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Basic Study

PPAR-alpha activation and DPP-4 inhibition target gut dysbiosis and inflammation to treat NAFLD in diet-induced obese mice

Dysbiosis and NAFLD treatment in HF-fed mice

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Abstract

BACKGROUND

Obesity and comorbidities onset encompass gut dysbiosis, altered intestinal permeability, and endotoxemia. Treatments that target gut dysbiosis can cope with obesity and NAFLD management. PPAR-alpha activation and DPP-4 inhibition alleviate NAFLD, but the mechanism may involve gut microbiota modulation and merits further investigation.

AIM

To address the effects of PPAR-alpha activation and DPP-4 inhibition (isolated or combined) upon the gut-liver axis, emphasizing inflammatory pathways in NAFLD management in high-fat-fed C57BL/6J mice.

METHODS

Male C57BL/6J mice were fed a control diet (C, 10% of energy as lipids) or a high-fat diet (HFD, 50% of energy as lipids) for 12 wk, when treatments started, forming the groups: C, HF, HFA (HFD + PPAR-alpha agonist WY14643, 2.5mg/Kg body mass), HFL (HFD + DPP-4 inhibitor linagliptin, 15mg/Kg body mass), and HFC (HFD + the combination of WY14643 and linagliptin).

RESULTS

The HFD was obesogenic compared to the C diet. All treatments elicited significant body mass loss, and the HFC group showed similar body mass to the C group. All treatments tackled oral glucose intolerance and raised plasma GLP1 concentrations. These metabolic benefits restored *Bacteroidetes*/*Firmicutes* ratio, resulting in increased goblet cells per area of the large intestine and reduced LPS concentrations in treated groups. At the gene level, treated groups showed higher intestinal *Mucin 2*, *Occludin*, and *Zo-1* expression than the HFD group. The reduced endotoxemia suppressed inflammasome and macrophage gene expression in the liver of treated animals. These

observations complied with the mitigation of liver steatosis and reduced hepatic triacylglycerol, reassuring the role of the proposed treatments on NAFLD mitigation.

CONCLUSION

PPAR alpha activation and DPP-4 inhibition (isolated or associated) tackled NAFLD in diet-induced obese mice by restoration of gut-liver axis. The reestablishment of the intestinal barrier and the rescued phylogenetic gut bacteria distribution mitigated liver steatosis through anti-inflammatory signals. These results can cope with NAFLD management by providing pre-clinical evidence that drugs used to treat obesity comorbidities can help to alleviate this silent and harmful liver disease.

Key Words: Nonalcoholic fatty liver disease; High-fat diet; PPAR-alpha; DPP4-inhibitor; Dysbiosis; Inflammation

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Core Tip: Chronic HF diet intake alters the phylogenetic microbiota composition, with harmful proinflammatory signals to the liver that elicit the nonalcoholic fatty liver disease (NAFLD) in mice. Here, we treated HF-diet induced obese mice with a PPAR-alpha agonist (WY14643), a DPP-4 inhibitor (linagliptin), or their association, focusing on the gut-liver axis modulation. The treatments rescued gut dysbiosis and endotoxemia due to increased tight junction gene expression, mucin production, and numerical density of goblet cells in the intestinal crypts. Treated mice benefited from downregulated *Tlr4*, *Cd206*, and *Nlrp3*, alleviating the fatty liver by anti-inflammatory signals like increased *Il-10* and *Il-13*.

INTRODUCTION

The prevalence of obesity has tripled worldwide during the last decades, representing large budgets to public health systems due to associated comorbidities [1, 2]. Excessive dietary saturated fat intake triggers insulin resistance, white adipocyte hypertrophy, low-grade inflammation (metainflammation), brown adipose tissue dysfunction (whitening), fatty liver, and, more recently described, alteration in the composition of the gut microbiota (dysbiosis) [3, 4].

The digestive tract is populated by several microorganisms, predominantly bacteria from the *Firmicutes* and *Bacteroidetes* phyla, influenced by the quality of the diet [5]. Excessive saturated fat in the diet increases the proportion of Gram-negative bacteria that have lipopolysaccharides (LPS) in the composition of their outer wall [6]. LPS is an endotoxin that compromises the integrity of the intestinal mucosa through alterations in the structural proteins of the tight junctions (TJs), resulting in increased intestinal permeability and migration of LPS to other tissues [5, 7].

The gut-liver axis comprises the anatomical communication of these two organs *via* the portal vein [8]. Recent studies by our group have demonstrated phylogenetic changes in the gut microbiota of mice after twelve weeks of high-fructose diet feeding, with a significant increase in liver steatosis and inflammation, indicative of the progression of NAFLD (fatty liver disease associated with metabolic dysfunction) to more harmful forms of liver diseases [9, 10]. Given this scenario, the identification of metabolic pathways that rescue gut dysbiosis and mitigate the liver changes arising from dietary excess of saturated fat is pertinent, considering the high prevalence of obesity and its deleterious progression to health and the fact that there is no, so far, a treatment directed exclusively to NAFLD [11].

Peroxisome proliferator-activated receptors (PPARs) are transcription factors involved with several metabolic pathways [12]. The pharmacological activation of PPAR-alpha isoform promotes reduced body mass, increased insulin sensitivity, formation of beige adipocytes [13], in addition to a significant reduction in NAFLD by increasing mitochondrial beta-oxidation [14]. Recently, PPAR-alpha deletion promoted intestinal

dysbiosis and inflammation in mice [15]. The dipeptidyl-peptidase-4 (DPP-4) inhibitor linagliptin extends the glucagon-like peptide-1 (GLP1) time of action with beneficial brown and white adipocyte remodeling, browning induction, and M2 macrophage polarization [16], besides enhanced liver vascularization, suppressed *de novo* lipogenesis, and endoplasmic reticulum stress alleviation [17].

This study aimed to address the effects of PPAR-alpha activation and DPP-4 inhibition (isolated or combined) upon the gut-liver axis, emphasizing inflammatory pathways in NAFLD management in high-fat-fed C57BL/6J mice.

MATERIALS AND METHODS

Animals and Diet: Adult male C57BL/6J mice were group-housed (n = 5 per cage), maintained under controlled temperature (21 ± 2°C) and humidity (60 ± 10%), with free access to water and diet, in a ventilated rack with cages for mice (NexGen mouse 500, Allentown, PA, USA). The environment comprised 12/12h light-dark and air renewal cycles (15min/h). The procedures followed the recommendations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and were approved by our local Ethics Committee (Institute of Biology, CEUA number 041/2018).

Experimental Protocol: Fifty adult male C57BL/6J mice (3 mo old) from the Central Biotery of the Federal Minas Gerais University took part in this study. Initially, the animals were randomly assigned to two nutritionally different groups:

1. Control group (C) – animals that received a control diet (14% of energy as protein, 10% as fat, and 76% as carbohydrates; total energy 15 KJ/g, n = 10).
2. High-fat group (HF) – animals that received a high-fat diet (14% of energy as protein, 50% as fat and 36% as carbohydrates; total energy 21 KJ/g, n = 40).

After twelve weeks, the C group and ten animals from the HF group continued the same food scheme for additional five weeks, whereas the remaining animals from the HF group were randomly subdivided according to the treatment, making up the groups:

3. HFA – received the PPAR-alpha agonist (WY14643, Sigma-Aldrich, 3.5 mg/Kg body mass) incorporated into the HF diet (n = 10) for five weeks.

4. HFL – received the DPP-4 inhibitor (Linagliptin, Boehringer Ingelheim, 15 mg/Kg body mass) incorporated into the HF diet (n = 10) for five weeks.

5. HFC – received the combination of PPAR-alpha agonist with a DPP-4 inhibitor (same doses of the groups in monotherapy) incorporated into the HF diet (n = 10) for five weeks.

The entire experimental protocol lasted for 17 wk (12 wk of obesity induction + 5 wk of treatment). The doses of WY-14643 and linagliptin were based on previous experiments carried out by our group [4, 10]. PragSoluções (Jaú, São Paulo, Brazil) produced the experimental diets according to the recommendations of the American Institute of Nutrition (AIN 93M) [18]. All groups were treated following the order in which the groups were described.

Food / Energy Intake and Body Mass (BM): The food intake was measured daily, subtracting the amount of diet offered on the previous day by the remainder verified on the following day. Energy intake comprised the product between food consumption and the energy contained in 1g of each diet (in KJ). Animals' body masses were addressed on a digital scale once a week (BL-3200H, precision 0.01g).

Metabolic Analysis: One week before the sacrifice, the animals accomplished the Oral Glucose Tolerance Test (OGTT). Under a 6-hour fast (time 0) and, after 15, 30, 60, and 120 minutes of the orogastric gavage of a glucose solution (2g/Kg body mass), blood samples were obtained from the caudal vein. A manual glucometer (Accu-Chek, Roche, São Paulo, SP, Brazil) checked the blood glucose levels at different times. The area under the curve (AUC) addressed the oral glucose tolerance (GraphPad Prism, version 8.3 for Windows, GraphPad Software, La Jolla, CA, USA).

Sacrifice and ELISA: Mice fasted for 6 h. Under intraperitoneal anesthesia with ketamine (240 mg/kg) and xylazine (30 mg/kg), blood samples, obtained by cardiac puncture, had the plasma separated after centrifugation (712 *xg*) to perform biochemical analyzes. The liver, the large intestine (cecum), and the small intestine (jejunum and ileum) were carefully dissected, weighed, and followed the protocols for different techniques.

Enzyme-linked immunosorbent assay (ELISA) was performed to measure plasma GLP1 (multi-species GLP1 ELISA Kit Cat. #EZGLP1T-36K, Millipore, Missouri, USA), and LPS (multi-species LPS ELISA Kit Cat. #SEB526Ge-96T, Cloud-Clone Corp., Katy, USA). A semiautomatic spectrophotometer and a commercial kit (K117, Bioclin, Quibasa, Belo Horizonte, MG, Brazil) were used to measure hepatic triacylglycerol (TAG) as previously described [19].

Histology: Liver and cecum fragments, fixed in Millonig buffered formalin (pH 7.2 - 7.4), were subsequently dehydrated, diaphanized, included in Paraplast Plus (Sigma-Aldrich, St. Louis, MO, USA) and sectioned (5 µm thick) with a microtome. Slides stained with hematoxylin and eosin (liver) or Alcian Blue (Sigma Chemical Company - pH 2.5) plus Periodic Acid-Schiff (PAS, intestine - Sigma Chemical Company) were photographed using the Leica DMRBE microscope (Wetzlar, Germany) and the Infinity Lumenera digital camera (Ottawa, ON, Canada). The images were blinded analyzed with STEPanizer (www.stepanizer.com) as described below:

Hepatic stereology: Five animals per group and ten images per animal were analyzed. The volume density of liver steatosis (Vv [liver, st]) was estimated by the point-counting technique, following the formula: Vv [liver, st] = Pp [liver, st] / PT (Pp is the number of points that reach the fat droplets, PT is the total of test points). The images were analyzed with STEPanizer using a 36 points test system [20].

Gut stereology: The number of goblet cells per area (Q_A [goblet]) was estimated using the STEPanizer. All goblet cells within the test area were counted, except those touching the forbidden lines. The result was divided by the test area in mm^2.

16S rDNA PCR Amplification: The feces found in mice cecum were used to extract microbial DNA using the kit "QIAamp Fast DNA stool mini kit" (Qiagen, Düsseldorf, Germany), according to the manufacturer's instructions. DNA quantification, purity, and concentration were addressed using Qubit (Life Technologies, Carlsbad, California, US) and horizontal electrophoresis (1% agarose gel). Real-time quantitative PCR assays were used for the relative quantification of specific phyla of microorganisms (*Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria*) in fecal microbiota from mice guts by detecting 16S rRNA genes. To the relative quantification, the abundance of different phyla was normalized by ΔΔCt of the total bacteria amount in the samples [21]. The indicators used are described in Table 1.

Real-time reverse transcriptase-polymerase chain reaction (RT-qPCR)

Total RNA was extracted from 50 mg of liver and 70 mg of the small intestine (jejunum and ileum), using Trizol reagent (Invitrogen, CA, USA). Afterward, the addition of 200µL of chloroform was followed by centrifugation (1200 *g* for 10 min at 4°C for liver samples or 12,000 g for 15 min at 4°C for intestine samples). The RNA extract portion was separated, and 500µL of isopropanol was added for 10 min (liver samples) or 15 min (intestine samples) for RNA to precipitate. Then, the samples were centrifuged (1200 *g* for 10 min at 4°C for liver samples and 12,000 *g* for 10 min at 4° for intestine samples). Isopropanol was removed, the pellet formed was resuspended with 500µL of 75% ethanol for liver samples or 70% ethanol (ice-cold) for intestine samples and then centrifuged (1200 *g* for 5 min at 4°C for liver samples and 10,000 *g* for 5 min at 4°C for intestine samples).

Ethanol was removed and the pellet resuspended in 20µL (liver)/50µL (intestine) of deionized water (MilliQ). The samples were submitted to a dry bath (50°C for 5 min) and quantified in Nanovue equipment (GE Life Sciences). For RNA transcription into complementary DNA (cDNA), 1.0µg RNA was treated with DNAse I (Invitrogen, CA, USA). First-strand cDNA synthesis was performed using Oligo (dT) primers for reverse

transcriptase mRNA and Superscript III (both from Invitrogen). qPCR was performed using a CFX96 recycler (Bio-Rad, Hercules, CA, USA) and the SYBRGreen mix (Invitrogen, Carlsbad, CA, USA). *Beta-actin* was used to correct the expression of the target genes in liver samples and *Gapdh* for intestine samples. Primer sequences are found in Table 2 (liver) and 3 (intestine). All gene symbols are italicized (the first letter capitalized) and protein symbols in uppercase [22].

Data Analysis: Sample size calculation considered that, in metabolic and molecular biology analyses, if something increases or decreases in five replicates, the probability of occurrence is $P = (1/2)^5 = 0.05$. So, a minimum of 5 replicates was adopted for the analyses [23]. The data are shown as mean and standard deviation (SD). During the first 12 wk, the statistical analysis comprised the student's T-test and Welch's correction. At the treatment phase, data were analyzed using the Brown-Forsythe and Welch one-way ANOVA with the Dunnett T3 post hoc test [24]. The *P-value*<0.05 was considered significant (GraphPad Prism version 8.3 for Windows, GraphPad Software, La Jolla, CA, USA).

RESULTS

Treatment reduced body mass (BM) without altering energy intake

The animals from C and HF groups had equal BM at baseline. All animals tolerated well the diets and treatments. The protocol was maintained as previously stated. In the 13th week, the HF group had overweight compared to the C group (+30%), which lasted until the end of the experiment. Figure 1A depicts these results. Although the food intake did not differ between the groups (Figure 1B), the energy intake in the HF-fed groups was higher than in the C group (Figure 1C).

All proposed treatments rescued glucose tolerance and raised GLP1 concentrations

Figure 2A shows the OGTT curve, in which the HF group showed a significant increase in fasting glucose (T0) compared to the C group. This difference remained until the end

of the test (T120). The C and the treated groups rescued the baseline blood glucose levels in the other evaluation times (T30, T60, and T120). On the other hand, the HF group could not reach baseline glucose levels, indicating a delay after glucose overload, implying oral glucose intolerance, confirmed by the higher AUC for OGTT in the HF group than in the C group (+23%). In contrast, all treated groups showed lower AUC than the HF group, indicating the oral glucose intolerance alleviation.

Plasma GLP1 concentrations diminished in the HF group compared to the C group (Figure 2C). As expected, treatment with linagliptin enhanced GLP1 concentrations in the HFL group (+13%), which also happened in the HFC group and after the single treatment with the PPAR-alpha agonist in the HFA group compared to the HF group (Figure 2D).

The treatments recovered the microbiota composition and reversed endotoxemia in HF-fed mice

The 16S rRNA genes of cecal gut bacteria amplification were measured at the end of the experiment to evaluate the microbiota composition. The HF group had *Firmicutes* phylum increase coupled with a decrease in the *Proteobacteria* and *Bacteroidetes* phyla compared to the C group, as shown in Figure 3A. However, all treatments reversed these phylogenetic alterations in treated groups. The treatments restored the amount of *Bacteroidetes*, resembling the C group, and caused a significant decrease in *Proteobacteria*, which can play a decisive role in the beneficial effects observed by the proposed treatments. Changes in microbiota composition in the HF group triggered increased LPS concentrations (+10%, Figure 3B), while the HFL and HFC groups showed significantly reduced plasma LPS concentrations (-11%, for HFL vs. HF, and -12%, for HFC vs. HF).

The hepatic mRNA expression of both *Lbp* (Figure 3C) and *Tlr4* (Figure 3D) genes increased in the HF group compared to the C group, while the treated groups significantly reduced their expression.

DPP-4 inhibitor and PPAR-alpha agonist improved the intestinal barrier structure and protection

The HF group had an 80% decrease in intestinal *Mucin2* gene expression about the C group. Conversely, the HFA, HFL, and HFC groups showed a significant increase in *Mucin2* expression (+439%, for HFA vs. HF; +345%, for HFL vs. HF; +670%, for HFC vs. HF, Figure 4A).

In agreement with the previous result, the HF group also had reduced intestinal *Occludin* (-78%) and *Zo-1* (-38%) expression compared to the C group. On the other hand, the HFA showed a 238% increase in *Occludin* expression, whereas the HFL and HFC groups had a >500% increase in expression of this gene (Figure 4B). Regarding the *Zo-1* gene expression, all treated groups had a significant increase (+166% for HFA vs. HF; +397% for HFL vs. HF; and +102% for HFC vs. HF, Figure 4C).

The high intake of saturated fat altered the histochemical pattern of the intestinal mucosal cells, revealed by the reaction with Alcian Blue and PAS. Figure 4D shows decreased mucus in the HF group, while the treatments elicited an increase in mucus production in the apical region of the crypts, followed by an increased presence of goblet cells (mucus-producing cells). Gut stereology confirmed these observations with the results of Q_A [goblet] that showed a reduction in the HF group compared to the C group (-44%), while the HFA (+68%), HFL (+47%), and HFC (+56%) groups showed an increase in the number of goblet cells per tissue area (Figure 4E).

DPP-4 inhibitor and PPAR-alpha activation mitigated liver steatosis

HF-fed mice exhibited noticeable microvesicular liver steatosis, while mice from all treated groups showed liver steatosis mitigation, with the liver parenchyma resembling the C group (Figure 5A).

Following these histological findings, the Vv [liver, st] in the HF group was higher than in the C group (+38%, Figure 5B). All treated groups drastic reductions in liver steatosis (-72% for HFA vs. HF; -50% for HFL vs. HF; and -77% for HFC vs. HF).

In compliance with the stereological findings, the hepatic TAG levels increased in the HF group (+38%). Conversely, the treated groups showed lower hepatic TAG concentrations than the HF group (-11% for HFA; -16% for HFL; and -13% for HFC, Figure 5C).

DPP-4 inhibition and PPAR-alpha activation attenuated macrophage activation and reduced liver inflammation in HF-fed mice

The HF group showed higher *Cd206* expression, a specific marker for macrophages, than the C group (+103%, Figure 6A). In contrast, the treated groups showed reduced *Cd206* expression (-67% for HFA vs. HF; -65% for HFL vs. HF; and -74% for HFC vs. HF).

Along with the *Cd206* results, the HF diet significantly reduced the *Il-10* expression compared to the C group (-54%), whereas the combined treatment yielded an 80% decrease in HFC *IL-10* expression compared to HF (Figure 6B). The *Nlrp3* behaved like the *Cd206* gene, with higher expression in the HF group than in the C group (+128%), and expressive reduction in the HFA (-72%), HFL (-88%), and HFC (-85%) compared to the HF group (Figure 6C).

Regarding the *Il-13* cytokine, the HF group exhibited a significant increase compared to the C group (+290%). Only the HFA and HFC treatments were able to reduce the expression of this cytokine in comparison with the HF group (-39% and -67%).

DISCUSSION

The excessive saturated fat intake caused overweight, oral glucose intolerance, gut dysbiosis, morphological and functional intestinal barrier alterations. Hence, HF animals had endotoxemia with proinflammatory signals to the liver, causing substantial NAFLD. The single treatment with the PPAR-alpha agonist, DPP-4 inhibitor, and their combination yielded beneficial results: rescuing of body mass, oral glucose tolerance, gut goblet cells numerical density per area, TJs gene expression, phylogenetic

microbiota distribution, and LPS concentrations. Thus, treated animals showed liver steatosis and inflammation mitigation due to gut dysbiosis and endotoxemia control.

The chronic ingestion of a diet with high content of saturated fats increases the body mass and impairs glucose and lipid metabolism [25]. In agreement, the HF-fed mice had overweight and oral glucose intolerance, with difficulty in rescuing the glycemic levels during the OGTT compared to the C group. These metabolic alterations were paralleled by gut dysbiosis in the HF group, confirming that dietary patterns interfere with the gut-liver axis, favoring the NAFLD pathogenesis and progression.

The integrity of the intestinal barrier structure and function relate to the gut microbiota composition. Gut microbiota comprises a great diversity of symbiotic bacteria, whereas the intestinal barrier relies on junctional proteins that make this epithelium less permeable to pathogens and toxins. In this way, both prevent metabolic dysregulation and contribute to maintaining gut homeostasis [26-28].

Dysbiosis caused by chronic consumption of fats is usually characterized by an increase in the *Firmicutes* to the *Bacterioidetes* phylum, as shown by the HF group. These two phyla constitute more than 90% of the phylogenetic category already known and characterized in the intestine of experimental models [29]. Conversely, all treated groups showed an expressive reduction in the quantitative percentage of *Firmicutes*, especially the groups treated with the DPP-4 inhibitor and the combined therapy.

The rescuing of gut dysbiosis in the treated groups resulted in marked improvements in TJs gene expression. HF diet also impairs the junctional components present in the intestinal epithelium, making it more permeable and consequently more susceptible to translocation of microorganisms and toxins into the systemic circulation. The increase in intestinal permeability is known as the leaky gut [28, 30].

The leaky gut was rescued through enhanced expression of TJs genes in all treated groups. MUCIN2 knockout mice exhibit alterations in TJs structural proteins, besides mitochondrial damage, and inflammation, collaborating with the leaky gut [31]. The treatments significantly augmented *Mucin2*, the major goblet cell gene responsible for mucin secretion, besides the TJs *Occludin* and *Zo-1* gene expression [32]. PPAR-alpha has

recently been described as essential for lipid droplet formation and crypt expansion in the intestine during chronic HF diet intake [33]. Sitagliptin, a DPP-4 inhibitor, exerted protective effects on the intestinal barrier (high *occludin* and *Zo-1*) by GLP-2 induction in experimental colitis [34]. Herein, the combination of these drugs resulted in the highest intestinal expression of *Mucin2* and *Occludin*, indicating that adequate function of goblet cells and well-preserved TJs may underlie the mechanisms involved in the HFC group beneficial results.

HF diet intake also impairs the lining epithelium of the intestinal mucosa. The goblet cells found in the intestinal villi and crypts are mucin producers that play a crucial role in protecting and lubricating the intestinal mucosa. The amount of goblet cells indirectly reflects the ability to secrete mucus [28]. Herein, the HF diet caused a reduced number of goblet cells per area of the gut crypt, while all treatments reversed this alteration, with the normalization of numerical density of goblet cells and *Mucin2* gene expression. Hence, treatments collaborated with intestinal barrier integrity and reduced the translocation of endotoxins, such as LPS, to the systemic circulation, rescuing from a condition called metabolic endotoxemia [35].

The high translocation of endotoxins derived from the gut microbiota induces Toll-Like-like receptors (TLR) activation. LPS is the most common pathogen-associated molecular pattern (PAMP), and its binding to TLR4 is catalyzed by lipopolysaccharide-binding protein (LBP), mainly expressed in the liver and adipose tissue [35].

LPS stems from the destruction of the bacterial cell wall. A rise of LPS in the systemic circulation triggers the release of proinflammatory cytokines and an inadequate amplification of the immune response, causing tissue damage [29]. An increase in plasma levels of LPS was evidenced in the HF group, characterizing the involvement of the epithelium with a consequent increase in intestinal permeability, making this epithelium more permeable to the entry of microorganisms. Additionally, we showed that hepatic *Lbp* and *Tlr4* gene expression increased in the HF group, inducing the translocation of cytokines associated with inflammation and changing the hepatic lipid

metabolism, configuring itself as a potent inducer of fatty liver and contributing to the pathogenesis of NAFLD.

In contrast, the treatments normalized *Lbp* and *Tlr4* expressions, both emerging as targets to treat the NAFLD. The DPP-4 inhibitor alogliptin suppressed TLR4 *via* ERK activation, resulting in decreased matrix metalloproteinases and proinflammatory cytokines in U937 histiocytes [36]. Sitagliptin has previously attenuated NAFLD by suppressing inflammation and insulin resistance due to TLR4/NF-kB pathway downregulation in diabetic rats [37]. PPAR-alpha activation by WY-14643 mitigated liver steatosis in high-fructose-fed mice by reducing LPS concentrations, improving intestinal barrier ultrastructure, upregulating hepatic beta-oxidation, and suppressing lipogenesis in mice [10].

A recent study showed that systemic LBP blockade or decreased LBP levels in the liver normalized glucose homeostasis, mainly by reducing fasting glucose levels, without changing adiposity or liver steatosis [38]. In this study, the group treated with the PPAR-alpha agonist as monotherapy did not reduce LPS concentrations. However, it had reduced *Lbp* expression, with anti-inflammatory and anti-steatotic effects like the other treatments.

In this context, macrophages play a role in acute and chronic inflammatory liver diseases. Macrophages have receptors, such as CD163 and CD206, which participate in the phagocytosis of harmful substances. CD206, due to its high affinity for macrophages, is a potential biomarker of hepatic macrophage (Kupffer cells) activation, an indicator of inflammation and fibrosis in chronic liver diseases [39]. In response to LPS and other stimuli, the metabolic profile of macrophages and dendritic cells stimulates the glycolytic pathway resulting in the metabolic accumulation of citrate and succinate that, in turn, regulate the gene expression of cytokines such as interleukin 10 (IL-10) [40]. Recent evidence shows that IL-10 may play a dual role in some contexts by stimulating the immune response rather than suppressing it. However, the cytokine IL-10 emerges as an anti-inflammatory mediator determining the protection of its host in responses to pathogens [41].

Our results showed that *Cd206* macrophages were activated in the HF group, reinforcing the idea that chronic consumption of this diet activates inflammatory pathways that contribute to the development of liver disease. On the contrary, the groups treated with PPAR-alpha activator and DPP-4 inhibitor had reduced expression of *Cd206* macrophages, conferring a protective effect against the activation of inflammatory pathways. Recent evidence showed that highly fibrous livers had a higher density of *Cd206* macrophages [39]. Regarding *Il-10*, its gene expression was reduced in the HF group. However, only the combined treatment increased *Il-10* expression, implying that the isolated treatments might have acted through another anti-inflammatory pathway. IL-13 overexpression in HF diet-fed mice is a pathway related to fatty liver and insulin resistance onset. The combined treatment and the single PPAR-alpha activation markedly reduced hepatic *Il-13* expression, collaborating with reduced macrophage activation and glycemic homeostasis, alleviating the fatty liver [42].

Concerning inflammasome, NLRP3 is present mainly in immune and inflammatory cells such as macrophages, monocytes, dendritic cells, and neutrophils after activation by inflammatory stimuli. NLRP3 is activated by numerous PAMPs such as hyperglycemia, fatty acids, bacterial toxins, bacterial and viral nucleic acids, among others [43]. Studies in macrophages and animal models have shown that oxidized low-density lipoproteins and cholesterol crystals trigger NLRP3 activation. In macrophage and type 2 diabetes animal models, glucose and free fatty acids trigger inflammasome activation, damaging glucose metabolism and favoring insulin resistance [44]. Thus, NLRP3 may contribute to the appearance and progression of several diseases related to metabolic syndrome.

In this study, we showed that treatment with the PPAR-alpha agonist and DPP-4 inhibitor showed a potent anti-inflammatory effect in the liver, as they demonstrated a reduced gene expression of *Nlrp3* and *Cd206*. The blockade of NLRP3 has recently improved NAFLD and mitigated liver fibrosis in two models of steatohepatitis [45], highlighting the proposed treatments as viable tools to control the fatty liver. Two DPP-

4 inhibitors have previously suppressed NLRP3 in human macrophages by downregulating the TLR4-IL-1beta pathway [46]. Sitagliptin alleviated liver injury caused by thioacetamide in mice by decreasing NLRP3 and exerting anti-apoptotic effects [47]. Regarding PPAR-alpha activation, the oleoylethanolamide protected against LPS-induced liver injury in mice by NLRP3 suppression [48]. However, the present study is the first to report the effects of both drugs and their combination on the gut-liver axis in HF-fed mice. Figure 7 summarizes our main findings.

Some limitations of the present study comprise the absence of female mice evaluation to state possible sexual dimorphism, plasma glucose concentrations were not measured, and we could not determine LBP concentrations but evaluated its gene expression. Future research should also include the evaluation of genera and families of *Proteobacteria* phylum as it seems to be involved in the harmful evolution of NAFLD.

CONCLUSION

In conclusion, HF-fed mice showed impairment in the intestinal barrier and alteration in the phylogenetic diversity of the gut microbiota, leading to dysbiosis, contributing to the influx of LPS into the liver. Impaired gut-liver axis favor liver damage, making it more susceptible to NAFLD by proinflammatory signals (*Tlr4* and *Nlrp3* upregulation). Treatment with the PPAR-alpha agonist and the DPP-4 inhibitor modulated the gut microbiota and rescued the intestinal barrier gene expression and goblet cells numerical density, reducing endotoxemia and liver steatosis by anti-inflammatory signaling. Given the beneficial effects found by the treatments, they become possible in the therapeutic strategy for the NAFLD spectrum of diseases.

ARTICLE HIGHLIGHTS

Research background

Gut microbiota can be modified by dietary composition and play a role in fatty liver pathogenesis through endotoxemia. Peroxisome proliferator-activated receptor (PPAR)-alpha activation has previously rescued the gut-liver axis with anti-steatotic effects in

high-fructose-fed mice, whereas high-dose dipeptidyl peptidase-4 (DPP-4) inhibitor (linagliptin) inhibited hepatic lipogenesis in high-fat-fed mice. A combination of these drugs could restore the gut-liver axis in obese mice.

Research motivation

Nonalcoholic fatty liver disease (NAFLD) is highly prevalent among obese individuals and can evolve into harmful liver diseases. Currently, there is no treatment directly prescribed to counter the NAFLD. Herein, we propose a drug combination (PPAR-alpha agonist plus DPP-4 inhibitor) that can alleviate fatty liver by modulating the gut-liver axis with anti-inflammatory properties.

Research objectives

To evaluate the effects of the monotherapy with a PPAR-alpha agonist (WY14643), a DPP-4 inhibitor (linagliptin), or their association on the gut-liver axis, highlighting the intestinal barrier, endotoxemia, and inflammatory pathways in the livers of high-fat-fed mice. These pre-clinical insights can help to establish new strategies in the treatment of NAFLD.

Research methods

Mice were fed a control diet (C, 10% of energy as lipids) or a high-fat diet (HF, 50% of energy as lipids) for 12 wk. Then the HF group was randomly divided into four groups: HF, HF-A (treated with the PPAR-alpha agonist), HFL (treated with the DPP-4 inhibitor), and HFC (treated with the combination of both drugs). Treatment lasted for five weeks. The gut-liver axis was assessed with histological, biochemical, stereological, and molecular techniques.

Research results

The HF diet yielded overweight, oral glucose intolerance, altered gut microbiota composition, decreased the numerical density of goblet cells, and tight junction's gene

expression with increased plasma lipopolysaccharide (LPS) concentrations and increased fatty liver. The combined treatment rescued all these metabolic alterations by restoring gut microbiota and intestinal barrier markers, resulting in decreased LPS concentrations and fatty liver by anti-inflammatory signals. Further studies may focus on the *Proteobacteria* phylum, whose alteration can trigger harmful signaling to the liver.

Research conclusions

The combination of PPAR-alpha activation with DPP-4 inhibition yielded marked anti-steatotic effects by modulating the gut-liver axis with reduced endotoxemia and amelioration of the intestinal barrier histology and gene expression. In turn, the livers of obese mice treated with the drug combination benefited from downregulation of *Tlr4* and *Nlrp3* pathways and exhibited hepatic parenchyma like the C group. Of note, we used stereology to estimate the numerical density of goblet cells in cecum slides stained with alcian blue and PAS. This technique can assess the effect of different interventions on the intestinal barrier and leaky gut in further studies.

Research perspectives

This study brings novelty to the treatment of NAFLD, a challenge to the scientific community. Our results confirm the importance of the gut-liver axis to the pathogenesis of fatty liver and propose a combined treatment that targets gut microbiota composition, endotoxemia, and intestinal barrier alterations to alleviate fatty liver by local anti-inflammatory effects in HF-fed mice.

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1	Isabela Macedo Lopes Vasques-Monteiro, Flávia Maria Silva-Veiga, Carolline Santos Miranda, Édira Castello Branco de Andrade Gonçalves et al. "A rise in Proteobacteria is an indicator of gut-liver axis-mediated nonalcoholic fatty liver disease in high-fructose-fed adult mice", Nutrition Research, 2021 Crossref	264 words — 5%
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