Gut-liver axis modulation in fructose-fed mice: a role for PPAR-alpha and linagliptin

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Abstract

Fructose dietary intake affects the composition of the intestinal microbiota and influences the development of hepatic steatosis. Endotoxins produced by gram-negative bacteria alter intestinal permeability and cause bacterial translocation. This study evaluated the effects of gut microbiota modulation by a purified PPAR-alpha agonist (WY14643), a DPP-4 inhibitor (linagliptin), or their association on intestinal barrier integrity, endotoxemia, and hepatic energy metabolism in high-fructose-fed C57BL/6 mice. Fifty mice were divided to receive the control diet (C group) or the high-fructose diet (HFRU) for 12 weeks. Subsequently, the HFRU group was divided to initiate the treatment with PPAR-alpha agonist (3.5 mg/kg/BM) and DPP-4 inhibitor (15 mg/kg/BM). The HFRU group had glucose intolerance, endotoxemia, and dysbiosis (with increased Proteobacteria) without changes in body mass in comparison with the C group. HFRU group showed damaged intestinal ultrastructure, which led to liver inflammation and marked hepatic steatosis in the HFRU group when compared to the C group. PPAR-alpha activation and DPP-4 inhibition countered glucose intolerance, endotoxemia, and dysbiosis, ameliorating the ultrastructure of the intestinal barrier and reducing Tlr4 expression in the liver of treated animals. These beneficial effects suppressed lipogenesis and mitigated hepatic steatosis. In conclusion, the results herein propose a role for PPAR-alpha activation, DPP-4 inhibition, and their association in attenuating hepatic steatosis by gut-liver axis modulation in high-fructose mice model. These observations suggest these treatments as potential targets to treat hepatic steatosis and avoid its progression.

Introduction

Non-transmissible chronic diseases (NCDs) are becoming increasingly important in global public health. Although NCDs may be related to genetic factors and dietary habits, they represent a significant and modifiable factor for human health. Fructose intake has increased in recent years due to ultra-processed food intake and causes lipotoxicity with ectopic lipid deposition (Lambertz et al. 2017).

Non-alcoholic fatty liver disease (NAFLD) is characterized by intrahepatic lipid accumulation and is considered the hepatic component of the metabolic syndrome (Paniagua et al. 2014). An innovative concept proposes that the gut-liver axis plays a crucial role in the genesis and progression of NAFLD, as excessive fructose consumption alters the composition of the gut microbiota
(dysbiosis), with increased lipopolysaccharides (LPS) production (Zhou et al. 2014, Leung et al. 2016). High LPS damages mucosal integrity by changes in tight junction proteins such as zonula occuludens-1 (ZO-1), resulting in increased intestinal permeability (Matsushita et al. 2016) and the influx of LPS and bacterial DNA to the bloodstream, triggering inflammation, and into the liver through the portal vein, the so-called endotoxemia (Kirpich et al. 2015).

LPS stimulates liver inflammation through toll-like receptor 4 (TLR4), with impairment of hepatic insulin signaling, hepatic stellate cells (HSC) activation, and the progression of NAFLD to more severe forms such as steatohepatitis (NASH) and cirrhosis (Friedman 2008, Liu et al. 2014). Chronic excessive fructose intake increases postprandial endotoxemia, TLR4 liver expression, and plasma LPS (Liu et al. 2014, Zhou et al. 2014). Given this scenario, the use of drugs that reverse dysbiosis and avoid hepatic changes, resulting from excessive dietary fructose, is relevant.

In this context, peroxisome proliferator-activated receptors (PPARs) are transcription factors found in the intertwining of various metabolic pathways (Souza-Mello 2015). The pharmacological activation of the alpha isoform (PPAR-alpha) promotes the body mass reduction, relief of insulin resistance, the formation of beige adipocytes (Rachid et al. 2015), and a significant reduction of NAFLD by increased mitochondrial beta-oxidation (Veiga et al. 2017). Recently, the deletion of PPAR-alpha was shown to promote gut dysbiosis and inflammation of the large intestine in mice (Manoharan et al. 2016).

Linagliptin is a selective inhibitor of dipeptidyl peptidase-4 (DPP-4) that suppresses the rapid degradation of the glucagon-like peptide-1 (GLP-1), a gastrointestinal hormone (incretin) that increases glucose-dependent insulin secretion. DPP-4 inhibitors decrease GLP-1 degradation and thus increase its endogenous circulating levels (Campbell & Drucker 2013). Linagliptin has recently induced browning of the s.c. white adipose tissue and macrophage polarization to M2 state in high-fat-fed mice (Zhuge et al. 2016, de Oliveira Correia et al. 2019), besides mitigating hepatic steatosis in db/db mice (Michurina et al. 2016). Figure 1 illustrates the main sites of actions attributed to the PPAR-alpha agonist and the DPP-4 inhibitor.

There are no reports in the literature about the association of these two treatments (PPAR-alpha agonist and linagliptin) on the gut-liver axis and the hepatic outcomes. This study evaluated the effects of gut microbiota modulation by a purified PPAR-alpha agonist (WY14643), a DPP-4 inhibitor (linagliptin), or their association on intestinal barrier integrity, endotoxemia, and hepatic energy metabolism in high-fructose-fed C57BL/6 mice.

Materials and methods

Animals and diet

Adult male C57BL/6 mice were kept under controlled temperature and humidity conditions (60 ± 10%), with free access to water and diet, in pathogen-free cages. The environment was subjected to 12 h light:12 h darkness cycle and air renewal cycles (15 min/h). All procedures were performed according to the recommendations of the NIH Guide for the Care and Use of Laboratory Animals (NIH publication No 85-23, revised 1996). The experimental protocol (CEUA number 041/2018) was

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**Figure 1**

Main sites of actions of WY14643 and linagliptin. (A) Main effects attributed to PPAR-alpha activation on the liver, adipose tissue, pancreas, heart, and muscle. (B) Main effects attributed to DPP-4 inhibition on the liver, adipose tissue, kidney, blood vessels, and pancreas. A full color version of this figure is available at https://doi.org/10.1530/JOE-20-0139.
Treatments to NAFLD by microbiota modulation

Experimental protocol

Fifty-three-month-old mice were used in this study. Initially, the animals were randomly divided into two nutritionally different groups for 12 weeks:

1. Control group (C) – animals that received a standard rodent diet throughout the experimental period (n=10);
2. High-fructose group (HFRU) – animals that received a high-fructose rodent diet (50% of the energy from fructose) (n=40).

After 12 weeks, the animals of the HFRU group were subdivided according to the treatment, making up the groups:

1. HFRU – continued to receive the HFRU diet (n=10);
2. HFRUA – had treatment with the PPAR-alpha agonist (WY14-643, Sigma-Aldrich, 3.5 mg/kg body mass) incorporated into the HFRU diet (n=10);
3. HFRUT – received treatment with Trayenta (linagliptin, Boehringer Ingelhelm, 15 mg/kg body mass) incorporated into the HFRU diet (n=10);
4. HFRUC – had treatment with combined PPAR-alpha and Trayenta (same doses of the groups in monotherapy) incorporated into the HFRU diet (n=10).

The experimental protocol was performed for 17 weeks. Experimental diets were produced by PragSoluções (Jaú, São Paulo, Brazil) according to the recommendations of the American Institute of Nutrition (AIN 93M). Glucose analysis, ELISA, and RT-PCR were carried out in six animals per group, whereas stereology was carried out in five animals per group.

Food and energy intake, water intake, and body mass (BM)

Food intake was measured daily by subtracting the amount of diet offered for each group on the previous day by the remainder verified on the following day. Energy intake was calculated as the product of food consumption by the energy offered by 1 g of each diet (in kJ). The water intake was measured and recorded twice a week through the difference between the amount offered (200 mL of water) for the rest of the previous day, verified in a beaker. Throughout the experimental period, the BM was evaluated weekly.

Glucose analysis

One week before the killing, the oral glucose tolerance test (OGTT) was performed, in which blood glucose was measured with a manual glucometer (Accu-Chek, Roche, São Paulo, SP, Brazil) after a 6-h fast (time 0) and after orogastric gavage of glucose solution (2 g/kg body mass), at times 15, 30, 60 and 120 min.

Euthanasia

The mice were fasted for 6 h and then underwent deep anesthesia with ketamine (240 mg/kg) and xylazine (30 mg/kg). The liver and intestine were carefully dissected, weighed, and followed the protocols for different techniques. Blood samples were obtained by cardiac puncture, and plasma was obtained after centrifugation to perform ELISA to measure GLP-1 (multi-species GLP-1 ELISA Kit Cat. #EZGLPIT-36K, Millipore) and LPS (multi-species LPS ELISA Kit Cat #SEBS26Ge-96T, Cloud-Clone Corp, Katy, USA). Inter-assay and intra-assay CV% fell within the recommended range for GLP1: inter-assay CV%, 5.05% (manufacturer’s recommendation <12%), and intra-assay CV%, 1.46% (manufacturer’s recommendation 1–2%); and for LPS: inter-assay CV%, 11.30% (manufacturer’s recommendation <12%), and intra-assay CV% 2.03% (manufacturer’s recommendation <10%).

Hepatic stereology

Liver fragments from all hepatic lobes were fixed in Millonig buffered formalin (pH 7.2–7.4), and subsequently dehydrated, diaphanized and included in Paraplast Plus (Sigma-Aldrich), sectioned (5 µm thick) and stained with hematoxylin and eosin. Digital photomicrographs of liver tissue were obtained (Leica DMRBE microscope, Infinity Lumenera digital camera, Ottawa, ON, Canada). Five animals per group and ten random non-consecutive images per animal were analyzed. The volume density of hepatic steatosis (Vv (st)) was estimated by point-counting: Vv (st, liver) = Pp (st, liver)/P (Pp is the number of points that reach the fat droplets, P is the total of test points), a technique as reproducible and as reliable as hepatic triacylglycerol to estimate the degree of hepatic steatosis (Catta-Preta et al. 2011). The images were analyzed with STEPanizer (www.stepanizer.com) using a 36 points frame (Tschanz et al. 2011).

Transmission electron microscopy (TEM)

Cecum fragments (1 mm³, at least three animals per group) were fixed in 2.5% glutaraldehyde (Merck) in

References

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Liver immunofluorescence (REELIN, ALPHA-SMA, PLIN-2)

Liver sections were deparaffinized and hydrated and, then, antigenic recovery was performed using citrate buffer, pH 6.0 at 60°C for 20 min and blocked with ammonium chloride, 2% glycine, followed by incubation for 2 h with the following primary antibodies (diluted in 1% PBS/BSA, 1:100): anti-alpha-smooth muscle actin (ab7817, monoclonal mouse, Abcam); anti-REELIN (ab78540, monoclonal mouse, Abcam); and anti-PLIN2 (CSB-PA920084, monoclonal rabbit, Cusabio). Subsequently, samples were treated with Alexa-488 or Alexa-546 fluorophore-conjugated secondary antibody (Invitrogen donkey anti-IgG mouse, Molecular Probes) for 1 h, then washed in PBS, followed by staining of the nuclei with DAPI. Slides were mounted with SlowFade (Invitrogen, Molecular Probes). Images were taken using a Confocal Laser Scanning Microscope (Nikon Confocal Laser Scanning Microscope, model C2, Nikon Instruments, Inc.).

RT-PCR

Liver

Total RNA was extracted from about 50 mg of liver tissue using Trizol reagent (Invitrogen). Subsequently, 200 μL of chloroform was added, followed by centrifugation (12,000 g for 10 min at 4°C), and the RNA extract portion was set aside. To this portion, 500 μL of isopropanol was added, which was allowed to react for 10 min to precipitate RNA and then centrifuged (1200 g for 10 min at 4°C). After that isopropanol was removed, and the pellet formed was resuspended with 500 μL of 75% ethanol and then centrifuged (12,000 g for 5 min at 4°C). Ethanol was removed and the pellet was resuspended in 20 μL 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 cDNA, 1.0 μg RNA was treated with DNase I (Invitrogen). First-strand cDNA synthesis was performed using Oligo (dT) primers for reverse transcriptase mRNA and Superscript III (both from Invitrogen). RT-PCR was performed using a CFX96 recycler (Bio-Rad) and the SYBRGreen mix (Invitrogen). Beta-actin was used to correct the expression of the target genes, and primer sequences are found in Table 1. All gene symbols are italicized (the first letter capitalized) and protein symbols in uppercase.

Intestine

Total RNA was extracted from about 70 mg of gut tissue using Trizol reagent (Invitrogen). Subsequently, 200 μL of chloroform was added, followed by centrifugation (12,000 g for 15 min at 4°C), and the portion corresponding to the RNA extract was set aside. After 15 min, 500 μL of isopropanol was added to precipitate RNA and then centrifuged (12,000 g for 10 min at 4°C). After that isopropanol was removed, and the pellet formed was resuspended with 500 μL of 70% ethanol (ice-cold) and then centrifuged (10,000 g for 5 min at 4°C). Ethanol was removed and the pellet was resuspended in 50 μL of deionized water (MilliQ). From this point, the protocol was identical to the previously described RT-PCR for liver. GAPDH was used to correct the target gene expression, and primers sequences are detailed in Table 1.

16S rDNA PCR amplification

The feces present in mice cecum were used for microbial DNA extraction using the commercial kit (QIAamp Fast DNA stool mini kit, Qiagen) following the manufacturer’s instructions. DNA quantification, purity, and concentration were analyzed using Qubit (Life Technologies) 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. In order to perform relative quantification, the abundance of different phyla was normalized by ΔΔCt of the total amount of bacteria present in the sample (Livak & Schmittgen 2001). The indicators used are described in Table 2.

Data analysis

Data were expressed as mean and s.d. The differences between groups in the first 12 weeks used the Student’s
t-test and Welch correction. After treatment, data were analyzed by the Brown–Forsythe and Welch correction one-way ANOVA with the post hoc test of Dunnett T3. The P-value <0.05 was considered significant (GraphPad Prism version 8.3 for Windows, GraphPad Software).

Results

Food and energy intake, water intake and body mass (BM)

The animals from C and HFRU groups had no difference regarding BM at baseline. However, after the initiation of the treatment (13th week), the treated groups had a reduction in BM compared with their counterparts (–7% for HFRUA vs HFRU; –3% for HFRUT vs HFRU; and –6% for HFRUC vs HFRU), which lasted until the end of the experiment. These results are described in Fig. 2A.

Glycemic response

Figure 3A shows the OGTT curve, in which the HFRU group showed a significant increase in fasting glucose (T0) compared to the C group. This difference was maintained until the end of the test (T120). C and treated groups re-established baseline blood glucose levels in the remaining assessment periods (T30, T60, and T120). Conversely, the HFRU group did not manage to reach baseline glucose levels, indicating a delay in glucose normalization after glucose overload.

As shown in Fig. 3B, the analysis of the area under the curve (AUC) for OGTT showed that HFRU exhibited higher AUC than the C group (+36%), indicating oral glucose intolerance in the HFRU group. In contrast, the treated groups showed lower AUC than the HFRU group (–26% for HFRUA vs HFRU; –37% for HFRUT vs HFRU; and –20% for HFRUC vs HFRU).

Plasma GLP-1 concentrations were reduced by chronic HFRU diet intake when compared to the C group (–2%, Fig. 3C). As expected, treatment with linagliptin enhanced GLP-1 concentrations in the HFRU group and

Table 1  Forward and reverse sequences of RT-qPCR primers – liver and intestine.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-actin</td>
<td>5′-TGTTACAACCTGGGACGACA</td>
</tr>
<tr>
<td>Cpt-1a</td>
<td>3′-GGGGTTGCAAAGGTCTCAA</td>
</tr>
<tr>
<td>Dpp-4</td>
<td>5′-GCAGAGACGCCAATAAGGA</td>
</tr>
<tr>
<td>Fas</td>
<td>3′-GGCTTTCGACCAGGAAGAC</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5′-TGAGGAAAGGCACCTACACT</td>
</tr>
<tr>
<td>Mucin</td>
<td>3′-GAATTTGCGTGGAACAGTGAA</td>
</tr>
<tr>
<td>Ppar-alpha</td>
<td>5′-TCGACTGGTTCTTCTTGAT</td>
</tr>
<tr>
<td>Ppar-gamma</td>
<td>3′-AGCATGCCTCAGGTCCTGT</td>
</tr>
<tr>
<td>Srebp-1c</td>
<td>5′-AGCAGCCCTAGACCCAAACA</td>
</tr>
<tr>
<td>Tlr4</td>
<td>3′-GCGGAAGGTATTATGTGTGA</td>
</tr>
<tr>
<td>Zo-1</td>
<td>5′-AGGACACCAAAGACCTGTGAG</td>
</tr>
</tbody>
</table>

For all genes: melting temperature (58–60°C) and product length (75–150 bp).

Cpt-1a, carnitine palmitoyl transferase-1a; Dpp-4, dipeptidyl peptidase-4; Fas, fatty acid synthase; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Ppar, peroxisome proliferator-activated receptors; Srebp-1c, sterol regulatory element-binding protein-1c; Tlr4, toll-like receptor 4; Zo-1, zonula occludens-1.

Table 2  Initiators of the phylum or class of microorganisms of intestine microbiota.

<table>
<thead>
<tr>
<th>Phylum or class</th>
<th>Initiators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacteria</td>
<td>F: 5′-TACGCGCCGACAAGCACA-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-ACTGGCTACGGGAGCGGAG-3′</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>F: 5′-TGAGGAAAGGCACCTACACT-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-CTCTTTCGACCAGGAAGAC-3′</td>
</tr>
<tr>
<td>Class-γ-proteobacteria</td>
<td>F: 5′-TCTGCTACGTGCTGTGTA-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-CTTGCCAGCTAGATGAT-3′</td>
</tr>
<tr>
<td>Eubacteria (all bacteria)</td>
<td>F: 5′-ATCTGCTACGGGAGCGGAG-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-ATTACGCGCCGCTGCG-3′</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>F: 5′-TAAACATTGGGAGGAATGCAG-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-ACCATGACACCTGTGAC-3′</td>
</tr>
</tbody>
</table>
the HFRUC group in comparison with the HFRU group (+13 and +10%, Fig. 3C). Also, PPAR-alpha activation yielded raised GLP-1 concentrations in HFRUA compared with HFRUt (+6%, Fig. 3C).

**PPAR-alpha activation and DPP-4 inhibition restore microbiota composition and mitigate endotoxemia in HFRU-fed mice**

The microbiota composition (Fig. 4A) was evaluated by amplification of the 16S rRNA genes of cecal gut bacteria at the end of the experiment. The most noticeable features were the increase in Proteobacteria species parallel to a decrease in Bacteroidetes species in the HFRU group in comparison with the C group. Importantly, all treatments restored the values of Bacteroidetes species, resembling C group, and were associated with a significant decrease in Proteobacteria species, suggesting that this phylum might have a decisive role in the beneficial effects observed by the proposed treatments.

Altered microbiota composition in the HFRU group caused higher LPS levels (+10%, Fig. 4B), as well as higher Tlr4 gene expression than C group (+120%, Fig. 4B). In contrast, treated groups showed reduced plasma LPS concentrations (−9% for HFRUA vs HFRU; −9% for HFRUT vs HFRU; −10% for HFRUC vs HFRU) and lower Tlr4 gene expression than HFRU group: HFRUA (−40%), HFRUT (−50%), and HFRUC (−14%).

**Treatments rescued intestinal ultrastructural damage caused by excessive dietary fructose**

Transmission electron microscopy analysis (Fig. 5) revealed that the C group presented with microvillus at the apical surface of the absorptive epithelial cell of the intestine, implying a large area of nutrients absorption, besides active mucus layer, numerous mitochondria (arrowhead), cellular and nuclear membranes integrity. On the contrary, the HFRU group showed lipid inclusions (asterisk) within the gut epithelial cells, nearly absence of mitochondria, altered mucus layer (black arrow), and irregular microvillus, sometimes showing fission, denoting damage in cell functioning. The treated groups showed less perceived lipid inclusions, restoration of mitochondrial content, and structure (arrowhead), besides well-preserved microvillus and mucus layer (black arrow).
Treatments restore intestinal permeability produced by excessive dietary fructose

Mucin mRNA expression showed a reduction in the HFRU group compared to the C groups (−50%, Fig. 6A). Conversely, the treated groups HFRUA and HFRUC showed increased Mucin mRNA expression in comparison with HFRU (+210% for HFRUA vs HFRU; +445% for HFRUC vs HFRU, Fig. 6A).

ZO-1 is considered a tight junction protein and contributes to the function of the intestinal mucosa barrier. Therefore, the results revealed that Zo-1 mRNA expression levels were reduced in the HFRU group compared to C groups (−52%, Fig. 6B). Conversely, after treatment, Zo-1 expression increased in the HFRUA, HFRUT and HFRUC groups (+41, +45, and +120%, P<0.0001, Fig. 6B).

In agreement with a proinflammatory state triggered by excessive fructose intake, the HFRU group exhibited higher Dpp-4 mRNA expression than the C group (+83%, Fig. 6C). All treatments significantly reduced the Dpp-4 gene expression (−32% for HFRUA vs HFRU; −50% for HFRUT vs HFRU; and −58% for HFRUC vs HFRU, Fig. 6C).

**PPAR-alpha agonist, DPP-4 inhibition and their combination attenuated hepatic steatosis by inducing beta-oxidation**

The HFRU diet yielded expressive hepatic lipid deposition, as shown in Fig. 7A, which was attenuated by the different treatments proposed in this study. The HFRU group presented with increased micro and macrovesicular steatosis when compared to the C group (+320%, Fig. 7B). However, the different treatments were effective in reducing hepatic steatosis in comparison with the HFRU group (−90% for HFRUA vs HFRU; −53% for HFRUT vs HFRU; and −26% for HFRUC vs HFRU, Fig. 7B).
Both genes related to beta-oxidation had lower expression in the HFRU group than in the C group: \( \text{Ppar}^\alpha ( -25\% , \text{Fig. 7C} ) \) and its transcript \( \text{Cpt-1a}^\alpha ( -30\% , \text{Fig. 7D} ) \). In contrast, the treated groups had increased expression of \( \text{Ppar}^\alpha \) gene (+225\% for HFRUA vs HFRU; 162\% for HFRUT vs HFRU; and +193\% for HFRUC vs HFRU, \text{Fig. 7C} ) and \( \text{Cpt-1a}^\alpha \) gene (+92\% for HFRUA vs HFRU; +44\% for HFRUT vs HFRU; and +60\% for HFRUC vs HFRU, \text{Fig. 7D} ) in comparison with their counterparts.

Treatments reduced hepatic lipogenesis and countered inflammation

Lipogenesis increased in the HFRU group, which showed higher \( \text{Ppar}^\gamma (+228\% , \text{Fig. 8A}) \), and its transcript \( \text{Srebp-1c} (+57\% , \text{Fig. 8B}) \) and \( \text{Fas} \) gene expression (+37\% , \text{Fig. 8C} ) than the C group. On the other hand, treatments suppressed the lipogenic genes (\text{Fig. 8A, B and C}): \( \text{Ppar}^\gamma , \text{Srebp-1c} \) and \( \text{Fas} \) genes had reduced expression in HFRUA (−69; −37; and −24\% ) HFRUT (−61; −47; and −91\% ); and HFRUC (−63; −65; and −82\% ).

In agreement with the increased hepatic lipogenesis, high consumption of fructose augmented \( \text{Tlr4} \) gene expression in the liver when compared to the C group (+382\% , \text{Fig. 8D} ), suggesting that \( \text{Tlr4} \) may contribute to increasing the hepatic steatosis. Conversely, the HFRUA, HFRUT, and HFRUC groups showed a reduction in \( \text{Tlr4} \) expression in comparison with the HFRU group (−14; −33; and −73\% , \text{Fig. 8D} ).

Immunofluorescence

Positive immunostaining for \text{ALPHA-SMA} and \text{REELIN} were found in the livers of the HFRU group (\text{Fig. 9} ), which might suggest HSCs activity and the progression of NAFLD to NASH. The treated groups, however, showed immunoreactions, like the C group, corroborating the results of light microscopy of well-preserved hepatic parenchyma after the treatments.

HFRU animals also showed positive PLIN2 labeling (\text{Fig. 9} ), indicating the high instability of their lipid droplets. Again, the treated groups presented with a pattern of immunostaining compatible with that presented by the control group, corroborating the significant reduction in the percentage of hepatic steatosis, estimated by point-counting technique.
Discussion

Disorders of glucose and lipid metabolism favor the occurrence of diseases that represent serious health problems in developed and underdeveloped countries. The data in this article confirms that a diet rich in fructose promotes changes in the gut-liver axis (Matsushita et al. 2016, Do et al. 2018), favoring the pathogenesis and progression of NAFLD. Our observations of dysbiosis, high plasma LPS concentration and augmented intestinal permeability in the HFRU group comply with the increased liver Tlr4 expression and aggravated NAFLD. Conversely, the groups treated with PPAR-alpha and linagliptin (isolated or associated) showed improvement in glucose metabolism, dysbiosis tackling, reduced LPS levels, and rescued intestinal permeability, leading to the NAFLD control even in mice fed with the HFRU diet chronically.

Chronic HFRU diet intake did not cause overweight but led to oral glucose intolerance. The PPAR-alpha agonist led to reduced body mass from the first week until the end of treatment, but linagliptin did not change body mass in this experimental model. Both treatments and their association rescued the oral glucose intolerance, which can be attributed to the enhanced GLP-1 levels,
an incretin that drives glucose-stimulated insulin secretion in pancreatic islets (Shigeto et al. 2015). The increased GLP-1 half-life and concentration are related to the inhibition of the DPP-4 enzyme, the primary mechanism of action of linagliptin (Drucker & Nauck 2006). However, the PPAR-alpha agonist WY14643 also increased the GLP-1 levels of animals fed with the HFRU diet, suggesting that a possible inhibition of the DPP-4 might be obtained through the PPAR-alpha anti-inflammatory effects (Larter et al. 2012, Takahashi et al. 2017).

Dysbiosis caused by chronic fructose consumption is usually characterized by an increase in the Firmicutes/Bacteroidetes ratio (Jumpertz et al. 2011), as shown in our results. These two phyla constitute more than 90% of the phylogenetic categories already known and characterized in the gut of experimental models of obesity induced by a high-fat diet (Shin et al. 2015, Candido et al. 2018). The deregulation in their proportion can induce inflammation, increase oxidative stress, and favor lipogenesis, triggering hepatic steatosis through mechanisms associated with changes in intestinal metabolites (Jumpertz et al. 2011). The HFRU group showed an increase in Firmicutes and Proteobacteria phyla, parallel to a decrease in Bacteroidetes. Conversely, the treatments rescued the Bacteroidetes proportion and markedly reduced both Firmicutes and Proteobacteria phyla, agreeing with observations in obese individuals and mice after bariatric surgery (Shao et al. 2017, Pajecki et al. 2019). This fact suggests that Proteobacteria modulation may have a role in the endpoints observed herein.

Proteobacteria belong to a phylum composed of gram-negative bacteria (Rizzatti et al. 2017). Interestingly, the leading bacterial products involved in the pathogenesis of NAFLD are LPS, which is commonly present on the cell membrane of gram-negative bacteria and binds to TLR4 (Spraak et al. 2009). The HFRU group exhibited the highest LPS concentrations and TLR4 expression, which could be associated with the most frequent Proteobacteria presence. This scenario induced cytokine translocation, critically contributing to inflammation and insulin resistance, altering hepatic lipid metabolism (Leung et al. 2016), configuring itself as a potent inducer of hepatic steatosis.

A previous study confirmed a link between Proteobacteria and liver fibrosis on the basis that mice fed a control diet and transplanted with gram-negative flora showed increased liver damage compared to the control (De Minicis et al. 2014). Our results in the HFRU group agree with other studies that have shown Proteobacteria as the main phylum that contribute to liver fibrosis and liver damage (Caussy et al. 2018, Panasevich et al. 2018). In contrast, PPAR-alpha activation, DPP-4 inhibition, and their association have drastically reduced Proteobacteria phylum. These observations suggest that the treatments proposed in this experiment might have important antifibrotic properties through Proteobacteria modulation.

Fructose impairs the integrity of the intestinal barrier, making it more susceptible to the influx of toxins (Zhou et al. 2016). The HFRU group showed increased plasma LPS and hepatic Tlr4 expression, suggesting endotoxemia. The increase in endotoxins translocation and plasma levels contribute to inflammation and degradation of the intestinal mucosal barrier (Rahman et al. 2016, Volynets et al. 2017). High levels of endotoxemia are related to increased intestinal permeability and decreased expression of tight junction proteins, such as ZO-1, which is expressed in the apical membrane and has a role in the leak pathway regulation (Pearce et al. 2018). Importantly, the HFRU group showed reduced Zo-1 expression coupled with high plasma LPS levels, but both conditions were reverted by the proposed treatments, with the augmentation of Zo-1 expression potentialized when treatments were associated (HFRUC group). These observations put forward that the PPAR-alpha agonist and the DPP-4 inhibitor could act as essential microbiota modulators.

There is no previous report on the literature associating oral linagliptin administration with gut microbiota profile. Linagliptin is the unique DPP-4 inhibitor with a predominant fecal excretion in humans and rodents when administered orally (Bleich et al. 2010, Fuchs et al. 2012). This fact might imply a direct contact of the lower intestine microbiota with unchanged linagliptin (Bleich et al. 2010), besides the enhanced GLP1 levels, which has been previously associated with increased Bacteroidetes presence after sitagliptin + metformin administration to Zucker rats (Reimer et al. 2014).

Regarding PPAR-alpha, previous evidence shows that this transcription factor has a central role in suppressing intestinal inflammation through IL-22 and antimicrobial peptides regulation, besides contributing to host homeostasis and intestinal barrier immunity maintenance (Manoharan et al. 2016). The deletion of PPAR-alpha in mice led to a higher susceptibility to intestinal inflammation (Esposito et al. 2014). In contrast, PPAR-alpha activation is crucial to induce Th2 lymphocytes, linked to anti-inflammatory effects in the Peyer’s patches of mice (Di Paola et al. 2018). These anti-inflammatory effects might influence microbiota modulation.

As for large intestine ultrastructure, the goblet cells secrete mucin-like glycoproteins, whose primary function
is to protect and lubricate the intestinal mucosa (Navarro-Garcia et al. 2010, Birchenough et al. 2015). In this regard, the chronic fructose intake led to altered mucus layer and compromised microvillus ultrastructure, besides lipid inclusions and rare mitochondria within the colonocytes. Both treatments and their association resulted in the recovery of the colonocytes ultrastructure, highlighting that treatments could impact not only the ultrastructure of the cell but also their function.

Concerning the amount of Mucin, the PPAR-alpha activation seems to increase its gene expression to a more significant extent than the DPP-4 inhibition. Importantly, the combination of both treatments had additive effects in this parameter as the HFRUC group showed the highest Mucin expression. The crypts dynamics is also impaired by increased intestinal epithelium TLR4 expression (Burgueno & Abreu 2020), while rats fed with the HF diet showed intestinal barrier dysfunction together with enhanced oxidative stress and intestinal inflammation mediated by TLR4 (Su et al. 2019). Herein, all treatments normalized intestinal Tlr4 expression in the intestine, correlating with the well-preserved ultrastructure, crypts, and mucus layer. The HFRU group showed dysbiosis, molecular and ultrastructural intestinal damage, endotoxemia, and high hepatic Tlr4 expression, picturing a frame in which dysbiosis contributes to the NAFLD pathogenesis, the multiple-hit (Buzzetti et al. 2016). Fructose-rich diets generally induce systemic insulin resistance and fasting hyperinsulinemia, thus promoting insulin-mediated activation of Srebp-1c and hepatic de novo lipogenesis (Aroor et al. 2018). The type of lipid that predominates in the liver influences its PPAR-alpha expression and susceptibility to steatosis (Echeverria et al. 2016). Thus, fatty livers are characterized by polyunsaturated fatty acids n-3 and PPAR-alpha depletion concomitant with increased lipogenic and inflammatory genes Srebp-1c and Nf-kb expression (Hernandez-Rodas et al. 2017).

PPAR-alpha activation has been proven as a promising approach to tackle hepatic steatosis by triggering an improvement in the mitochondrial entry of fatty acids for beta-oxidation and lipogenesis suppression (Veiga et al. 2017). Moreover, the restoration of PPAR-alpha expression by the administration of natural activators suppressed lipogenesis and countered hepatic inflammation in HF-fed mice (Hernandez-Rodas et al. 2017, Valenzuela & Videla 2020). Herein, we showed potent anti-inflammatory effects after PPAR-alpha treatment because HFRUA mice showed reduced hepatic Tlr4 expression coupled with reduced plasma LPS concentrations.

It can be argued that DPP-4 inhibition exerts anti-inflammatory effects by suppressing TLR4 in macrophages from humans and mice (Hiromura et al. 2018), besides reducing the reactive oxygen species (Sato et al. 2019), improving insulin signaling and vascular function through nitric oxide (Aroor et al. 2018). Linagliptin has also been associated with a polarization of macrophages with an effective anti-inflammatory M2 response and browning of s.c. white adipose tissue, inducing thermogenesis and reducing body mass, both of which are related to hepatic steatosis mitigation (Zhuge et al. 2016, de Oliveira Correia et al. 2019).

The group that received the combination of treatments might benefit from an interaction between PPAR-alpha activation and DPP-4 inhibition, as it was shown that PPAR-alpha is required for the upregulation of GLP-1 receptor in pancreatic islets in mice treated with metformin (Maida et al. 2011). Herein, both groups treated with the PPAR-alpha agonist (HFRUA and HFRUC) showed enhanced GLP-1 levels and DPP-4 inhibition, suggesting an intersection between PPAR and incretin pathways.

The gut microbiota composition and the endotoxemia can also contribute to hepatic fibrosis pathogenesis. Chronic disorders of hepatic homeostasis usually encompass liver damage and inflammation, leading to the activation of HSCs, an essential surrogate for NAFLD progression to NASH (Spruss et al. 2009). HSCs are fat-storing mesenchymal cells located adjacent to the Disse space. After a liver injury, the cells become active and acquire myofibroblastic morphology, characterized by exacerbated proliferation and secretion of the extracellular matrix. The stored vitamin A is lost, and there is an increase in the expression of ALPHA-SMA (Sato et al. 2003, Friedman 2008). The latter is an essential marker of HSC activity. The positive immunostaining for REELIN has also been associated with the progression of NAFLD to NASH (Lua et al. 2016), implying that the HFRU-fed animals had a more harmful type of liver disease.

As for the high-fructose model, the administration of GW501516 (a PPAR-beta agonist) improved inflammation, kept the HSCs at the quiescent stage, suppressed lipogenesis, and rescued insulin resistance by renin-angiotensin system modulation (Magliano et al. 2015). Recently, the use of linagliptin alone or associated with empagliflozin had an anti-fibrotic effect in a model of C57BL6 mice model of NASH (Jojima et al. 2016).
The present results confirmed the presence of hepatic steatosis in mice induced by the chronic HFRU diet intake. This phenomenon may be related to the excessive release of LPS induced by the altered gut microbiota, contributing to the pathogenesis of NAFLD. The treated groups, however, had a significant improvement in hepatic steatosis, dysbiosis, recovery of intestinal ultrastructure with reduced endotoxemia, which reflected in the better energy metabolism of the hepatocytes. Figure 10 illustrates the main findings of this study.

Conclusion

In conclusion, mice fed with the HFRU diet showed dysbiosis and impaired intestinal barrier, contributing to the influx of LPS into the liver with the following hepatic steatosis and positive immunostaining that suggests HSCs activity, configuring marked damage to the liver structure and function. The treatment with PPAR-alpha agonist and DPP-4 inhibitor managed to modulate the gut microbiota and rescue the intestinal barrier, with reduced endotoxemia and hepatic steatosis. Given the various beneficial effects presented by the treatments, they become potential approaches in the therapeutic strategy for the NAFLD spectrum of diseases.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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