with chronic low-grade inflammation. Effects of glucagon-like peptide-1, the target in the gastrointestinal tract for anti-obesity drugs such as Liraglutide, were not properly associated with inflammation markers. This study investigated the effects of Liraglutide on metabolic and gastrointestinal parameters in a rat model of obesity **Methods:** Twenty-six Wistar rats with obesity were randomly distributed to receive saline (n=10), 400 µg (n=8), or 1,200 µg of Liraglutide/kg/day (n=8), subcutaneously for 30 consecutive days, once a day. Weight gain, feeding efficiency, caloric consumption, gastric motility, adiposity, histomorphometric, murinometric, biochemical parameters, and cytokines TNF-α and TGF-β1 in duodenal tissue were measured. Data were analyzed by ANOVA followed by Bonferroni post hoc or Kruskal-Wallis test followed by Dunn's multiple comparison test. **Results:** Liraglutide-treated animals had better feeding efficiency, and higher caloric intake in a dose-dependent manner. Higher doses slowed gastric emptying and diminished the amplitude of gastric contractions. These effects were accompanied by decreases in intestinal muscle layer thickness and crypt depth. Liraglutide significantly reduced retroperitoneal and visceral white adipose tissue depots. High-dose treatment decreased levels of TNF-α and enhanced levels of TGF-β1 in duodenal tissue. Liraglutide treatment provided significant reductions in total cholesterol, triglyceride, and hepatic transaminases. **Conclusions:** Liraglutide reduced fat accumulation, improved metabolic parameters, and down-regulating levels of inflammatory signaling in duodenal tissue. Liraglutide at high doses controlled obesity-related outcomes and such effects seemed to be driven by its action on glucagon-like peptide-1 receptors in the gastrointestinal tract, slowing gastric motility.

1| STUDY DESIGN

2| METHODS



3| MAIN RESULTS

Liraglutide at high doses controlled obesity-related outcomes in rats, mostly:

- Decreasing feed efficiency and caloric intake;
- Slowing gastric emptying and decreased amplitude of gastric contractions;
- Lowering intestinal muscle layer thickness and crypt depth;
- Reducing visceral and retroperitoneal white adipose tissue depots;
- Down-regulating the expression of inflammatory signaling in the gastrointestinal tract;
- Improving several metabolic parameters.

1. INTRODUCTION

Obesity is a chronic, complex, and multifactorial disease triggered by mechanisms that impair the homeostatic control of energy intake and energy expenditure, thus resulting in excessive fat accumulation[1]. Indeed, adipocyte hypertrophy, hypoxia, and cell death occur because of increased adipose depots. The mechanism is complex and partially understood; however, evidence reveals the role of adipocytes in the secretion of high levels of pro-inflammatory markers, including TNF-α, and other chemokines[2,3]. Excess adipose mass upregulates the production of adipokines and these pro-inflammatory proteins typically act to promote metabolic alterations, particularly in the context of obesity[4].

Apart from the role played by adipokines in the pathogenesis of obesity, other factors influence weight gain, including food intake, caloric content, and nutrient absorption[5]. These processes involve the gastrointestinal (GI) tract, a neuroendocrine organ that acts to regulate hunger and satiety[6]. Hence, the stomach controls the rate at which calories reach the duodenum, triggering GI peptide secretions, particularly glucagon-like peptide-1 (GLP-1), a target for the latest anti-obesity drugs[7].

GLP-1 analogs, such as Liraglutide, were firstly developed to ameliorate glycemic control and, secondarily, to reduce body weight[8]. Liraglutide is 97% equivalent to the endogenous human GLP-1 and has a prolonged half-life of 13 hours, thus being resistant to the enzyme dipeptidyl peptidase-4 (DPP-4) degradation. Liraglutide binds to GLP-1 receptors in the peripheral and central nervous system, pancreas, GI tract, kidney, and heart[9].

The mechanism consists of the reduction of food intake since GLP-1 analogs act through both peripheral and central pathways to increase satiety and fullness. However, apart from weight loss and glycemic control, multiple physiological effects are expected since the receptors are widely expressed in many tissues and organs[10]. Among the physiological effects, the GI tract has shown itself to be potentially sensitive to GLP-1 analogs which have been recognized by their action on appetite and food intake, GI secretion, and motility[11]. This is of paramount importance considering that GI motility disturbances could be associated with chronic low-grade inflammation linked to obesity[12].

The GI tract is critically involved in the maintenance of energy homeostasis; however, there is limited data available concerning the effect of GLP-1 analogs on GI motility. *In vitro* and preclinical studies suggest GLP-1 analogs have anti-inflammatory effects acting either directly on immune cells expressing GLP-1 receptors or even indirectly, promoting glycemic control and weight loss[13].

The ability to assess GI motility accurately has been of remarkable value to understanding the physiology and pathophysiology, the impact of disease, or the effect of drugs on this parameter. Animal models provide the basis for understanding the physiology and pathophysiology of the GI tract, allowing the assessment of the effects of drugs on motor function. In addition, non-invasive and real-time monitoring of GI motility is ideally interesting. Alternating Current Biosusceptometry (ACB) has been established as an attractive method since it does not interfere with regular motor function[14].

This study aims to investigate the effects of Liraglutide on metabolism, gastrointestinal morpho-functional motility, and inflammatory gut cytokines in a rat model of obesity.

2. MATERIALS AND METHODS

2.1 Rat model of obesity

The animal experiments were performed in compliance with the ARRIVE guidelines 2.0[15] and were approved by the Ethics Committee for Animal Research from the Federal University of Mato Grosso, Brazil (protocol number 23108.064761/2021-20). All the animals had free access to water and a standard diet (Nuvilab CR-1, Quimtia®, Brazil) and were housed in a temperature-controlled room (23 ± 2 ºC) on a 12-h light/dark cycle.

A transgenerational rat model of obesity based on newborn administration of monosodium glutamate (MSG) was adopted[16]. Briefly, male newborn Wistar rats were injected subcutaneously with 4.0 mg/g body weight of MSG (Sigma-Aldrich, USA) on days 2, 4, 6, 8, and 10 after birth. All offspring were weaned at postnatal day 21 and kept under controlled conditions until postnatal day 90 when the obesity was determined by Lee Index[17] as follows:

$$LeeIndex = \frac{\sqrt[3]{Body\ Weight\ (g)}}{Nose\text{-}AnalLenght(mm)} \times \mathbf{10^4}$$

Animals with Lee Index ≥ 0.300 were classified as obese F1 parenteral generation and then they were mated with non-obese adult female rats. Upon pregnancy confirmation, rats were housed individually until the offspring were born. Lee Index was applied again and all the male rats with obesity (F2 generation) were included in the experiments.

2.2 Experimental protocol

Twenty-six male rats with obesity (aged 90 days) from F2 generation were randomly included into three experimental groups: a) control group (n = 10), injected with 0.06 mL/day saline; b) obese low-dose Liraglutide group (n = 8; LD-LG), injected with 400 µg Liraglutide /kg/day; c) obese high-dose Liraglutide group (n = 8; HD-LG), injected with 1200 µg Liraglutide/kg/day. Liraglutide (Saxenda®, Novo Nordisk, Denmark) was injected subcutaneously for 30 consecutive days, once a day, at 8:00 a.m. Doses were calculated according to previous studies that reported the effectiveness of Liraglutide in the treatment of obesity in rat models[18].

2.3 Weight gain, feed efficiency, and caloric consumption

Weight Gain Rate (WGR) is the weight gained for each animal from day 0 (before) to 30 days after the treatment, and it was calculated as follows:

$$Average\ Weight\ Gain\ Rate(AWGR,\ \%) = \frac{Final\ Weight\ (g) - Initial\ Weight\ (g)}{Initial\ Weight(g)} \times 100$$

Feed efficiency was measured by the Coefficient of Feeding Efficiency (CFE) as the ratio between the weight gain per amount of food ingested for 30 days[19]:

$$Coefficient\ of\ Feeding\ Efficiency\ (CFE) = \frac{Final\ Weight\ (g) - Initial\ Weight\ (g)}{Amount\ of\ Food\ (g)}$$

Caloric consumption was determined taking into account the centesimal composition of the standard diet (Nuvilab CR-1, Quimtia®, Brazil) composed of 7% moisture, 93% mineral matter, 26% crude protein, 3% ether extract, 44% crude fiber, 13% of carbohydrates, and 4.002 kcal/g of gross energy, as follows:

Caloric consumption (kcal/day) = Food consumption (g) x Gross energy (kcal)

2.4 Gastric emptying and contractility recordings

Non-invasive recordings of gastric motility were performed by the Alternating Current Biosusceptometry (ACB) technique, as reported in several studies elsewhere[14,20]. The ACB sensor (Br4-Science®, Brazil) is a set-up of induction coils to monitor the displacement of magnetic markers throughout the gastrointestinal tract. Magnetic signals are triggered by susceptible materials, such as manganese ferrites, as a response to a magnetic field[14]. The magnetic markers used for monitoring the gastric emptying and contractility of all experimental animals consisted of 0.40 g ferrite powder ($MgZnFe_2O_3$; Thornton Eletronica, Brazil) blended with 1.60 g laboratory standard chow.

Gastric emptying measurements were performed on day 28 after treatment. Animals fasted overnight and before the monitoring, they were fed with the magnetically labeled chow described above. Afterward, the ACB sensor was gently placed on the animal's abdominal surface for the monitoring session that lasted 1 min and was repeated every 15 min for 6 h[14]. For this protocol, there was no need for anesthesia and the animals were kept awake during all measurements. Statistical moment[21] was applied to calculate the Mean Gastric Emptying Time (MGET), which represents the amount of magnetically labeled chow that empties from the stomach as a function of time t (min).

Gastric contractility recordings were done on day 30 after treatment. The animals fasted for 12 hours and then were fed the magnetic chow. From then on, the animals were anesthetized with 75.0 mg/kg Ketamine (Cetamin®, Syntec, Brazil) and 2.5 mg/kg Acepromazine (Acepran®, Vetnil, Brazil) given intraperitoneally. With the animals on a

supine position, the ACB sensor was placed upon the abdominal surface for continuous monitoring of the gastric contractility in real-time[20]. Contractility recordings lasted 20 min and were captured at the sampling rate of 20 Hz, stored in ASCII, and digitized using a multichannel recorder (MP100 System; Biopac Inc., Santa Barbara, CA, USA).

Contractility signals were analyzed in MatLab® (R2015a, Natick, MA, USA) by visual inspection followed by *Butterworth* band-pass filters with a cutoff frequency of 0.3-1.5 Hertz (Hz), and Fast Fourier Transform (FFT). Dominant frequency was identified as the highest peak in each FFT and was expressed as cycles per minute (cpm). The amplitude (A) of gastric contraction was calculated as the ratio between the intensity of the highest frequency peak (P) and lowest frequency peak (P′) and expressed in decibels (dB) as $A=10\log_{10}(P/P')$.

2.5 Sample collection, murinometric, and serum biochemical assays

Animals were killed with anesthetic overdose (300 mg/kg ketamine and 30 mg/kg xylazine, intraperitoneally) followed by decapitation to collect whole blood and organs. Blood samples were collected and centrifuged at 3,500 rpm for 10 min at 4°C to collect serum and stored at –80°C until further analysis. In addition, the stomach, cecum, liver, heart, kidneys, retroperitoneal adipose tissue (AT), visceral AT, and epididymal AT were also collected.

The organs were immersed in saline, had excess moisture dried with paper towels, and were immediately weighed to calculate the Relative Organ Weight (ROW), as follows[19]:

$$Relative\ Organ\ Weight\ (ROW,\ \%) = \frac{Organ\ Weight\ (g)}{Animal\ Weight\ (g)} \times 100$$

The adiposity level was determined by the Adiposity Index calculated as the sum of the Retroperitoneal White Adipose Tissue (RWAT), Visceral WAT (VWAT), and

Epididymal WAT (EWAT) depot weights, and expressed as a percentage of total body weight for each animal:

$$Adiposity\ Index\ (\%) = \frac{RWAT+VWAT+EWAT\ (g)}{Final\ Weight\ (g)} \times 100$$

To evaluate the tissue cytokine profile, a duodenal sample of each animal was removed, mechanically macerated, homogenized in lysis buffer containing protease inhibitors, and centrifuged for protein extraction. The supernatant was analyzed by the flow cytometry method (FACSCelesta®, BD Biosciences, USA). Cytometric Bead Array kit (BD Biosciences, USA) was used for the dosages of the Tumor Necrosis Factor-alpha (TNF-α) and Transforming growth factor-beta 1 (TGF-β1), following the protocols recommended by the manufacturer. The data were obtained using CBA analysis software (BD Biosciences, USA) and expressed as pg/mL.

To evaluate the histomorphometry of gastric and duodenal tissues, samples were carefully collected and fixed with 10% phosphate-buffered formalin for 24 h, and then dehydrated with serial alcohol, diaphonized in xylol, and embedded in paraffin. Paraffin blocks were cut into 4-mm-thick sections and stained with hematoxylin and eosin (H&E). The images were captured using a light microscope Nikon Eclipse Si (Nikon Instruments Inc., U.S.A.) in objective x10, equipped with a 12.0 MP c (Camera Prime Cam Intervision Plus 12, Prime Life Science Corp., USA), and analyzed using ImageJ software (National Institutes of Health, USA). The thickness of the gastric muscular and mucosa, villus height, crypt depth, and the thickness of the muscular duodenal layer were analyzed.

Serum biochemical parameters were measured using routine automated laboratory methods with commercial enzymatic assays to determine total cholesterol (TC, mg/dL), high-density lipoprotein-cholesterol (HDL-C, mg/dL), non-high-density lipoprotein-cholesterol (NHDL-C, mg/dL), triglyceride (TG, mg/dL), glutamate oxalate transaminase (SGOT, U/L), and glutamate pyruvate transaminase (SGPT, U/L).

2.6 Statistical analysis

Data were expressed as mean ± standard deviation (SD). One-way ANOVA followed by Bonferroni post hoc test was used. Histomorphometric data were given as a median and min-max range. Kruskal-Wallis test followed by Dunn's multiple comparison test was

applied to analyze the differences between groups. All these analyses were performed using the statistical software GraphPad Prism 8 (GraphPad Software, Boston, MA, USA). P-values < 0.05 were considered statistically significant.

3. RESULTS

Our data showed that daily treatment with both Liraglutide doses had no effects on weight gain (Fig. 1A), but reduced feeding efficiency compared with the control (Fig. 1B). The caloric consumption was increased in rats treated with 400 µg/kg/day (Fig. 1C).

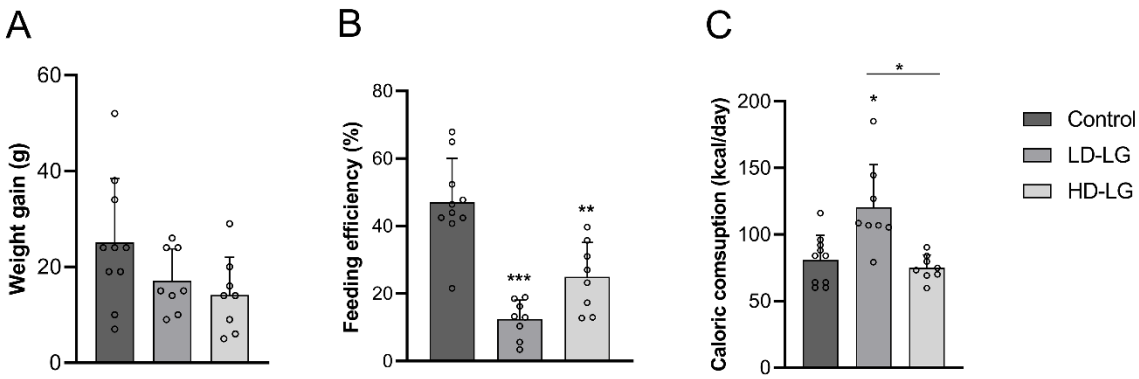


Figure 1. Effect of Liraglutide on body weight, food intake, and caloric consumption after 30 days of treatment with 400 µg/kg/day (LD-LG) or 1200 µg/kg/day (HD-LG). $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.0001$. Liraglutide-treated rats (LD-LG and HD-LG) *vs.* control or indicated group, by One-way ANOVA, followed by Bonferroni post-hoc test. Data are given as mean ± standard deviation (SD).

The results showed a significant increase in the gastric emptying time for Liraglutide-treated rats at 1200 µg/kg/day (Fig. 2A), as well as a clear decrease in the amplitude of the gastric contractions (Fig. 2B). Moreover, Liraglutide treatment changed the frequency of gastric contractions at the end of the experiments (Fig. 2C).

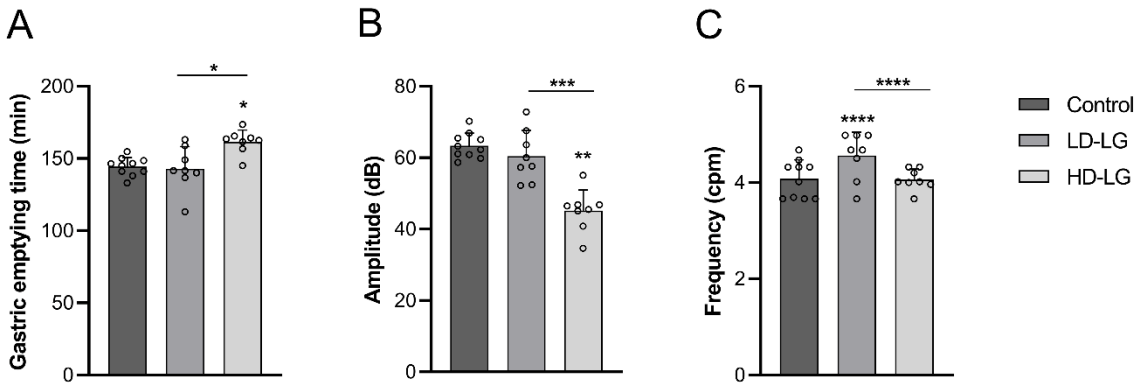


Figure 2. Effect of Liraglutide on gastric motility parameters after treatment with 400 µg/kg/day (LD-LG) or 1200 µg/kg/day (HD-LG). (A) Gastric emptying time increases after treatment; (B) Amplitude of gastric contractions was reduced after HD-LG treatment, and (C) frequency of contractions also increased during treatment. * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.05$ Liraglutide-treated rats (LD-LG and HD-LG) *vs.* control or indicated group, by One-way ANOVA followed by Bonferroni post-hoc test. Data are given as mean ± standard deviation (SD).

We also assessed the effects of a 30-day Liraglutide treatment on gastric and duodenal tissue (Table 1). The gastric muscular layer thickness was significantly decreased with Liraglutide at doses of 400 µg/kg/day and 1200 µg/kg/day. However, gastric muscular mucosa thickness was reduced with Liraglutide treatment at the highest dose. The treatment did not modify the muscular thickness and villus height of duodenal tissue, despite a significant decrease in crypt depth.

Table 1. Morphometric parameters from gastric and duodenum tissues in experimental groups 30 days after Liraglutide treatment at 400 µg/kg/day (LD LG) or 1200 µg/kg/day (HD-LG).

	Control	**LD-LG**	**HD-LG**
Stomach			

Muscular thickness (µm)	158.11 (80.72 - 267.80)	120.85* (78.92 - 244.43)	106.63* (56.46 - 213.11)
Mucosa thickness (µm)	529.36 (341.80 - 747.20)	528.80 (366.30 - 722.60)	468.22† (254.13 - 789.91)
Duodenum			
Muscular thickness (µm)	115.60 (49.83 - 202.74)	110.60 (56.72 - 163.82)	105.03 (64.50 - 148.14)
Villus height (µm)	433.70 (343.14 - 530.90)	404.78 (338.44 - 570.10)	460.83 (361.06 - 530.08)
Crypt depth (µm)	242.00 (172.42 - 293.00)	207.09* (155.80 - 261.00)	204.21* (146.21 - 278.70)

Data are given as a median and min-max range. *$p < 0.0001$ Liraglutide treated rats (LD-LG and HD-LG) *vs* vehicle *(*control), †$p < 0.0001$ Liraglutide treated rats (HD-LG) *vs* LD-LG and control by Kruskal-Wallis followed by Dunn's multiple comparisons.

Liraglutide affects body fat distribution. Daily treatment with 1200 µg/kg/day significantly decreased the adiposity index (Fig. 3A) and also reduced the retroperitoneal depot (Fig. 3B). Both doses reduced the visceral depots (Fig. 3C), and had no effects on epididymal adipose depots (Fig. 3D) of the animals.

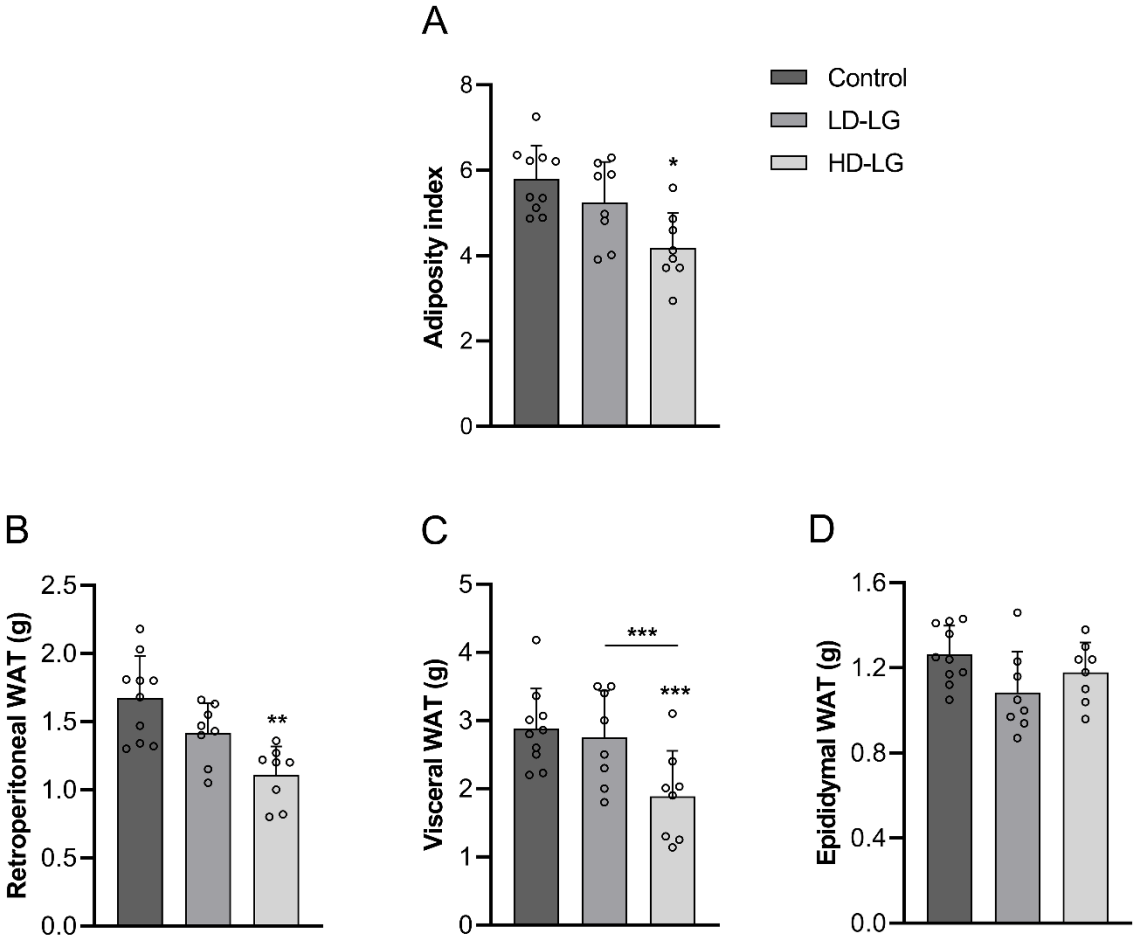


Figure 3. Effect of Liraglutide on body fat distribution after 30 days of treatment with 400 µg/kg/day (LD-LG) or 1200 µg/kg/day (HD-LG). (A) Adiposity index decreases after treatment; (B) retroperitoneal white adipose tissue (WAT), (C) visceral WAT were reduced during treatment, (D) epididymal (WAT) was not affected. $^{*}p < 0.01$, $^{**}p < 0.001$, $^{***}p < 0.05$, Liraglutide-treated rats (LD-LG and HD-LG) *vs* control or indicated group, by One-way ANOVA, followed by Bonferroni post-hoc test. Data are given as mean ± standard deviation (SD).

We found that Liraglutide at a dose of 1200 µg/kg/day (HD-LG) reduced the duodenal concentration of TNF-α (Fig. 4A) and slightly increased levels of TGF-β1 (Fig. 4B) on the duodenal tissue at the end of experiments.

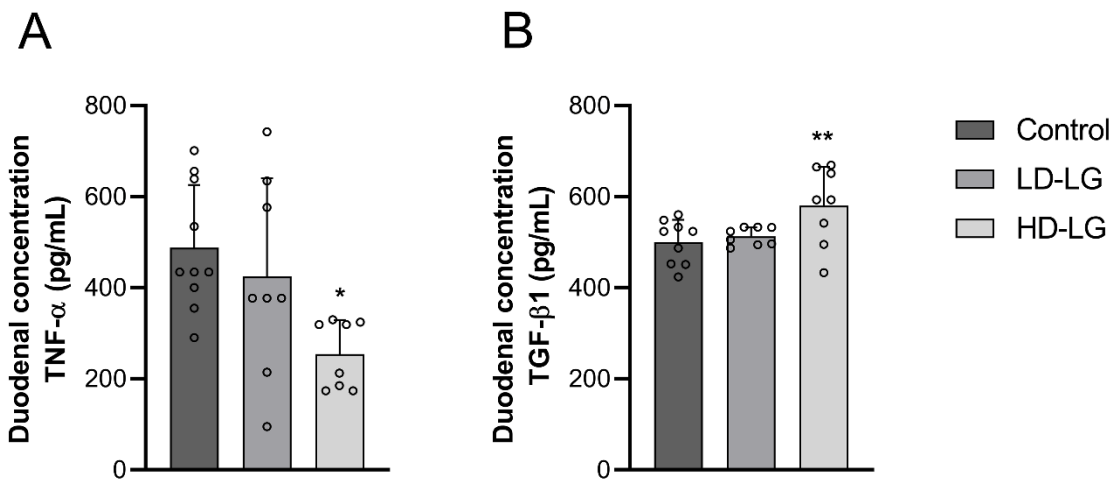


Figure 4. Effect of Liraglutide on duodenal inflammatory markers TNF-α (A) and TGF-β1 (B). * $p < 0.01$, ** $p < 0.05$, Liraglutide-treated rats (HD-LG) *vs.* control, by One-way ANOVA, followed by Bonferroni post-hoc test. Data are given as mean ± standard deviation (SD).

Liraglutide administered once daily at doses of 400 µg/kg/day (LD-LG) or 1200 µg/kg/day (HD-LG) unveils interesting effects on murinometric and biochemical parameters assessed at the end of treatment (Table 2). Liver, heart, and white adipose tissue weights, besides small intestine length, were affected by Liraglutide in a dose-dependent manner. Additionally, cholesterol (HDL-C, NHDL-C), triglycerides (TG), and hepatic enzymes were significantly affected by the treatment in comparison with the control group.

Table 2. Murinometric and serum biochemical parameters in experimental groups 30 days after saline or Liraglutide treatment at 400 µg/kg/day (LD -LG) and 1200 µg/kg/day (HD-LG).

	Control (n=10)	**LD-LG (n=8)**	**HD-LG (n=8)**
Body weight (g)			
Initial body weight	413.60 ± 15.30	430.10 ± 36.10	425.40 ± 21.70
Final body weight	453.00 ± 23.10	445.40 ± 30.92	443.40 ± 16.17
Relative Organ Weight (%)			
Stomach	0.60 ± 0.21	0.72 ± 0.17	0.65 ± 0.20
Cecum	1.23 ± 0.36	1.55 ± 0.35	1.25 ± 0.58
Liver	2.50 ± 0.12	2.26 ± 0.26	2.21 ± 0.26†
Kidney	0.58 ± 0.04	0.54 ± 0.06	0.56 ± 0.07
Heart	0.30 ± 0.04	0.25 ± 0.01§	0.28 ± 0.03
White adipose tissue	5.81 ± 0.82	5.24 ± 0.95	4.18 ± 0.82*
Serum biochemical			
TC (mg/dL)	68.90 ± 15.60	62.90 ± 9.32	62.25 ± 16.20
HDL-C (mg/dL)	47.90 ± 8.72	27.85 ± 4.05 ††	26.50 ± 6.75 ††
NHDL-C (mg/dL)	21.01 ± 7.50	37.00 ± 10.03†	35.75 ± 13.80†
TG (mg/dL)	86.63 ± 15.60	59.12 ± 16.44†††	43.62 ± 12.51†††
SGOT (U/L)	53.50 ± 7.60	29.62 ±15.11§	58.87 ± 13.15§
SGPT (U/L)	62.30 ± 9.65	51.14 ±5.66	49.62 ±10.30†

Data are given as mean ± standard deviation (SD). TC, Total cholesterol; HDL-C, high-density lipoprotein-cholesterol; NHDL-C, non-high-density lipoprotein-cholesterol; TG, triglyceride; SGOT, glutamate oxalate transaminase; SGPT, glutamate pyruvate transaminase. $^{\dagger}p < 0.05$ Liraglutide treated rats (HD-LG) *vs.* control, $^{\S}p < 0.01$, Liraglutide treated rats (LD-LG) *vs* vehicle *(*control) and HD-LG, $^{*}p < 0.001$, Liraglutide treated rats (HD-LG) *vs.* control, $^{\dagger\dagger}p < 0.0001$ Liraglutide treated rats (LD-LG and HD-LG) *vs.* control, $^{\S\S}p < 0.01$, Liraglutide treated rats (LD-LG) *vs* HD-LG and control, $^{\dagger\dagger\dagger}p < 0.001$ Liraglutide treated rats (LD-LG and HD-LG) *vs.* control by One-way ANOVA followed by Bonferroni post-hoc test.

4. DISCUSSION

Our study showed that the administration of Liraglutide once daily modulates several obesity-related parameters in a dose-dependent manner. Male rats with obesity treated with Liraglutide at 1200 µg/kg/day had decreased feed efficiency and caloric intake compared with control or low-dose groups, similar to what had been shown in previous rodent studies[22].

Additionally, gastric emptying has proven to be one of the factors that impact weight loss in response to Liraglutide treatment[23]. Our data showed that slower gastric emptying time was accomplished by a decrease in the amplitude of gastric contractions for high-dose treatment. As assessed in human studies, Liraglutide has delayed gastric emptying and diminished small intestine motility, hence leading to increased transit time[24]. Although GLP-1 analogs also have inhibitory effects on gastric emptying in rodents, the contribution of such mechanism for weight loss or food intake seems to be less effective than the activation of central anorectic pathways, thus improving obesity-related metabolic parameters[25].

Gastrointestinal motility plays an important role in the regulation of digestive processes. Circular and muscular layers act to coordinate the mixing and propelling movements of the luminal content[26]. The muscle layer thickness was decreased in Liraglutide-treated rats, which may explain the lower amplitude of gastric contractions and, consequently, the slower gastric emptying. A decrease in duodenum crypt depth may be linked to the Liraglutide's capability to modulate the level of proliferative cells in the crypt[27], possibly associated with the absorptive process that was not evaluated in this study. Hence, it is reasonable to assume that the evidence available points to a mechanism for weight loss that seems to be related to decreased appetite and feeding efficiency, in addition to slowing gastrointestinal motor functions.

Liraglutide also promotes a reduction in fat tissue in rodents[28]. Our study revealed a significantly lower adiposity index resulting from reduced retroperitoneal and visceral white adipose tissue depots in high-dose Liraglutide treatment. Visceral fat is recognized as an important factor that impacts obesity since this tissue produces inflammatory cytokines and adipokines that worsen metabolic obesity-related diseases[1]. Reduction of body fat seems to be an important advantage of Liraglutide as an anti-obesity drug, since excessive adiposity results in increased levels of free fatty acids, leading to fat storage in non-adipose tissues, such as the liver and pancreas.

Liraglutide can also significantly down-regulate the expression of inflammatory signaling mediators' pathways[2,11]. We found out that high-dose Liraglutide treatment decreases duodenal levels of TNF-α. It has been accepted that chronic low-grade systemic inflammation related to obesity worsens metabolic syndrome triggered by excessive levels of pro-inflammatory cytokines, including TNF-α, thus causing dysfunction of lipogenic and lipolytic pathways[1,2].

Conversely, intestinal levels of TGF-β1 were enhanced after treatment with high-dose Liraglutide. TGF-β1 is associated with mucosal integrity for the maintenance of intestinal epithelial homeostasis[29]. Thus, such an increase in TGF-β1 levels might be beneficial since it acts by recruiting tight junction proteins and molecules of adherence to repair the epithelial barrier and restore its function.

We also noticed that Liraglutide treatment has led to a reduction in liver weight for rats with obesity. Obesity-related nonalcoholic fatty liver (NAFLD) is a progressive and chronic disorder triggered by a partially understood and complex process that leads to an influx of free fatty acids into the liver resulting in morphological and metabolic changes in the organ. Studies suggest that weight loss associated with GLP-1 analogs ameliorates diet-induced NAFLD in rodent models[30]. Liraglutide treatment also provided significant reductions in total cholesterol, triglyceride, and hepatic transaminases, despite the level of SGOT in the high-dose group not changing which might be indicative of damage in other organs provoked by Liraglutide. There is growing evidence supporting the fact that Liraglutide exerts protective effects on hepatic and cardiovascular functions due to its anti-inflammatory and antioxidant activity independent of the changes in body weight[30].

The main limitations of our study were the lack of drug acclimation, in order to mimic the clinical practice, and the short experimental period (30 days), which did not allow for to evaluation of long-term effects on the parameters.

In summary, our study showed that Liraglutide at high doses effectively controlled obesity-related outcomes. The ability to reduce caloric intake seems to be driven by its action on GLP-1 receptors in the gastrointestinal tract, thus slowing gastric motility. Liraglutide reduced fat accumulation, improved metabolic parameters, reduced levels of TNF-α, and also enhanced levels of TGF-β1, hence ameliorating low-grade inflammation in the GI tract. These findings could not only promote its clinical use but also open new perspectives on the long-term beneficial effects of GLP-1 analog therapy for the treatment of obesity.

Authors' contribution:

J.W. G-N, L.A.C., and M.F.A. designed the study, analyzed gastric motility data, and wrote the paper; D.M.N.C., L.A.G., W.D.L.A., and M.P.R.M. collected data, analyzed murinometric and histomorphometric data, and wrote the manuscript draft. All authors had full access to all the data and reviewed and approved the final version of this manuscript.

Conflicts of interest:

J.W. G-N serves as a speaker for Novo Nordisk. The other authors have no conflicts of interest to declare.

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6. CONSIDERAÇÕES FINAIS

A Liraglutida apresenta vários benefícios, tanto do ponto de vista celular como em parâmetros morfológicos, funcionais e imunológicos. Tem efeito sobre as células epiteliais intestinais, influenciando os processos de morte celular e o arranjo do citoesqueleto da F-actina. Vale ressaltar que esta droga tem um efeito negativo na migração das células epiteliais intestinais.

A Liraglutida age, ainda, promovendo emagrecimento e diminuição da ingesta calórica, reduz acúmulo de gordura e melhora parâmetros metabólicos. Estes efeitos parecem ser impulsionados pela ação da Liraglutida nos receptores de GLP-1 no trato gastrointestinal, onde atua retardando a motilidade gástrica. A Liraglutida diminui a expressão da sinalização inflamatória, melhorando a inflamação de baixo grau no trato gastrointestinal.

Essas descobertas podem fortalecer seu uso clínico, mas também abrem novas perspectivas sobre os efeitos benéficos a longo prazo da terapia com análogos de GLP-1 para o tratamento da obesidade.

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