European Journal of Pharmacology 863 (2019) 172699

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Full length article

Therapeutic effect of treatment with metformin and/or 4-hydroxychalcone in male Wistar rats with nonalcoholic fatty liver disease

Selene de Jesús Acosta-Cota[a], Elsa Maribel Aguilar-Medina[a], Rosalío Ramos-Payán[a], José Guadalupe Rendón Maldonado[a], José Geovanni Romero-Quintana[a], Julio Montes-Avila[a], Juan I. Sarmiento-Sánchez[b], Carolina Gabriela Plazas-Guerrero[a], Marcela J. Vergara-Jiménez[c], Araceli Sánchez-López[d], David Centurión[d], Ulises Osuna-Martínez[a,*]

[a] Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Sinaloa, Av. las Américas y Blvd, Universitarios S/N, CP 80010, Culiacán, Sinaloa, Mexico
[b] Facultad de Ingeniería Civil, Universidad Autónoma de Sinaloa, Av. Las Américas y Blv, Universitarios S/N, CP 80010, Culiacán, Sinaloa, Mexico
[c] Facultad de Ciencias de la Nutrición y Gastronomía, Av. Cedros S/N, CP 80019, Culiacán, Sinaloa, Mexico
[d] Departamento de Farmacobiología, Cinvestav Sede Sur, Czda. de los Tenorios 235, Col. Granjas Coapa, Del. Tlalpan, C.P. 14330, Ciudad de México, Mexico

ARTICLE INFO

Keywords:
NAFLD
Metformin (PubChem CID 4091)
4-Hydroxychalcone (PubChem CID 5282361)
Therapeutic effect

ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease in the world. Despite the impact of this pathology in the population, nowadays there is no specific treatment for this disease, focusing its treatment on risks factors. However, it is imperative the existence of a specific treatment, due to this, the aim of this study was to determine the therapeutic effect of treatment with metformin, 4-hydroxychalcone or co-treatment on male Wistar rats with NAFLD. Wistar rats were divided into two groups with free access to either tap water or 50% sucrose (NAFLD) during 25 weeks. After 20 weeks of induction each were divided into four groups that received daily p.o. administration of: i) saline solution (1 ml); ii) metformin (200 mg/kg/day); iii) 4-hydroxychalcone (80 mg/kg/day) and i.v.) co-treatment (metformin plus 4-hydroxychalcone at the doses mentioned above), for 5 weeks. In healthy rats: metformin and co-treatment modified food and total caloric intake and induced diarrhea; but none of the treatments changed the other parameters evaluated. Meanwhile in rats with NAFLD: i) metformin inhibited hepatic total cholesterol and TGF-β, increased diarrhea frequency, and slightly decreased liver steatosis, and fibrosis; ii) 4-hydroxychalcone decreased IL-6, TNF-α and TGF-β, increased IL-10, and markedly decreased liver steatosis and fibrosis; and iii) co-treatment markedly decreased food intake, total caloric intake, and body weight, increased diarrhea; increased IL-10, showing and intermediate effect on decrease TNF-α, TGF-β, liver steatosis and fibrosis. Our results showed that 4-hydroxychalcone treatment was the most effective among the treatments tested against NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by abnormal lipid accumulation in hepatocytes; promotes from progression of simple liver steatosis to nonalcoholic steatohepatitis (NASH) with presence of inflammation and, in more severe cases, to liver fibrosis, cirrhosis and hepatocellular carcinoma (Croci et al., 2019; Sanches et al., 2015). Nowadays, it is the most common liver disease in the world affecting up to a quarter of the adult population; and over 65% of obese individuals and the majority of patients with type 2 diabetes (Croci et al., 2019). Moreover, several lines of evidence have revealed a high relationship of NAFLD with obesity, visceral adiposity, insulin resistance and metabolic syndrome; and contributes to the development of type 2 diabetes and cardiovascular disease (Cicero et al., 2019; Croci et al., 2019; Fazel et al., 2016).

Despite the impact of this pathology on the world population, there is currently no specific treatment for this disease, being its management focused on treating the risk factors. Moreover, the main objectives of the treatment are to slow or stop the progression of the disease, decrease steatosis, reduce the levels of transaminases, and to prevent inflammation and fibrosis (Dietrich and Hellerbrand, 2014). Regarding the treatment, insulin sensitizing agents, antilipemic agents, antioxidants, tumor necrosis factor inhibitors, among others, have been used (López-Oliva and Muñoz-Martínez, 2014).

In the absence of effective treatment for NAFLD, the effect of treatments used for NAFLD risk factor has been evaluated, such as

* Corresponding author.
E-mail address: ulises.osuna@uas.edu.mx (U. Osuna-Martínez).

https://doi.org/10.1016/j.ejphar.2019.172699
Received 16 August 2019; Received in revised form 24 September 2019; Accepted 25 September 2019
Available online 26 September 2019
0014-2999/

metformin which has been evaluated: (1) in an *in vitro* study on HepG2 cells (Ramachandran and Saraswathy, 2014) where shown exerts liver protection against acetaminophen as a consequence to increase antioxidant responsive elements; (2) in murine models with different results, such as decreased hepatic steatosis (Liu et al., 2014; Woo et al., 2014), transaminases (Raso et al., 2009), pro-inflammatory cytokines (Raso et al., 2009; Woo et al., 2014), suppresses hepatic oxidative stress (Xu et al., 2016); and protection against CCl_4-induced fibrosis by suppression of transforming grow factor beta 1 (TGF-β1) (Fan et al., 2017); and (3) in humans (Doycheva and Loomba, 2014; Haukeland et al., 2009); however, few studies have been carried out, their results are inconclusive, and the doses used can lead to the development of side effects.

Otherwise, researches have continued searching for possible pharmacological molecules with therapeutic action, among them is 4-hydroxychalcone (4-HC) that has been shown to exert, antioxidant (Kucerova-Chlupacova et al., 2018), anti-inflammatory (Qu et al., 2014) and hepatoprotective (Ebadollahi-Natanzi et al., 2011) activity. This allows us to infer that this hydroxychalcone could have beneficial effects on NAFLD, decreasing the inflammatory process, which is characteristic of the progression of the disease and is a preamble to the development of fibrosis.

Considering that metformin and 4-HC have been shown to exert their effects through different mechanisms of action, the primary aim of this study was to evaluate the therapeutic effect of metformin and 4-HC, as monotherapies and co-treatment on male Wistar rats with NAFLD.

2. Materials and methods

2.1. Reagents

Compound 4-hydroxychalcone (molecular confirmation tested by RMN ^1H) was synthetized by condensation of acetophenone and *p*-hydroxybenzaldehyde, using a Lewis acid ($BF_3{\cdot}OEt_2$) as a catalyst and 1,4-dioxane as solvent (Narender and Papi Reddy, 2007). Metformin tablets (850 mg) were purchased from "Laboratories AMSA". One tablet was pulverized and diluted in 4 ml of saline solution (concentration of 200 mg in 0.94 ml).

2.2. Experimental animals

Male Wistar rats weighing 70–90 g (4 weeks old, n = 48) were used. The rats were donated by the animal research facility located at Cinvestav Sede Sur. The animals were housed in plastic cages in a special temperature-controlled room (22 ± 2 °C, 50% humidity) on a 12 h light – 12 h dark cycle (with light beginning at 7:00 a.m.), with food (Purina Lab Diet 5012) and water freely available. All methods and protocols of the current investigation were approved by "Comité de Bioética de la Facultad de Ciencias Químico Biológicas" on 6th March 2017, and followed the regulations established by the Mexican Official Norm for the Use and Welfare of Laboratory Animals (NOM-062-ZOO-1999), in compliance with the Guide for the Care and Use of Laboratory Animals in U.S.A. (2011).

2.3. Induction of NAFLD

There are different animal models for NAFLD induction, where obesity is an important factor for its development; among them those which include dietary changes, are considered the most related with the etiology in humans (Rosini et al., 2012). These studies include long-term ingestion of diets with high: fat, cholesterol, sugar (30–55% sucrose and/or fructose), or combinations thereof (Cole et al., 2018).

The induction of NAFLD on Wistar rats was as previously reported (Acosta-Cota et al., 2019); the animals had free access to water with 50% sucrose (wt/vol) for a period of 20 weeks, after this time the animals developed NASH (grade 6 of NAFLD), with presence of steatosis, inflammation and fibrosis. Whereas healthy groups had free access to tap water.

2.4. Treatment with metformin and/or 4-hydroxychalcone

Each one of the first two groups of animals (n = 48) were divided into 4 groups that received daily p.o. administration of metformin (200 mg/kg; n = 12); 4-HC (80 mg/kg; n = 12); co-treatment that consists of metformin and 4-HC (200 mg/kg and 80 mg/kg, respectively; n = 12); or vehicle (saline solution; 1 ml; n = 12) during five weeks. These doses were chosen based on previous reports (Li et al., 2016; Qu et al., 2014). The doses were adjusted daily, according to any change in body weight of rat over the whole period of treatment for each group. It is important to mention, that during chronic administration of metformin, 4-HC, and co-treatment the side effect of diarrhea was monitored in all experimental groups. This was because it is the main side effect presented with metformin treatment (Bonnet and Scheen, 2017).

2.5. Assessment of macronutrients and total caloric intake

To know the caloric intake of the experimental groups during the administration of treatments, we calculated weekly the amount of food and water consumed by them, during the five weeks of treatments. Once we had these data, we calculated the macronutrients and total caloric intake with the nutritional composition of the standard laboratory diet (Purina Lab Diet 5012) and the sucrose content in the water (50% wt/vol).

2.6. Assessment of anthropometric parameters

Considering that NAFLD is closely related to the presence of obesity, we evaluated the changes on body weight and intraabdominal fat. To achieve this, the animals were weighted weekly with a precision electronic scale throughout the 25 weeks of intervention. Moreover, after animals were euthanized, adipose tissue from peritoneal cavity was excised and weighed, then intraabdominal fat was calculated using the following formula: [(tissue weight/body weight) x 100].

2.7. Assessment of liver damage

As a measurement of liver damage, after animals were euthanized, the liver was removed and weighed [(tissue weight/body weight) x 100]. Then, hepatic triglycerides and total cholesterol were quantified, according to the methodology used by Zhou et al. (2017). Briefly, 150 mg of liver were collected, minced and transferred into a test tube with 3 ml of chloroform/methanol (2:1 vol/vol). The mixture was homogenized for 2 min, sonicated for 30 s and shaken for 2 h. After, 1 ml of ddH_2O was added, and the samples were centrifuged for 20 min at 2700 G. The bottom layer was carefully collected into a new test tube, and incubated overnight (12 h). The next day liver lipids were dissolved with in absolute ethanol (1.5 ml) and sonicated. Finally, triglycerides (TG) and total cholesterol (TC) were quantified using an enzymatic colorimetric method with a commercial kit (GPO-PAP (TRGIS) and CHOL; Randox Laboratories, Crumlin, UK) following manufacture instructions and using a spectrophotometer with 500 nm filter. Hepatic TG and TC are expressed as mg/g of tissue.

Additionally, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity were quantified at the end of the study by enzymatic colorimetric assay using a commercial kit (GPT AA and GTO AA; Wiener Laboratories, Rosario Argentina) following manufacturer instructions and using a spectrophotometer with 340 nm filter (Spectronic Genesys 5) at 37 °C. Serum values of AST and ALT are expressed as IU/l.

2.8. Histopathological analysis

After euthanasia, the liver was excised and one of the lobes was immerse in buffered formalin: formaldehyde 10% 100 ml/l (J.T. Baker), NaH_2PO_4 4 g/l (Vetec), Na_2HPO_4 6.5 g/l (Fermont); pH 7.4. Tissue sections were cut into pieces of 0.5 cm × 2.0 cm, and were paraffin embedded (Leica Paraplast). Subsequently, the samples were cut using a microtome (Leica RM2125 RTS) with a thickness of 5–7 μm, placed on 2 slides per sample and stained with hematoxylin and eosin (H&E) or Masson trichrome (MT).

The determination of NAFLD degree in its development (from 0 to 8) was made according to the histological score system of the clinical research network of NASH (LaBrecque et al., 2012). This procedure was performed by counting the number of hepatocytes without steatosis, hepatocytes with steatosis, and ballooning cells present in 50 fields in samples stained with H&E, analyzed at 40x. While, fibrosis was determined independently (F0 to F4) (Diehl and Day, 2017). The images were taken with an increase of 40x for staining with H&E, and an increase of 10x for staining with MT. The images were digitized using a "Primo Star Zeiss" video camera and the Zen2 Blue Edition Zeiss program. The images shown are representative for each group (n = 6).

2.9. ELISA assay

To verify changes on inflammatory process, serum interleukin-6 (IL-6), interleukin-10 (IL-10), TGF-β and tumor necrosis factor alpha (TNF-α), were analyzed using rat ELISA kits. The ELISA kits were obtained and used in accordance with directions of the manufacturer (Elabscience, Houston, Texas, USA). Serum values of IL-6, IL-10, TGF-β and TNF-α are expressed as pg/ml.

2.10. Experimental design

Forty-eight animals were divided into two groups. The first group (n = 24) received tap water, named as healthy group, and the second group (n = 24) received 50% sucrose (wt/vol), that developed NAFLD, during 20 weeks; after this time each were divided into four groups that received daily p.o. administration of: i) saline solution (1 ml); ii) metformin (200 mg/kg; n = 12); iii) 4-HC (80 mg/kg; n = 12) and iv) metformin plus 4-HC (200 and 80 mg/kg; respectively; n = 12) over 5 week period. It is important to mention that during these five weeks the animals continued with free access to tap water or 50% sucrose, respectively.

The measurement of body weight, and food and caloric intake were performed weekly, while the measurement of diarrhea was performed daily during the treatments. One day after finished the treatments, the animals were euthanized, and blood samples, intraabdominal fat and hepatic tissue were collected. Finally, the percentage by weight of liver tissue and intraabdominal fat, hepatic TG and TC, as well as serum AST, ALT, IL-6, IL-10, TGF-β and TNF-α levels were determined. In addition, the liver histology stained with H&E and MT was obtained (Fig. 1).

2.11. Statistical analysis

The acquired values were subjected to statistical analysis and were expressed as the mean ± standard error of the mean (S.E.M.), and the number of animals was represented by n. In accordance with the normality of the data, and an analysis of variance (ANOVA) was performed. Only to the values of frequency of diarrhea were non-parametric, due of that numerical variables were expressed as medians and upper/lower quartiles, and analysis of variance (ANOVA) on ranks was performed. Then the differences among the changes in all the analyzed parameters were evaluated using Tukey post hoc test. A P value < 0.05 was considered statistically significant. The results were analyzed using the statistical software Sigma plot (version 12.0) and were designed using Graph-Pad Prism (version 5.0).

3. Results

3.1. Effect of treatments on food, macronutrients and total caloric intake

As shown in Fig. 2, 50% sucrose ingestion significantly diminished food intake compared with all groups with tap water ingestion ($P < 0.001$; Fig. 2A). Moreover, among groups with tap water ingestion, metformin administration significantly increased food intake compared with the group with saline solution administration (1330.04 ± 39.89 *vs.* 1136.96 ± 44.51 g; $P = 0.005$; Fig. 2A); in contrast metformin plus 4-HC (co-treatment) administration significantly decreased food intake compared with group with: (i) saline solution administration (945.26 ± 39.01 *vs.* 1136.96 ± 44.51 g; $P = 0.006$; Fig. 2A), (ii) metformin administration (945.26 ± 39.01 *vs.* 1330.4 ± 39.89 g; $P < 0.001$; Fig. 2A); and (iii) 4-HC administration (945.26 ± 39.01 *vs.* 1233.94 ± 46.92 g; $P < 0.001$; Fig. 2A). Whereas on groups with 50% sucrose ingestion, the administration of metformin (366.9 ± 16.95 *vs.* 438.6 ± 20.59 g; $P > 0.05$; Fig. 2A) and 4-HC (363.7 ± 24.13 *vs.* 438.6 ± 20.59 g; Fig. 2A) decreased food intake, however these results were not statistically significant; while the administration of metformin with 4-HC (366.9 ± 16.95 *vs.* 285.57 ± 12.21 g; Fig. 2A) decreased significantly food intake, compared with the group with saline solution administration.

On the other hand, regarding the macronutrients intake (Table 1), the consume of carbohydrates was significantly lower on group with tap water ingestion and co-treatment compared with groups with tap water ingestion and treatment with metformin ($P < 0.001$) and 4-HC ($P = 0.015$); also was lower on group with 50% sucrose ingestion and co-treatment compared with group with: (1) tap water ingestion and co-treatment administration ($P = 0.023$), (2) 50% sucrose ingestion and saline solution administration ($P < 0.001$), and (3) 50% sucrose ingestion and metformin administration ($P = 0.023$). In contrast, group with 50% sucrose ingestion and treatment with saline solution consume significantly less carbohydrates compared with group with (1) tap water ingestion and saline solution administration ($P = 0.001$); and with groups with 50% sucrose ingestion and treatment with 4-HC ($P = 0.033$) and with co-treatment ($P < 0.001$); additionally the groups with 50% sucrose ingestion and treatment with monotherapies of metformin ($P < 0.001$) and 4-HC ($P = 0.003$) consume significantly more carbohydrates compared with group with tap water ingestion and treatment with metformin and 4-HC.

Among the carbohydrates consumed, the amount corresponding to sucrose was quantified, where all the groups with 50% sucrose ingestion consumed significantly more sucrose compared with all the groups with tap water ingestion ($P < 0.001$), whereas the consume of sucrose was significantly lower on group with 50% sucrose ingestion and co-treatment compared with groups with 50% sucrose ingestion and treatment with saline solution ($P < 0.001$) and metformin ($P = 0.01$) (Table 1).

Otherwise, among groups with tap water ingestion, the consume of proteins and fats was significantly increased on group with metformin administration compared with group with saline solution administration ($P = 0.005$); however on group with co-treatment the consume of proteins and fats was significantly decreased compared with the groups with treatment with saline solution ($P = 0.006$), metformin ($P < 0.001$) and 4-HC ($P < 0.001$). Besides, the consume of proteins and fats was significantly decreased on all groups with 50% sucrose ingestion compared with all groups with tap water ingestion ($P < 0.01$); and on group with 50% sucrose ingestion with metformin plus 4-HC administration compared with group with 50% sucrose ingestion and saline solution administration ($P = 0.046$) (Table 1).

3.2. Effect of treatments on anthropometric parameters

Fig. 2B shown the effect of treatments on body weight, during the 25 weeks of study. In it we observed that all groups with 50% sucrose

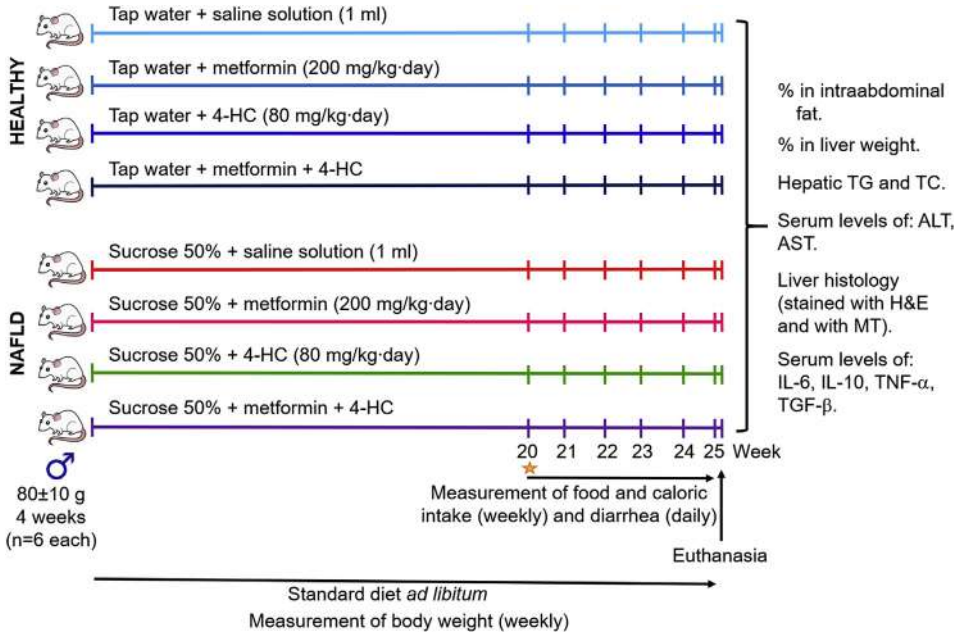

Fig. 1. Experimental design. All animals received standard diet *at libitum* plus tap water or 50% sucrose (with NAFLD) respectively during 20 weeks; after this time each were divided into four groups that received daily p.o. administration of: i) saline solution (1 ml); ii) metformin (200 mg/kg); iii) 4-hydroxychalcone (4-HC; 80 mg/kg) and iv) metformin + 4-HC (200 mg/kg; 80 mg/kg, respectively) over 5 weeks period. During these five weeks the animals continued with free access to tap water or 50% sucrose, respectively; besides during this time food and caloric intake were measured weekly, while diarrhea was measured daily. One day after finished treatments, the animals were euthanized, and blood samples, intraabdominal fat and hepatic tissue were collected to make different assays. ALT, alanine aminotransferase; AST, aspartate aminotransferase; H&E, hematoxylin and eosin; IL-6, interleukin-6; IL-10, interleukin-10; %, percentage; MT, Masson trichrome; TC, total cholesterol; TG, triglycerides; TGF-β, transforming grow factor beta; TNF-α, tumor necrosis factor alpha.

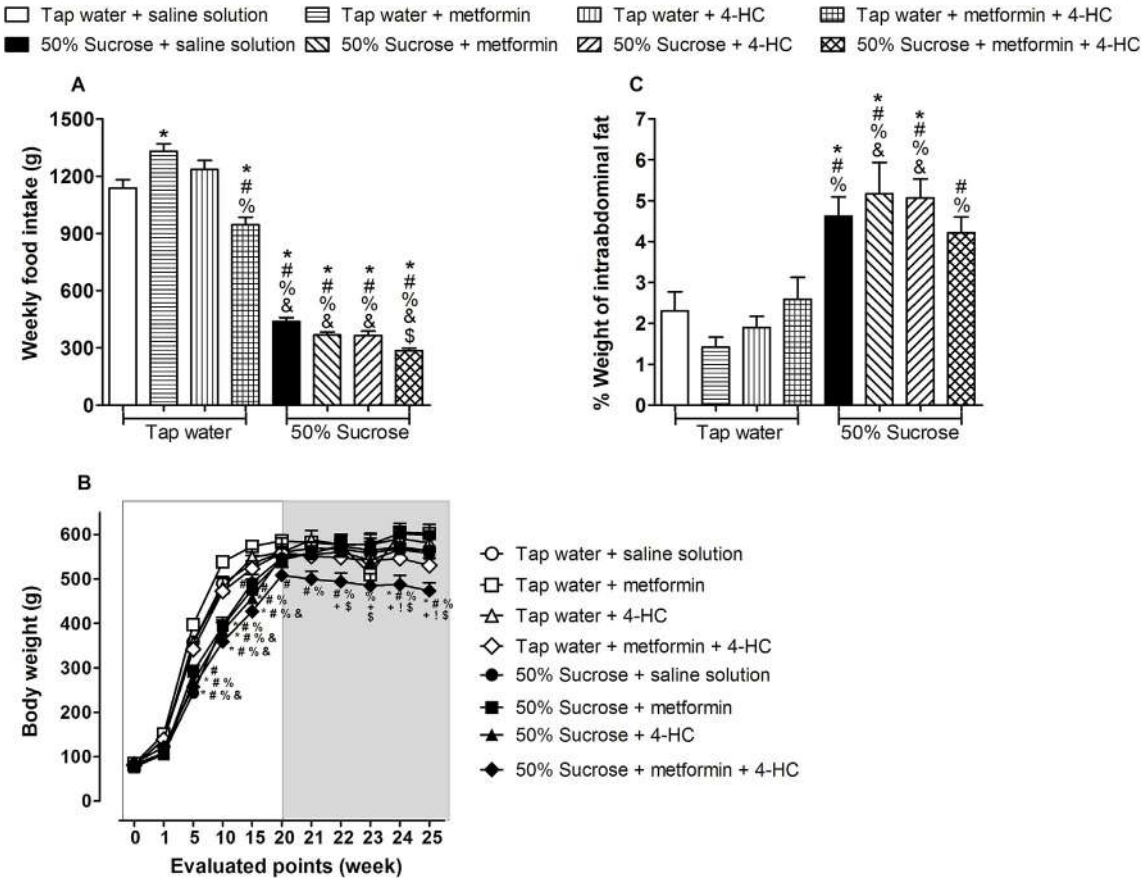

Fig. 2. Effect of treatments with metformin and/or 4-hydroxychalcone (4-HC) on food intake, body weight and percentage (%) weight of intraabdominal fat. The upper panel shows the effect of treatments on food intake (A) during the five weeks of treatment and % weight of intraabdominal fat (C); while bottom panel shows the effect on body weight (B) throughout the intervention period. Each bar or point represents the mean ± S.E.M. of n = 6 each group. One-way ANOVA and Tukey post hoc test were performed to quantify food intake and % weight of intraabdominal fat. Two-way repeated measures ANOVA and Tukey post hoc test were performed to quantify changes on body weight. *, $p < 0.05$ *vs.* tap water + saline solution; #, $p < 0.05$ *vs.* tap water + metformin; %, $p < 0.05$ *vs.* tap water + 4-HC; &, $p < 0.05$ *vs.* tap water + metformin + 4-HC; $, $p < 0.05$ *vs.* 50% sucrose + saline solution; +, $p < 0.05$ *vs.* 50% sucrose + metformin; ! $p < 0.05$ *vs.* 50% sucrose + 4-HC.

Table 1
Caloric intake during administration of treatments.

Group	Caloric intake (g) by				Total calories
	Sucrose	Carbohydrates	Proteins	Fats	
Tap water with					
Saline solution	20.1 ± 0.8	680.7 ± 26.7	307.2 ± 12.0	148.9 ± 5.8	5292.1 ± 207.2
Metformin	23.5 ± 0.7	796.3 ± 23.9	359.4 ± 10.8[a d]	174.2 ± 5.2[a d]	6190.8 ± 185.7[a]
4-HC	21.8 ± 0.8	738.8 ± 28.1	333.4 ± 12.7	161.6 ± 6.1	5743.5 ± 218.4
Metformin and 4-HC	16.7 ± 0.7	565.9 ± 23.4[b c]	255.4 ± 10.5[a b c]	123.8 ± 51[a b c]	4399.8 ± 181.6[b c]
50% Sucrose with					
Saline solution	641.8 ± 23.9[a b c d]	896.6 ± 28.6[a c d]	118.5 ± 5.6[a b c d]	57.5 ± 2.7[a b c d]	4577.5 ± 146[b c]
Metformin	583.8 ± 37.1[a b c d]	796.9 ± 46.2[d]	99.1 ± 4.6[a b c d]	48.1 ± 2.2[a b c d]	4016.7 ± 221.1[a b c]
4-HC	558.1 ± 37.7[a b c d]	769.4 ± 50.6[d]	98.3 ± 6.5[a b c d]	47.6 ± 3.2[a b c d]	3899.5 ± 254.2[a b c]
Metformin and 4-HC	465.9 ± 16.3[a b c d e f]	631.8 ± 20.7[b e f]	77.2 ± 3.3[a b c d e]	37.4 ± 1.6[a b c d e]	3172.7 ± 104.5[a b c d e]

Values reported as mean ± S.E.M. of n = 6 each group. One-way ANOVA and Tukey post hoc test were performed. a, $P < 0.05$ vs. tap water + saline solution; b, $P < 0.05$ vs. tap water + metformin; c, $P < 0.05$ vs. tap water + 4-HC; d, $P < 0.05$ vs. tap water + metformin + 4-HC; e, $P < 0.05$ vs. 50% sucrose + saline solution; f, $P < 0.05$ vs. 50% sucrose + metformin.

ingestion had significantly lower body weight on previous weeks of treatments, but then during the period of treatment were like body weight of healthy groups, with the exception of the NAFLD group and administration of metformin with 4-HC, which presented a significantly decrease on body weight compared with all healthy groups.

In other words, NAFLD group and treatment with saline solution had significantly lower body weight compared with: (i) healthy group with saline solution treatment, on weeks 5 ($P < 0.001$), 10 ($P < 0.001$) and 15 ($P = 0.03$); (ii) healthy group with metformin treatment, on weeks 5 ($P < 0.001$) and 10 ($P = 0.024$); (iii) healthy group with 4-HC treatment, on week 5 ($P = 0.002$); and (iv) healthy group with metformin plus 4-HC administration on week 5 ($P = 0.015$) (Fig. 2B). NAFLD group and metformin administration, presented significantly lower body weight compared with: (i) healthy group and saline solution administration on week 10 ($P = 0.005$); (ii) healthy group and metformin administration on weeks 5 ($P = 0.001$), 10 ($P < 0.001$), and 15 ($P = 0.004$); (iii) healthy group and 4-HC administration on weeks 10 ($P = 0.007$); and (iv) healthy group with metformin plus 4-HC administration on week 10 ($P = 0.042$) (Fig. 2B); however NAFLD group and metformin administration, presented significantly higher body weight compared with healthy group and metformin administration on week 23 ($P = 0.016$). NAFLD group and 4-HC administration, had significantly lower body weight compared with: (i) healthy group and saline solution administration on weeks 5 ($P = 0.011$), 10 ($P = 0.001$) and 15 ($P = 0.047$); (ii) healthy group and metformin administration on weeks 5 ($P < 0.001$), 10 ($P < 0.001$) and 15 ($P < 0.001$); and (iii) healthy group with 4-HC administration on weeks 5 ($P = 0.013$), 10 ($P = 0.005$) and 15 ($P = 0.008$) (Fig. 2B).

While NAFLD group and co-treatment, presented significantly lower body weight compared with: (i) healthy group and saline solution administration on weeks 5 ($P = 0.003$), 10 ($P < 0.001$), 15 ($P < 0.001$), 24 ($P = 0.031$) and 25 ($P = 0.01$); (ii) healthy group and metformin administration on weeks 5, 10 and 15 ($P < 0.001$), 20 to 22 ($P < 0.05$), 24 and 25 ($P < 0.001$); (iii) healthy group and 4-HC administration on weeks 5 ($P = 0.003$), 10 and 15 ($P < 0.01$) and from 21 to 25 ($P < 0.05$); (iv) healthy group with metformin plus 4-HC administration on weeks 5 ($P = 0.022$), 10 ($P < 0.001$) and 15 ($P = 0.004$); (v) NAFLD group and saline solution administration on weeks 22 ($P = 0.028$), 23 ($P = 0.007$), 24 and 25 ($P < 0.001$); (vi) NAFLD group and metformin administration on weeks 22–25 ($P < 0.05$); and (vii) NAFLD group and 4-HC administration on weeks 24 and 25 ($P < 0.05$) (Fig. 2B).

On the other hand, NAFLD groups and treatment with metformin or 4-HC had higher intraabdominal fat compared with all healthy groups ($P < 0.05$; Fig. 2C). NAFLD group with saline solution administration had higher intraabdominal fat compared with healthy groups and treatments with saline solution (4.62 ± 0.47 *vs.* 2.3 ± 0.47%; $P = 0.027$), metformin (4.62 ± 0.47 *vs.* 1.42 ± 0.25%; $P < 0.001$) and 4-HC (4.62 ± 0.47 *vs.* 1.59 ± 0.17%; $P = 0.005$) (Fig. 2C). While, NAFLD group with metformin plus 4-HC administration only had higher intraabdominal fat compared with healthy groups and treatments with metformin (4.33 ± 0.43 *vs.* 1.42 ± 0.25%; $P = 0.004$) and 4-HC (4.33 ± 0.43 *vs.* 1.59 ± 0.17%; $P = 0.028$) (Fig. 2C).

3.3. Effect of treatments on diarrhea frequency

The main side effect of metformin treatment is diarrhea (Bonnet and Scheen, 2017; Bouchoucha et al., 2011), while of 4-HC there are no reports of possible side effects that may cause. Considering the above mentioned and with the intention of research if 4-HC exerts this side effect, the frequency of diarrhea in the experimental groups was monitored during the time of treatment. Noticing that when were compared all groups (Table 2), the healthy group and metformin treatment had an increase on diarrhea frequency compared with healthy group and saline solution administration, healthy group and 4-HC administration, NAFLD group and saline solution or 4-HC administration, NAFLD group and metformin administration and NAFLD group and metformin plus 4-HC administration; besides NAFLD group and co-treatment had an increase on diarrhea frequency compared with the other three NAFLD groups, even though it was no statistically significant. While the group with tap water ingestion and co-treatment had an increase in diarrhea frequency compared with all other groups, but with a significantly increase compared with groups with 50% sucrose ingestion and treatment with: (i) saline solution, (ii) metformin and (iii) 4-HC ($P < 0.001$) (Table 2).

Table 2
Diarrhea frequency during administration of treatments.

Groups	Diarrhea frequency (days)
	Median (25th-75th percentile)
Tap water + saline solution	0 (0–1.25)
Tap water + metformin	2.5 (0.75–4.75)
Tap water + 4-HC	0.5 (0–1)
Tap water + metformin + 4-HC	5 (2.75–7.25)[e f g]
50% Sucrose + saline solution	0 (0–0)
50% Sucrose + metformin	0 (0–0.25)
50% Sucrose + 4-HC	0 (0–0)
50% Sucrose + metformin + 4-HC	2 (0–4)

Values reported as median (25th-75th percentile). n = 6, each group. ANOVA on ranks and Tukey post hoc test were performed. e, $P < 0.001$ vs. 50% sucrose + saline solution; f, $P < 0.001$ vs. 50% sucrose + metformin; g, $P < 0.001$ vs. 50% sucrose + 4-HC.

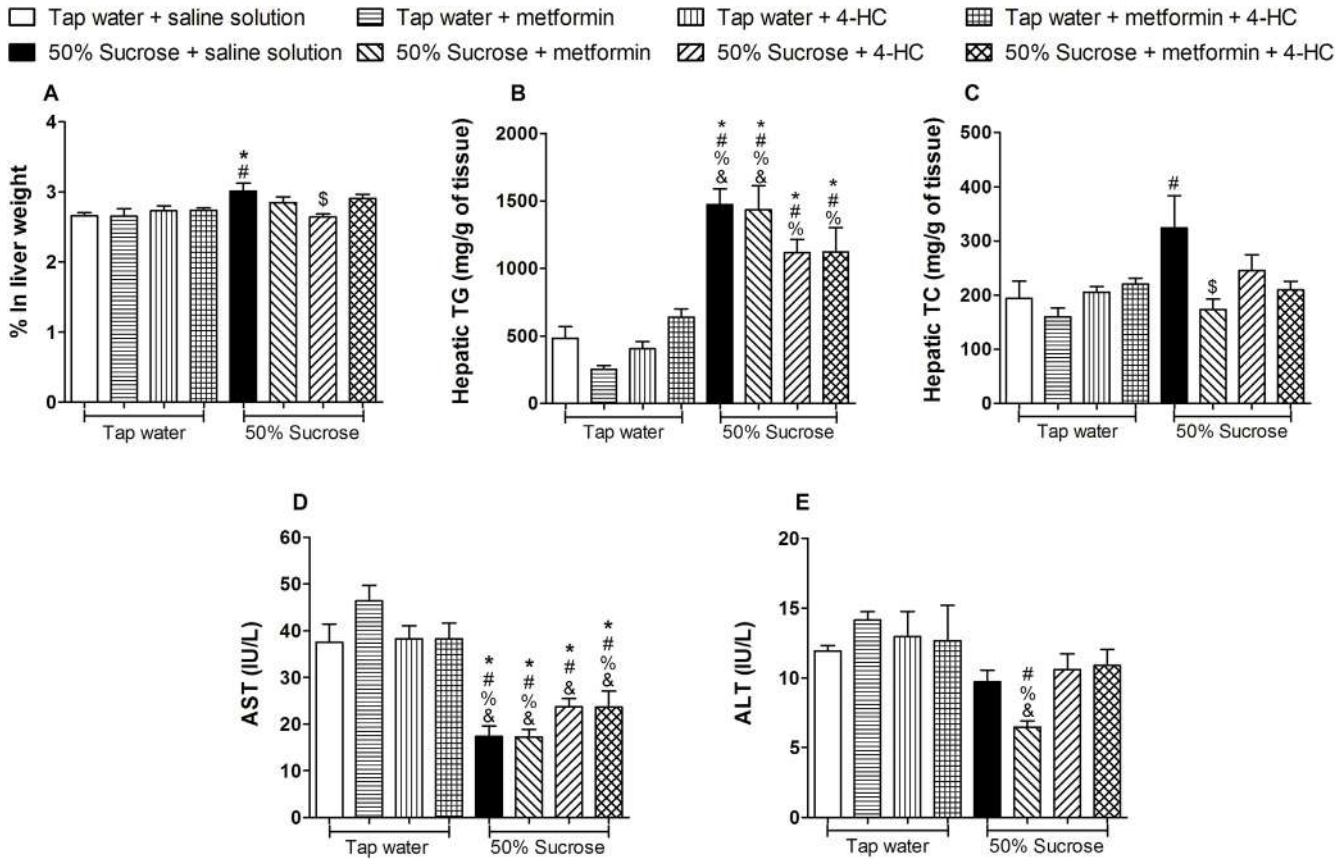

Fig. 3. Effect of treatments with metformin and/or 4-hydroxychalcone (4-HC) on percentage (%) in liver weight, hepatic triglycerides (TG), hepatic total cholesterol (TC), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). The upper panel shows the effect of treatments on % in liver weight (A), hepatic TG (B), and hepatic TC (C). The bottom panel shows the effect of treatments on AST (D), and ALT (E). Each bar represents the mean ± S.E.M. of n = 6 to % in liver weight and transaminases, and of n = 4 to hepatic TG and TC. One-way ANOVA and Tukey post hoc test were performed. *, $p < 0.05$ *vs.* tap water + saline solution; #, $p < 0.05$ *vs.* tap water + metformin; %, $p < 0.05$ *vs.* tap water + 4-HC; &, $p < 0.05$ *vs.* tap water + metformin + 4-HC; $, $p < 0.05$ *vs.* 50% sucrose + saline solution.

3.4. Effect of treatments on liver damage

Within the parameters evaluated to determine liver damage, the percentage in liver weight, hepatic TG and TC, as well as transaminases were found. In the first case it is observed that the NAFLD group and saline solution administration had a significantly increase on percentage in liver weight compared with: (1) healthy group and saline solution administration (3.01 ± 0.12 *vs.* 2.66 ± 0.04%; P = 0.036; Fig. 3A), and (2) healthy group with metformin administration (3.01 ± 0.012 *vs.* 2.65 ± 0.1%; P = 0.031; Fig. 3A). Moreover, only the treatment with 4-HC on NAFLD group prevented this increased, ergo, the NAFLD group and 4-HC administration had a significantly decreased on percentage in liver weight compared with NAFLD group and saline solution administration (2.64 ± 0.04 *vs.* 3.01 ± 0.12%; P = 0.024; Fig. 3A).

Regarding the effect observed on hepatic TG, NAFLD groups and treatments with saline solution and metformin had a significantly increased compared with all healthy groups (P < 0.01; Fig. 3B). NAFLD groups and treatments with 4-HC and co-treatment, had a significantly increased on hepatic TG compared with healthy groups and treatment with saline solution (P < 0.05), metformin (P < 0.001) and 4-HC (P < 0.01) (Fig. 3B). Whilst NAFLD groups and treatments with 4-HC (1115.78 ± 98.47 mg/g of tissue) and co-treatment (1121.25 ± 181.74 mg/g of tissue) had a tendency to decrease hepatic TG compared with NAFLD group and saline solution administration (1474.96 ± 116.46 mg/g of tissue), although they were not significant (P > 0.05).

On the other hand, on the effect observed on hepatic TC, NAFLD group and saline solution had a significantly increase compared with healthy group and metformin administration (323.82 ± 60.2 *vs.* 159.83 ± 16.5 mg/g of tissue; P = 0.009; Fig. 3C). In contrast, NAFLD group and metformin administration significantly decrease hepatic TC compared with NAFLD group and saline solution administration (173.89 ± 18.6 *vs.*323.82 ± 60.2 mg/g of tissue; P = 0.021; Fig. 3C). Additionally, NAFLD groups with 4-HC (245.32 ± 28.9 mg/g of tissue) and co-treatment (209.54 ± 15.74 mg/g of tissue) decreased hepatic TC compared with NAFLD group and saline solution administration (323.82 ± 60.22 mg/g of tissue), but these differences were not significant (P > 0.05).

Furthermore, in relation to transaminases, when compared serum AST levels (Fig. 3D), it was observed that 50% sucrose ingestion significantly decrease AST serum levels compared with tap water ingestion. This is, that the group with 50% sucrose ingestion and saline solution administration had a significantly decrease on AST compared with groups with tap water ingestion and treatments with saline solution (17.39 ± 2.2 *vs.* 37.58 ± 3.9 IU/l; P < 0.001); metformin (17.39 ± 2.2 *vs.* 46.42 ± 3.4 IU/l; P < 0.001); 4-HC (17.39 ± 2.2 *vs.* 35.08 ± 2.7 IU/l; P = 0.002) and metformin with 4-HC (17.39 ± 2.2 *vs.* 38.32 ± 3.3 IU/l; P < 0.001). NAFLD group and metformin administration (17.24 ± 1.7 IU/l) showed a significantly decrease on AST, compared with all healthy groups (P < 0.01). NAFLD group and 4-HC administration (23.58 ± 1.9 IU/l) showed a significantly decrease on AST, compared with the groups with tap water ingestion and treatment with saline solution (P = 0.028), metformin (P < 0.001) and metformin with 4-HC (P = 0.017). As well as, in NAFLD group and treatment with metformin and 4-HC (20.34 ± 3.3

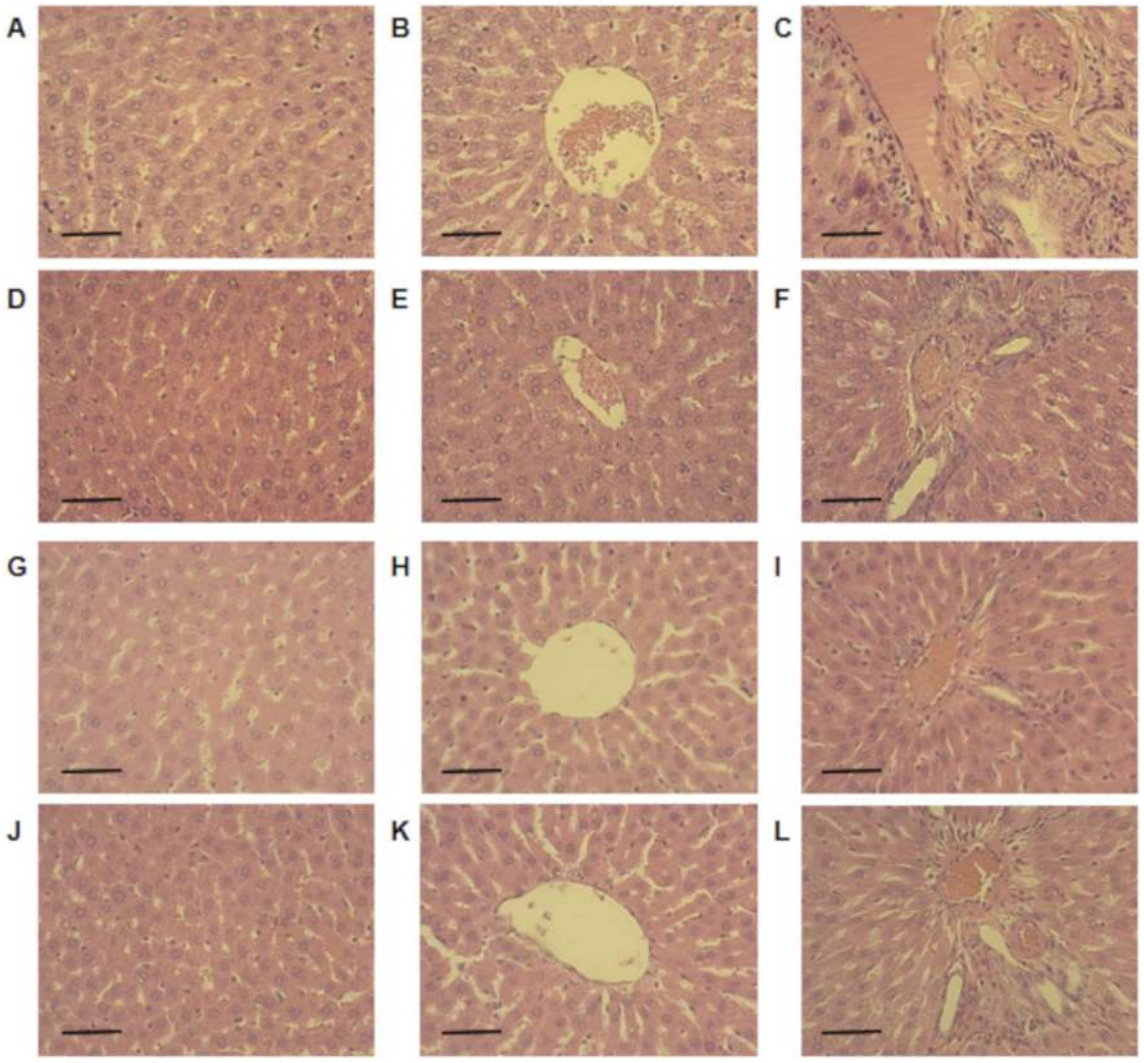

Fig. 4. Effect of treatments with metformin and/or 4-hydroxychalcone (4-HC) on hepatic morphology on healthy groups. Tissues stained with hematoxylin and eosin (H&E) were observed at 40x. Hepatic parenchyma (A, D, G, J). Central vein (B, E, H, K). Portal structure (C, F, I, L). Group treated with saline solution (A–C), metformin (D–F), 4-HC (G–I) and metformin + 4-HC (J–L). Representative images of each group (n = 6). Scale bars = 50 μm.

IU/l), compared with all healthy groups ($P < 0.05$) (Fig. 3D).

While, when compared serum ALT levels among all groups (Fig. 3E), it was observed a significantly decrease on NAFLD group and metformin administration compared with groups with tap water ingestion and treatment with metformin (6.48 ± 0.44 *vs.* 14.15 ± 0.6 IU/l; P = 0.002); 4-HC (6.48 ± 0.44 *vs.* 12.97 ± 1.8 IU/l; P = 0.015); and metformin with 4-HC (6.48 ± 0.44 *vs.* 12.67 ± 2.5 IU/l; P = 0.023).

3.5. Effect of treatments on liver morphology

In the healthy groups (Fig. 4) and treatment with metformin (Fig. 4D–F), 4-HC (Fig. 4G–I), and metformin plus 4-HC (Fig. 4J-L) was observed hepatic parenchyma with normal architecture with absence of steatosis (< 5%) and inflammatory infiltrate; central veins and portal structures, positioning them in grade 0 in the development of NAFLD. The above mentioned coincides with previous reports (Kierszenbaum, 2008). While the group with tap water ingestion and saline solution administration (Fig. 4A–C) showed in hepatic parenchyma (Fig. 4A) and around central veins (Fig. 4B) low microvesicular steatosis (mean of 5.48%) considered as grade 1 of steatosis. Even though the group with tap water ingestion and saline solution administration had access to a balance diet, probably the rats reached grade 1 of steatosis because they were sedentary; while the groups with treatment with metformin, 4-HC or metformin with 4-HC had grade 0 of steatosis because of the drugs effect.

On the other hand, the group with 50% sucrose ingestion and saline solution administration (Fig. 5A–C) showed severe microvesicular steatosis (65.48%) and limited macrovesicular steatosis (8.74%) mainly in parenchyma (Fig. 5A) and portal structures (Fig. 5C; mean of 74.22%); as well as many ballooned cells (2 per field) and presence of some inflammatory infiltrate (4 per sample, data not shown), positioning on grade 7 on the development of NAFLD (NASH). While the metformin administration (Fig. 5D–F) decrease the steatosis grade, because of this group showed severe microvesicular steatosis (52.25%) and limited macrovesicular steatosis (9.48%) mainly in parenchyma (Fig. 5D) and in less proportion around central veins (Fig. 5E) and in portal structure (Fig. 5F), with a mean of 67.91%; some ballooned cells (1 per field) and presence of one inflammatory infiltrate per sample (data not shown); positioning in grade 5 in the development of NAFLD (NASH).

However, 4-HC administration (Fig. 5G–I) further decreased the degree of liver damage that was induced by 50% sucrose intake, showing moderate microvesicular steatosis (55.16%) and limited macrovesicular steatosis (5.56%), mainly in parenchyma (Fig. 5G) and portal structures (Fig. 5I), with a mean of 60.66%; without presence of ballooned cells or inflammatory infiltrate; positioning in grade 2 in the

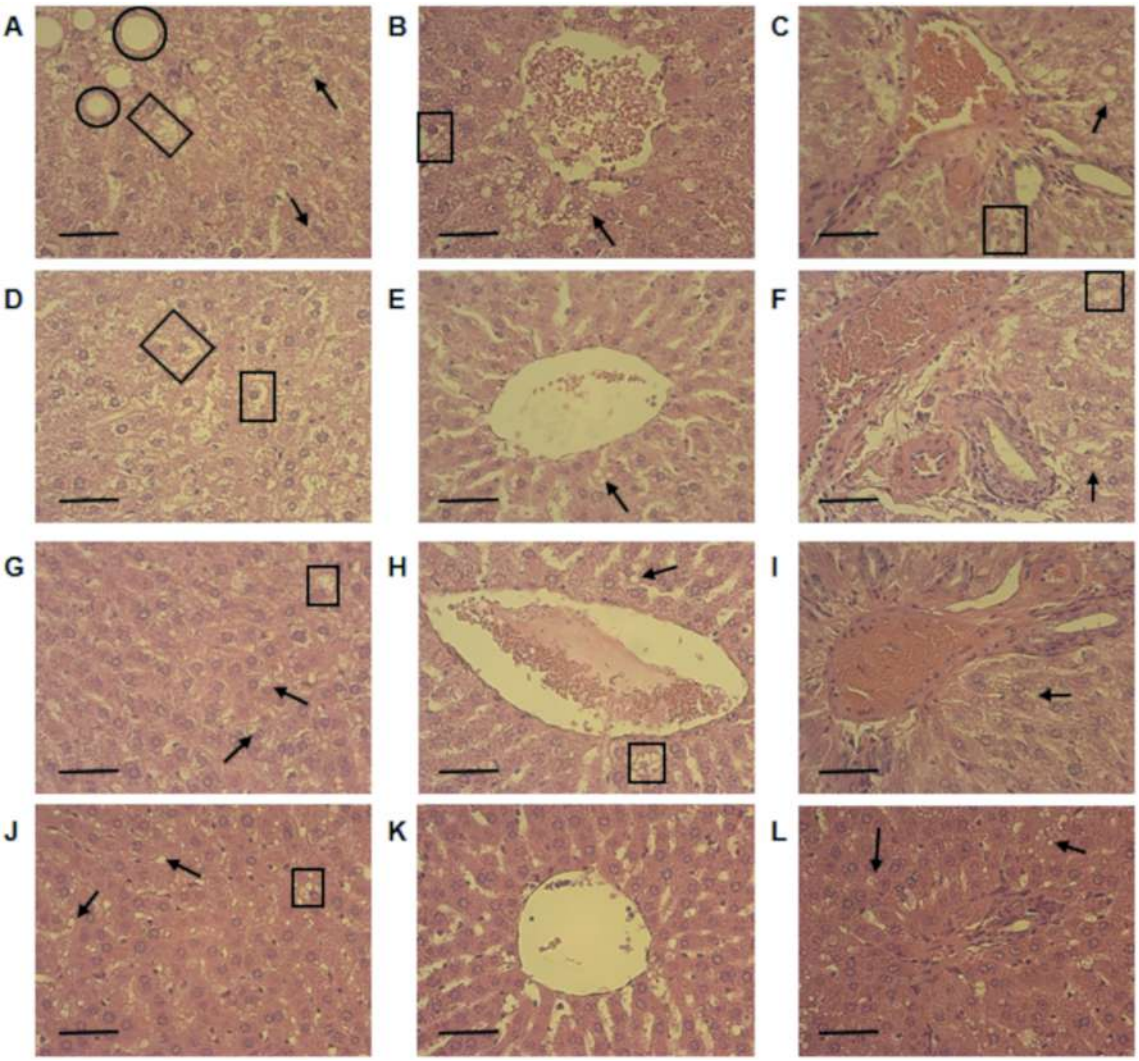

Fig. 5. Effect of treatments with metformin and/or 4-hydroxychalcone (4-HC) on hepatic morphology on groups with NAFLD. Tissues stained with hematoxylin and eosin (H&E) were observed at 40x. Hepatic parenchyma (A, D, G, J). Central vein (B, E, H, K). Portal structure (C, F, I, L). Group treated with saline solution (A–C), metformin (D–F), 4-HC (G–I) and metformin + 4-HC (J–L). The arrows point some hepatocytes with microvesicular steatosis, the squares frame some hepatocytes with macrovesicular steatosis, and the circles frame ballooning cells. Representative images of each group (n = 6). Scale bars = 50 μm.

development of NAFLD. A similar effect, was observed with the treatment with metformin and 4-HC (Fig. 5J-L), this group showed moderate microvesicular steatosis (47.73%) and limited macrovesicular steatosis (11.93%), mainly in parenchyma (Fig. 5J) and portal structure (Fig. 5L), with a mean of steatosis of 59.66%; without ballooned cells, but with one inflammatory infiltrate per sample (data not shown); positioning in grade 3 in the development of NAFLD.

3.6. Effect of treatments on changes in collagen formation in liver tissue

Where all samples from the groups with tap water intake were stained with MT, it became evident that there were no changes in the amount and distribution of collagen as a consequence of the treatment with saline solution (Fig. 6A–C), metformin (Fig. 6D–F), 4-HC (Fig. 6G–I) or metformin and 4-HC (Fig. 6J-L). In other words, these groups showed collagen around portal structures and central veins in a normal way; without showing significant accumulation of collagen in hepatic parenchyma (F0).

In contrast, when samples from the groups with 50% sucrose intake (NAFLD) were stained with MT. It became evident that the group with saline solution administration (Fig. 7A–C) three rats developed fibrosis in perisinusoidal spaces in parenchyma (F1); as well as two rats developed portal fibrosis with few septa (F2) and only one did not show modification on quantity and distribution of collagen (F0). While the treatment with metformin (Fig. 7D–F) decreased the degree of fibrosis induced by the 50% sucrose intake, because three rats presented portal fibrosis with septa (F1) and the three remaining rats did not develop fibrosis (F0).

However, the effect of treatment with 4-HC (Fig. 7G–I) on fibrosis was more remarkable than that presented by the treatment with metformin, this because only one rat develops portal fibrosis without septa (F1) and the five remaining rats did not develop fibrosis (F0). Finally, the group with metformin and 4-HC administration (Fig. 7J-L) was at a midpoint compared with monotherapies of metformin and 4-HC, due to that two rats developed fibrosis without septa (F1) and the other four rats did not develop fibrosis (F0).

3.7. Effect of treatments on serum cytokines

The ingestion of 50% sucrose significantly increased serum levels of IL-6 (Fig. 8A), TNF-α (Fig. 8B) and TGF-β (Fig. 8D), regardless of the treatment received ($P < 0.001$), compared with all groups with tap water ingestion; with the exception of the group with 50% sucrose ingestion and 4-HC administration which showed an increase in TNF-α

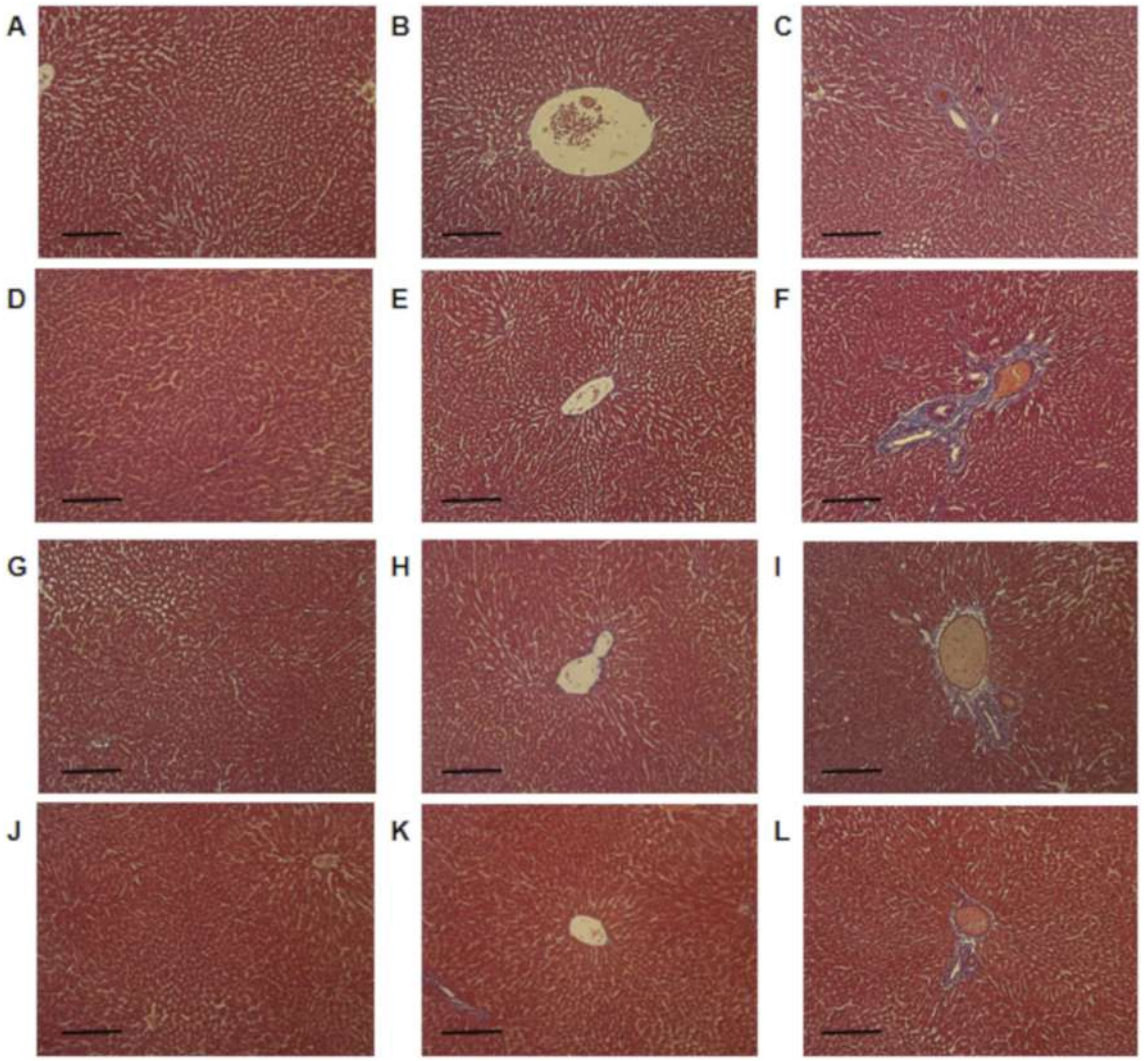

Fig. 6. Effect of treatments with metformin and/or 4-hydroxychalcone (4-HC) on changes of collagen hepatic tissue, on healthy groups. Tissues stained with Masson trichrome (MT) were observed at 10x. Hepatic parenchyma (A, D, G, J). Central vein (B, E, H, K). Portal structure (C, F, I, L). Group treated with saline solution (A–C), metformin (D–F), 4-HC (G–I) and metformin + 4-HC (J–L). Representative images of each group (n = 6). Scale bars = 200 μm.

only against the group with tap water ingestion and treatment with saline solution (95.13 ± 3.7 *vs*. 75.23 ± 2.15 pg/ml; P = 0.014; Fig. 8B). In contrast, the ingestion of 50% sucrose significantly decreased serum levels of IL-10 (Fig. 8C), regardless of the treatment received ($p < 0.01$), compared with all the groups with tap water ingestion; besides NAFLD group and saline solution administration had significantly lower IL-10 serum levels compared with the groups with 50% sucrose ingestion and treatment with 4-HC (255.22 ± 4.42 *vs*. 313.63 ± 6.48 pg/ml; P < 0.001; Fig. 8C) or metformin plus 4-HC (255.22 ± 4.42 *vs*. 304.95 ± 10.24 pg/ml; P = 0.005; Fig. 8C).

On the other hand, the NAFLD group with 4-HC administration was the only that significantly decreased IL-6 serum levels compared with NAFLD group and saline solution administration (94.17 ± 3.11 *vs*. 114.66 ± 4.19 pg/ml; P < 0.001; Fig. 8A). In addition, in TNF-α serum levels the treatment with 4-HC, in NAFLD group (Fig. 8B), significantly decreased it compared with NAFLD groups treated with: saline solution (95.13 ± 3.7 *vs*. 132.27 ± 3.87 pg/ml; P < 0.001), and metformin (95.13 ± 3.7 *vs*. 123.6 ± 2.68 pg/ml; P < 0.001); while treatment with both metformin and 4-HC, in NAFLD group, significantly decreased TNF-α serum levels compared with NAFLD group treated with saline solution (108.33 ± 2.4 *vs*. 132.27 ± 3.9 pg/ml; P = 0.002).

Moreover, it was observed that in NAFLD groups the treatment with 4-HC significantly decreased TGF-β serum levels compared with groups treated with saline solution (494.44 ± 16.1 *vs*. 648.24 ± 5.7 pg/ml; P < 0.001), metformin (494.44 ± 16.1 *vs*. 590.66 ± 14.3 pg/ml; P < 0.001), and metformin plus 4-HC (494.44 ± 16.1 *vs*. 564.02 ± 6.24 pg/ml; P = 0.002). While the monotherapy of metformin (590.66 ± 14.3 *vs*. 648.24 ± 5.7 pg/ml; P = 0.004) and treatment with both metformin and 4-HC (564.02 ± 6.2 *vs*. 648.34 ± 5.7 pg/ml; P < 0.001) significantly decrease TGF-β serum levels compared with NAFLD group and saline solution administration (Fig. 8D).

Finally, when comparing IL-10 serum levels among groups (Fig. 8C), it was observed that in NAFLD groups the treatment with 4-HC (313.63 ± 6.5 *vs*. 255.22 ± 4.4 pg/ml; P < 0.001) and treatment with both metformin and 4-HC (304.95 ± 10.2 *vs*. 255.22 ± 4.4 pg/ml; P = 0.005) significantly increased it compared with NAFLD group and saline solution administration. Additionally, on groups with NAFLD the treatment with metformin had a tendency to increase IL-10 serum levels compared with saline solution administration (286.48 ± 9.1 *vs*. 255.22 ± 4.42 pg/ml; P > 0.05; Fig. 8C) but was not significant.

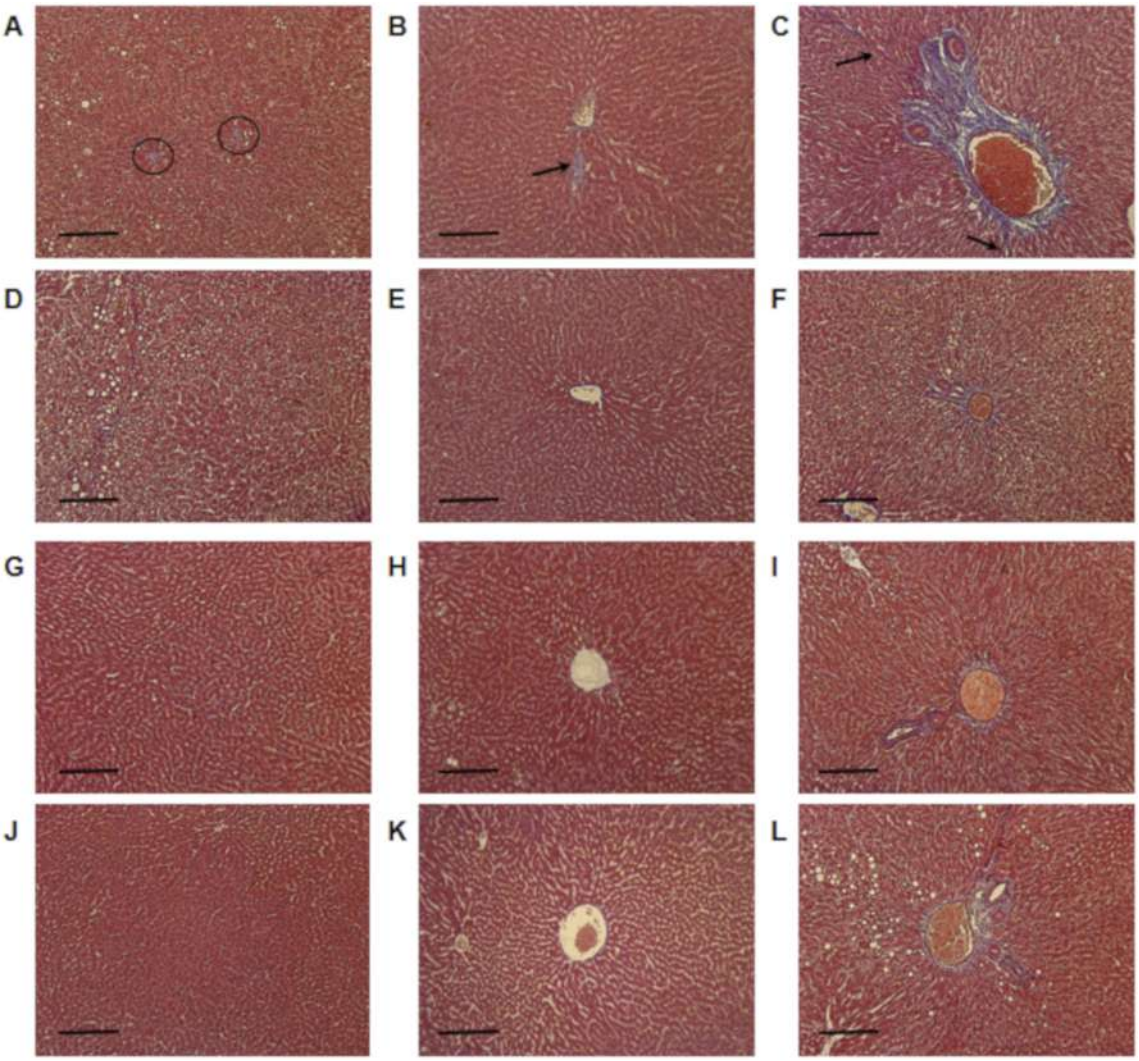

Fig. 7. Effect of treatments with metformin and/or 4-hydroxychalcone (4-HC) on changes of collagen in hepatic tissue, on groups with NAFLD. Tissues stained with Masson trichrome (MT) were observed at 10x. Hepatic parenchyma (A, D, G, J). Central vein (B, E, H, K). Portal structure (C, F, I, L). Group treated with saline solution (A–C), metformin (D–F), 4-HC (G–I) and metformin + 4-HC (J–L). The circles frame fibrosis perisinusoidal without septa; the arrows point fibrosis with septa. Representative images of each group (n = 6). Scale bars = 200 μm.

4. Discussion

4.1. Effect of treatments on eating habits and anthropometric parameters

Previous studies have been shown that metformin inhibit food consumption in rats with metabolic disorders (Jenkins et al., 2012; Lv et al., 2012), while in this study metformin slightly decreased food intake in rats with NAFLD, compared with NAFLD group and vehicle administration; the difference in these results may be due to the lower dose used. The aforementioned can be explained because metformin in the hypothalamus attenuates the monophosphate-activated kinase (AMPK) activity, which decreases neuropeptide Y, agouti-related protein (orexigenic), and increases proopiomelanocortin (anorectic) hypothalamic expression (Lee et al., 2012; Lv et al., 2012). Besides metformin can decreases food intake by improving leptin sensitivity (Malin and Kashyap, 2014) and increasing glucagon-like peptide 1 (GLP-1) levels (Lv et al., 2012; Malin and Kashyap, 2014), that in turn increases insulin (anorectic) secretion (Bouchoucha et al., 2011; McCreight et al., 2016). Nevertheless, it has been shown that metformin reduces leptin secretion *per se* prior to weight loss (Jenkins et al., 2012). This could explain our findings that metformin treatment increased food and total caloric intake in healthy rats compared with healthy group and vehicle administration. These contradictory effects of metformin may be related to different metabolic milieu.

This is the first study that shows 4-HC reduced food and total caloric intake in rats with NAFLD despite they were not significant, but without changes in body weight compared with rats with NAFLD and vehicle administration, probably because the dose used was low. However, there are few reports of this effect for *trans-chalcone*, which also has shown reduced food intake in rats (Karimi-Sales et al., 2018a; Karkhaneh et al., 2016; Najafian et al., 2010), but have not been reported the possible anorectic mechanism neither; nevertheless some authors suggest that *trans-chalcone* can improve leptin sensibility (Jalalvand et al., 2015; Karkhaneh et al., 2016), being a possible pathway to reduce food intake for 4-HC.

Interestingly, when animals received the co-treatment, food and total caloric intake was reduced both in healthy and NAFLD groups compared with its respective group with vehicle administration; and in rats with NAFLD the effect was greater than the monotherapies. Also, on the last two weeks of the co-treatment it was observed a decrease on body weight in rats with NAFLD compared with that which received vehicle; suggesting a possible synergistic effect on increasing leptin sensibility (Karkhaneh et al., 2016; Malin and Kashyap, 2014). However, further analysis will be necessary to understand this aspect.

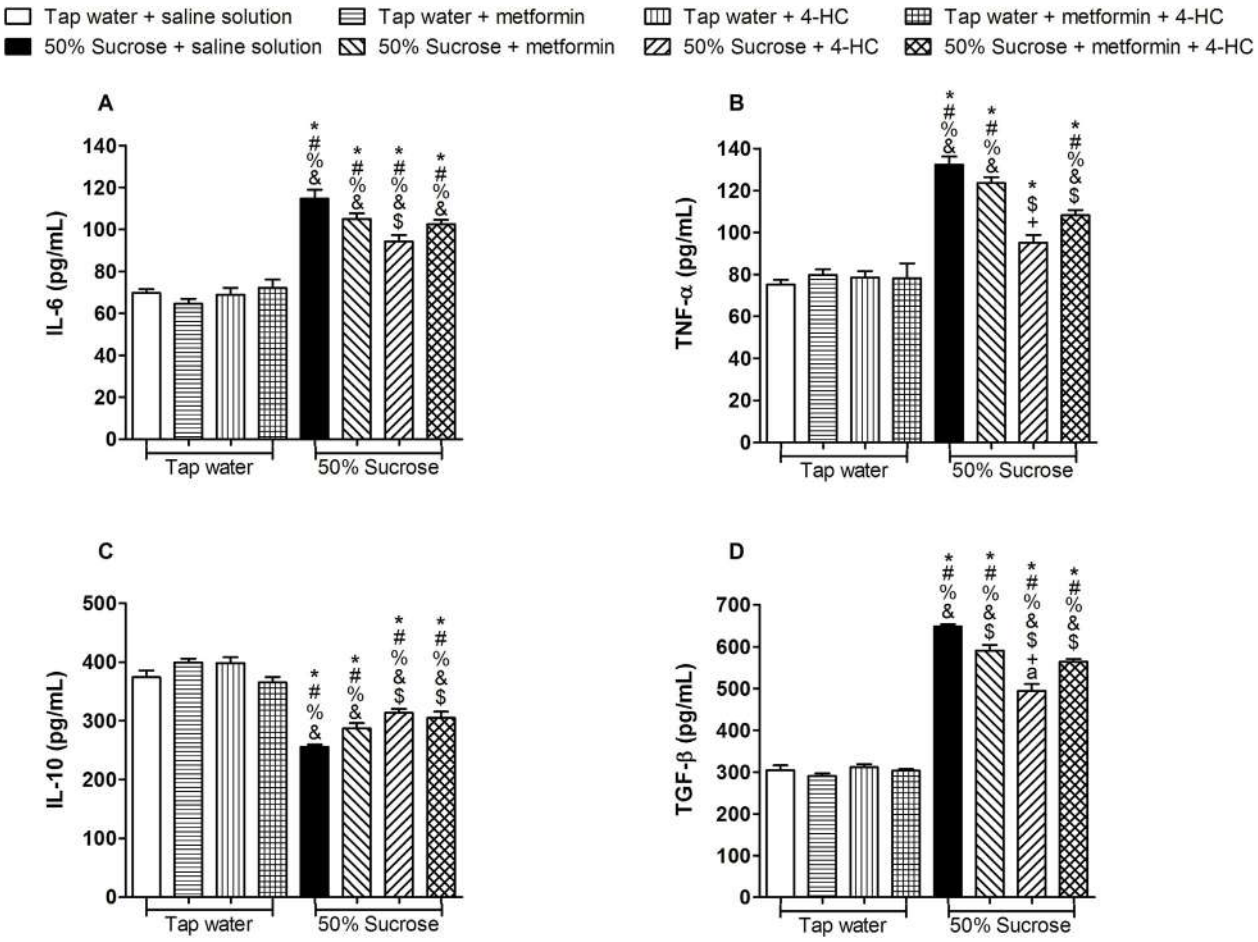

Fig. 8. Effect of treatments with metformin and/or 4-hydroxychalcone (4-HC) on serum levels of interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), interleukin-10 (IL-10) and transforming grow factor beta (TGF-β). Upper panel shows the effect of treatments on IL-6 (A) and TNF-α (B), and bottom panel shows the effect of treatments on IL-10 (C) and TGF-β (D). Each bar represents the mean ± S.E.M. of n = 6 each group. One-way ANOVA and Tukey post hoc test were performed. *, $p < 0.05$ *vs.* tap water + saline solution; #, $p < 0.05$ *vs.* tap water + metformin; %, $p < 0.05$ *vs.* tap water + 4-HC; &, $p < 0.05$ *vs.* tap water + metformin + 4-HC; $, $p < 0.05$ *vs.* 50% sucrose + saline solution; +, $p < 0.05$ *vs.* 50% sucrose + metformin; a, $p < 0.05$ *vs.* metformin + 4-HC.

Moreover, NAFLD groups did not show changes in body weight compared with healthy groups, except for the group with NAFLD and co-treatment which had the lowest food intake and body weight. However, NAFLD groups showed a significantly increase on percentage weight of intraabdominal fat, compared with healthy groups. These results could be explain due to the changes on caloric intake, where NAFLD groups had an increase on carbohydrates and sucrose intake, although had a decrease on proteins and fat intake, leading to an increase on metabolism of carbohydrates which promotes lipogenesis *de novo* (Ahmed et al., 2015), and promoting the increase of intraabdominal fat.

4.2. Effects of treatments on diarrhea frequency

Diarrhea is the most common gastrointestinal symptom of metformin (Bonnet and Scheen, 2017). Therefore, we monitored diarrhea frequency induced by the different treatments given to each group; finding that metformin increased this parameter in both healthy and NAFLD groups compared with vehicle (not significant). This can be explained because metformin can increase bile acid pool and GLP-1 levels, stimulates release of serotonin (5-HT), acts as agonist of 5-HT_3 receptors, and increase histamine levels in intestine (Cubeddu et al., 2000; McCreight et al., 2016; Yee et al., 2015).

In contrast, this study is the first shown that 4-HC treatment does not induce diarrhea in either healthy rats or rats with NAFLD. Being an important result, since approximately 30% of patients with metformin treatment have diarrhea and this leads to 5% discontinuing treatment (Bonnet and Scheen, 2017). Surprisingly, the co-treatment increased diarrhea frequency in both healthy and NAFLD groups compared with vehicle, respectively, being even twice that observed in groups with monotherapy of metformin. Being evident that it is not recommendable give the co-treatment due to the increase in this side effect; and further studies are needed to clarify the mechanism of action that leads to this synergistic effect.

4.3. Effect of treatments on improving liver damage and diminishing inflammation

In this study the development of NASH was a greater degree compared to previous study (Acosta-Cota et al., 2019), because the induction was for 5 weeks more compared to that study. That is to say, the group with 50% sucrose ingestion during 25 weeks developed grade 7 of NAFLD, where the highest grade that can be reached is 8 (LaBrecque et al., 2012).

Being the main effect of treatments the decrease on liver damage and inflammation. Where, in rats with NAFLD, metformin treatment decreased hepatic TC, steatosis and fibrosis, and TGF-β serum levels; these results are consistent with previous reports (Fan et al., 2017; Feng et al., 2019; Raso et al., 2009; Spruss et al., 2012). However, our results related with the effect of metformin treatment on IL-6 and TNF-α serum levels are similar with previous reports (Raso et al., 2009; Spruss et al., 2012; Woo et al., 2014) in which metformin significantly decreased

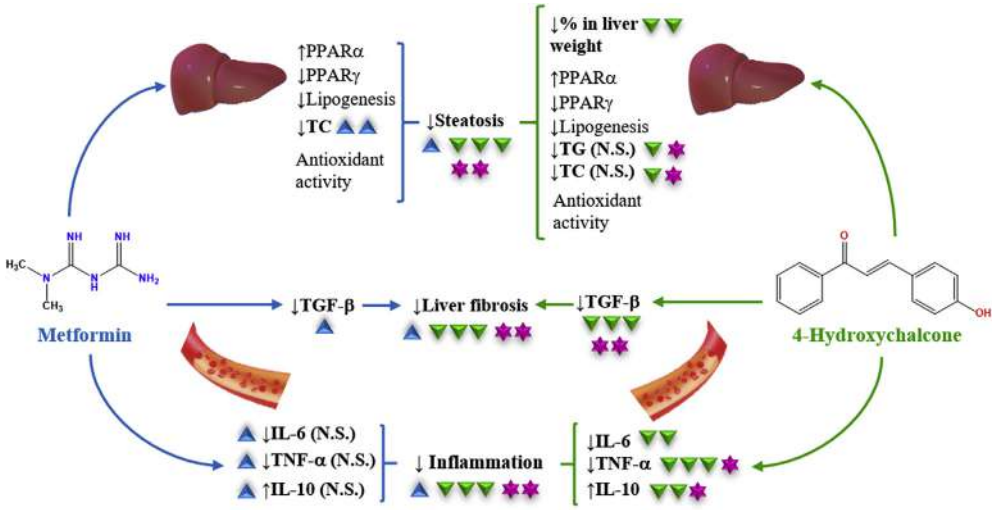

Fig. 9. Possible mechanisms of action of treatments with metformin and/or 4-hydroxychalcone (4-HC) on rats with nonalcoholic fatty liver disease. The effect of metformin on decreased steatosis can be probably because has antioxidant activity, increase hepatic peroxisome proliferator activated receptor (PPAR)-α, decrease PPAR-γ, and may decrease lipogenesis. This can explain our results of metformin on decrease hepatic total cholesterol (TC). Moreover, the effect of metformin on decreased liver fibrosis may be due to it decreased transforming grow factor beta (TGF-β), which is a key in the development of liver fibrosis. Metformin also had limited effect on decrease inflammation, because it decreased interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), and increased interleukin-10 (IL-10), however, there were not statistically significant. On the other hand, 4-HC showed the most marked therapeutic effect on decreased liver steatosis, liver fibrosis and inflammation. The effect of 4-HC on decrease liver steatosis is related with the effect shown in decrease percentage (%) in liver weight; both effects can be due to 4-HC has antioxidant activity and may be increase PPARα, and decrease PPARγ and lipogenesis; in turn this could explain our results, where 4-HC decreased hepatic triglycerides (TG) and TC, even though it were not statistically significant. Finally, contrary to expectations, the co-treatment showed an intermediate effect on decrease inflammation, liver steatosis and fibrosis, suggesting a possible antagonism, between metformin and 4-HC (future studies are required to understand the mechanism)., effect by metformin;, effect by 4-HC;, effect by co-treatment. One symbol means low effect, two symbols mean medium effect and three symbols mean high effect. Not significant (N.S.).

these parameters while in this study the effect was not significant, these variations may be due to the different dosages and models used. Moreover, we reported that metformin increased IL-10 serum levels (not significant). Taken together these results suggest that metformin has limited therapeutic effect on reducing liver steatosis, fibrosis and inflammation.

On the other hand, previous studies had shown that metformin activates hepatic AMPK leading to a decrease on lipogenesis, an increase in fat oxidation (Iranshahy et al., 2019; Lopez, 2018; Malin and Kashyap, 2014), an increase in hepatic peroxisome proliferator activated receptor (PPAR)-α and a decrease in PPAR-γ (Iranshahy et al., 2019; Raso et al., 2009); this together leads to decreased hepatic TC and steatosis. Moreover, metformin had an anti-inflammatory activity due to it increased IL-10 serum and decreased hepatic inflammatory infiltrate. Furthermore, several studies have shown that metformin improves liver antioxidant activity (Fig. 9) by a decrease of malondialdehyde, and an increase of superoxide dismutase, catalase and glutathione peroxidase in liver (Srividhya and Anuradha, 2002; Xu et al., 2016), which is an important role in the development of inflammation and in the activation of Kupffer cells and hepatic stellate cells (HSC) (López-Panqueva, 2013; Peverill et al., 2014). While Kupffer cells activated lead to an increase in TGF-β, which activate HSC and plays central role in the progression of liver fibrosis (Fan et al., 2017). The above mention is consistently with our results, because metformin decreased TGF-β and liver fibrosis.

The treatment with 4-HC showed the most marked therapeutic effect among the treatments tested. Thus, 4-HC treatment in NAFLD rats significantly decreased percentage in liver weight, hepatic TG, TC, steatosis and fibrosis; it also induced a marked decrease on hepatic and systemic inflammation, by diminish liver inflammatory infiltrates, decrease IL-6, TNF-α, TGF-β and increase IL-10 serum levels (Fig. 9). Admittedly, the degree of steatosis could be higher in other models, such as db/db mice (Fellmann et al., 2013), diet induced obesity and Spontaneously Diabetic Torii rats (Ohta et al., 2018), we suggest future studies with other NAFLD models. Despite, this study is the first to show that 4-HC has therapeutic effect on NAFLD. However, our results are similar with Qu et al. (2014) who reported that 4-HC decreased IL-1β, TNF-α and NF-κB in kidney cells. Furthermore, 4-HC has shown to stimulate PPAR-α (Mueller et al., 2011), and antioxidant activity (Kucerova-Chlupacova et al., 2018). Moreover, the chalcone L2H17 reduces the production of IL-1β, IL-6, TNF-α, suppresses NF-κB and reduces fibrosis in heart and kidney by decrease TGF-β and collagen IV (Fang et al., 2015); trans-chalcone decreases PPAR-γ and TNF-α and other chalcones decrease IL-6, TNF-α and increase PPAR-α (Karimi-Sales et al., 2018b).

Taken together these results and previous, we can suggest that the therapeutic effect of 4-HC on decreasing steatosis could be due to its action on PPAR-α and PPAR-γ, which are important in lipogenesis, inducing a lipogenesis decrease (Karimi-Sales et al., 2018b), that in turn could be observed in a decreased in hepatic TG, TC and steatosis. While the decrease in inflammatory cytokines (IL-6, TNF-α) and the increase in IL-10, are consistent with the diminish in liver inflammatory infiltrates; probably 4-HC also induce antioxidant activity in this model, suggesting this because there are different studies that report antioxidant activity of 4-HC (Díaz-Carrillo et al., 2018; Quian et al., 2011); all this, in turn can explain the attenuation of TGF-β and fibrosis (Fig. 9); because oxidant activity and inflammation play an important role in activate Kupffer cells and HSC.

Finally, the co-treatment showed an intermediate therapeutic effect on decrease hepatic steatosis, fibrosis and on anti-inflammatory effect, this between the monotherapies; suggesting a possible antagonism at this level. However, future studies are required to understand the possible molecular mechanism of action and antioxidant activity.

5. Conclusion

Our results suggest that monotherapy with 4-HC had therapeutic effect in male Wistar rats with NAFLD, by a marked decreased in the severity of hepatic steatosis, fibrosis and inflammation, and was the most effective among the treatments tested. Showing a possible new pharmacological therapy to NAFLD.

Funding

This study was supported by Autonomous University of Sinaloa through grant number PROFAPI2015/201.

CRediT authorship contribution statement

Selene de Jesús Acosta-Cota: Data curation, Formal analysis,

Investigation, Methodology, Writing - original draft. **Elsa Maribel Aguilar-Medina:** Investigation, Supervision, Resources, Project administration, Writing - review & editing. **Rosalío Ramos-Payán:** Resources, Supervision, Writing - review & editing. **José Guadalupe Rendón Maldonado:** Writing - review & editing. **José Geovanni Romero-Quintana:** Supervision, Writing - review & editing. **Julio Montes-Avila:** Resources, Methodology. **Juan I. Sarmiento-Sánchez:** Writing - review & editing. **Carolina Gabriela Plazas-Guerrero:** Methodology, Data curation. **Marcela J. Vergara-Jiménez:** Resources, Supervision, Writing - review & editing. **Araceli Sánchez-López:** Resources, Writing - review & editing. **David Centurión:** Resources, Writing - review & editing. **Ulises Osuna-Martínez:** Conceptualization, Investigation, Supervision, Resources, Funding acquisition, Methodology, Project administration, Writing - review & editing.

Declaration of competing interest

None declared.

Acknowledgements

The authors would like to thank Prof. Histotechnologist Elvic Noguera Corona for his valuable scientific assistance and advice in the processing of liver samples for histology, as well as the analysis of them.

References

Acosta-Cota, S.J., Aguilar-Medina, E.M., Ramos-Payan, R., Ruiz-Quinonez, A.K., Romero-Quintana, J.G., Montes-Avila, J., Rendon-Maldonado, J.G., Sanchez-Lopez, A., Centurion, D., Osuna-Martinez, U., 2019. Histopathological and biochemical changes in the development of nonalcoholic fatty liver disease induced by high-sucrose diet at different times. Can. J. Physiol. Pharmacol. 97, 23–36.

Ahmed, M.H., Husain, N.E., Almobarak, A.O., 2015. Nonalcoholic Fatty liver disease and risk of diabetes and cardiovascular disease: what is important for primary care physicians? J. Fam. Med. Prim. Care 4, 45–52.

Bonnet, F., Scheen, A., 2017. Understanding and overcoming metformin gastrointestinal intolerance. Diabetes Obes. Metab. 19, 473–481.

Bouchoucha, M., Uzzan, B., Cohen, R., 2011. Metformin and digestive disorders. Diabetes Metab. 37, 90–96.

Cicero, A.F.G., Sahebkar, A., Fogacci, F., Bove, M., Giovannini, M., Borghi, C., 2019. Effects of phytosomal curcumin on anthropometric parameters, insulin resistance, cortisolemia and non-alcoholic fatty liver disease indices: a double-blind, placebo-controlled clinical trial. Eur. J. Nutr. 1–7. https://doi.org/10.1007/s00394-019-01916-7.

Cole, B.K., Feaver, R.E., Wamhoff, B.R., Dash, A., 2018. Non-alcoholic fatty liver disease (NAFLD) models in drug discovery. Expert Opin. Drug Discov. 13, 193–205.

Croci, I., Coombes, J.S., Sandbakk, S.B., Keating, S.E., Nauman, J., Macdonald, G.A., Wisloff, U., 2019. Non-alcoholic Fatty Liver Disease: Prevalence and All-Cause Mortality According to Sedentary Behaviour and Cardiorespiratory Fitness. The HUNT Study. Progress in Cardiovascular Diseases.

Cubeddu, L.X., Bonisch, H., Gothert, M., Molderings, G., Racke, K., Ramadori, G., Miller, K.J., Schworer, H., 2000. Effects of metformin on intestinal 5-hydroxytryptamine (5-HT) release and on 5-HT3 receptors. Naunyn Schmiedeberg's Arch. Pharmacol. 361, 85–91.

Díaz-Carrillo, J.T., Díaz-Camacho, S.P., Delgado-Vargas, F., Rivero, I.A., López-Angulo, G., Sarmiento-Sánchez, J.I., Montes-Avila, J., 2018. Synthesis of leading chalcones with high antiparasitic, against Hymenolepis nana, and antioxidant activities. Braz. J. Pharm. Sci. 54.

Diehl, A.M., Day, C., 2017. Cause, pathogenesis, and treatment of nonalcoholic steatohepatitis. N. Engl. J. Med. 377, 2063–2072.

Dietrich, P., Hellerbrand, C., 2014. Non-alcoholic fatty liver disease, obesity and the metabolic syndrome. Best practice & research. Clin. gastroenterol. 28, 637–653.

Doycheva, I., Loomba, R., 2014. Effect of metformin on ballooning degeneration in nonalcoholic steatohepatitis (NASH): when to use metformin in nonalcoholic fatty liver disease (NAFLD). Adv. Ther. 31, 30–43.

Ebadollahi-Natanzi, A.R., Mahmoudian, S., Minaeie, B., Sabzevari, O., 2011. Hepatoprotective activity of phloretin and hydroxychalcones against Acetaminophen Induced hepatotoxicity in mice. Iran. J. Pharm. Sci. 7.

Fan, K., Wu, K., Lin, L., Ge, P., Dai, J., He, X., Hu, K., Zhang, L., 2017. Metformin mitigates carbon tetrachloride-induced TGF-beta1/Smad3 signaling and liver fibrosis in mice. Biomed. Pharmacother. = Biomedecine & pharmacotherapie 90, 421–426.

Fang, Q., Wang, J., Wang, L., Zhang, Y., Yin, H., Li, Y., Tong, C., Liang, G., Zheng, C., 2015. Attenuation of inflammatory response by a novel chalcone protects kidney and heart from hyperglycemia-induced injuries in type 1 diabetic mice. Toxicol. Appl. Pharmacol. 288, 179–191.

Fazel, Y., Koenig, A.B., Sayiner, M., Goodman, Z.D., Younossi, Z.M., 2016. Epidemiology and natural history of non-alcoholic fatty liver disease. Metab. Clin. Exp. 65, 1017–1025.

Fellmann, L., Nascimento, A.R., Tibirica, E., Bousquet, P., 2013. Murine models for pharmacological studies of the metabolic syndrome. Pharmacol. Ther. 137, 331–340.

Feng, W.H., Bi, Y., Li, P., Yin, T.T., Gao, C.X., Shen, S.M., Gao, L.J., Yang, D.H., Zhu, D.L., 2019. Effects of liraglutide, metformin and gliclazide on body composition in patients with both type 2 diabetes and non-alcoholic fatty liver disease: a randomized trial. J. Diabetes Investig. 10, 399–407.

Guide for the care and use of laboratory animals. In: Guide for the Care and Use of Laboratory Animals, 8th. Washington (DC).

Haukeland, J.W., Konopski, Z., Eggesbo, H.B., von Volkmann, H.L., Raschpichler, G., Bjoro, K., Haaland, T., Loberg, E.M., Birkeland, K., 2009. Metformin in patients with non-alcoholic fatty liver disease: a randomized, controlled trial. Scand. J. Gastroenterol. 44, 853–860.

Iranshahy, M., Rezaee, R., Karimi, G., 2019. Hepatoprotective activity of metformin: a new mission for an old drug? Eur. J. Pharmacol. 850, 1–7.

Jalalvand, F., Amoli, M.M., Yaghmaei, P., Kimiagar, M., Ebrahim-Habibi, A., 2015. Acarbose versus trans-chalcone: comparing the effect of two glycosidase inhibitors on obese mice. Arch. Endocrinol. Metab. 59, 202–209.

Jenkins, N.T., Padilla, J., Arce-Esquivel, A.A., Bayless, D.S., Martin, J.S., Leidy, H.J., Booth, F.W., Rector, R.S., Laughlin, M.H., 2012. Effects of endurance exercise training, metformin, and their combination on adipose tissue leptin and IL-10 secretion in OLETF rats. J. Appl. Physiol. 113, 1873–1883.

Karimi-Sales, E., Jeddi, S., Ebrahimi-Kalan, A., Alipour, M.R., 2018a. Trans-chalcone enhances insulin sensitivity through the miR-34a/SIRT1 pathway. Iran. J. Basic Med. Sci. 21, 359–363.

Karimi-Sales, E., Mohaddes, G., Alipour, M.R., 2018b. Chalcones as putative hepatoprotective agents: preclinical evidence and molecular mechanisms. Pharmacol. Res. 129, 177–187.

Karkhaneh, L., Yaghmaei, P., Parivar, K., Sadeghizadeh, M., Ebrahim-Habibi, A., 2016. Effect of trans-chalcone on atheroma plaque formation, liver fibrosis and adiponectin gene expression in cholesterol-fed NMRI mice. Pharmacol. Rep. : PR 68, 720–727.

Kierszenbaum, A.L., 2008. Histología y Biología Celular: Introducción a la anatomía patológica, Tercera ed. Elsevier Mosby, Barcelona, España.

Kucerova-Chlupacova, M., Dosedel, M., Kunes, J., Soltesova-Prnova, M., Majekova, M., Stefek, M., 2018. Chalcones and their pyrazine analogs: synthesis, inhibition of aldose reductase, antioxidant activity, and molecular docking study. Monatshefte für Chemie-Chem. Mon. 1–9.

LaBrecque, D., Abbas, Z., Anania, F., Ferenci, P., Ghafor-Kjan, A., Goh, K.L., Hamid, S.S., Isakov, V., Lizarzabal, M., Mojica-Pernaranda, M., Rivera-Ramos, J.F., Sarin, S., Stimac, D., Thomson, A., Umar, M., Krabshuis, J., LeMair, A., 2012. In: Gastroenterología, O.M.d. (Ed.), Enfermedad del hígado graso no alcohólico y esteatohepatitis no alcohólica.

Lee, C.K., Choi, Y.J., Park, S.Y., Kim, J.Y., Won, K.C., Kim, Y.W., 2012. Intracerebroventricular injection of metformin induces anorexia in rats. J. Diabetes Metab. 36, 293–299.

Li, X., Chen, Y., Zhao, Z., Lu, W., Zhou, T., 2016. Pharmacokinetic/pharmacodynamic analysis of metformin using different models in diabetic rats. Drug Res. 66, 547–554.

Liu, F., Wang, C., Zhang, L., Xu, Y., Jang, L., Gu, Y., Cao, X., Zhao, X., Ye, J., Li, Q., 2014. Metformin prevents hepatic steatosis by regulating the expression of adipose differentiation-related protein. Int. J. Mol. Med. 33, 51–58.

Lopez, M., 2018. Hypothalamic AMPK and energy balance. Eur. J. Clin. Investig. 48, e12996.

López-Oliva, E., Muñoz-Martínez, E., 2014. SREBP-1c, ChREBP y LXR: su influencia en el desarrollo de hígado graso no alcohólico. An. R. Acad. Farmac. 80, 14–48.

López-Panqueva, R.L., 2013. Hepatología para gastroenterólogos y hepatólogos. Rev. Colomb. Gastroenterol. 28, 161–168.

Lv, W.S., Wen, J.P., Li, L., Sun, R.X., Wang, J., Xian, Y.X., Cao, C.X., Wang, Y.L., Gao, Y.Y., 2012. The effect of metformin on food intake and its potential role in hypothalamic regulation in obese diabetic rats. Brain Res. 1444, 11–19.

Malin, S.K., Kashyap, S.R., 2014. Effects of metformin on weight loss: potential mechanisms. Curr. Opin. Endocrinol. Diabetes Obes. 21, 323–329.

McCreight, L.J., Bailey, C.J., Pearson, E.R., 2016. Metformin and the gastrointestinal tract. Diabetologia 59, 426–435.

Mueller, M., Beck, V., Jungbauer, A., 2011. PPARalpha activation by culinary herbs and spices. Planta Med. 77, 497–504.

Najafian, M., Ebrahim-Habibi, A., Yaghmaei, P., Parivar, K., Larijani, B., 2010. Core structure of flavonoids precursor as an antihyperglycemic and antihyperlipidemic agent: an in vivo study in rats. Acta Biochim. Pol. 57, 553–560.

Narender, T., Papi Reddy, K., 2007. A simple and highly efficient method for the synthesis of chalcones by using borontrifluoride-etherate. Tetrahedron Lett. 3177–3180.

Ohta, T., Yamada, T., Kamiya, T., Gotoh, T., Tsubaki, M., Shinohara, M., 2018. Quick-Fat diet inhibits the development of diabetes in Spontaneously Diabetic Torii (SDT) rats. Thai J. Pharm. Sci. (TJPS) 42.

Peverill, W., Powell, L.W., Skoien, R., 2014. Evolving concepts in the pathogenesis of NASH: beyond steatosis and inflammation. Int. J. Mol. Sci. 15, 8591–8638.

Qu, Q., Dai, B., Yang, B., Li, X., Liu, Y., Zhang, F., 2014. 4-Hydroxychalcone attenuates hyperaldosteronism, inflammation, and renal injury in cryptochrome-null mice. BioMed Res. Int. 2014 603415.

Quian, Y., Shang, Y., Teng, Q., Chang, J., Fan, G., Wei, X., Li, R., Li, H., Yao, X., Dai, F., Zhou, B., 2011. Hydroxychalcones as potent antioxidants: structure-activity relationship analysis and mechanism considerations. Food Chem. 126, 241–248.

Ramachandran, R., Saraswathy, M., 2014. Up-regulation of nuclear related factor 2 (NRF2) and antioxidant responsive elements by metformin protects hepatocytes against the acetaminophen toxicity. Toxicol. Res. 3, 350–358.

Raso, G.M., Esposito, E., Iacono, A., Pacilio, M., Cuzzocrea, S., Canani, R.B., Calignano, A., Meli, R., 2009. Comparative therapeutic effects of metformin and vitamin E in a

model of non-alcoholic steatohepatitis in the young rat. Eur. J. Pharmacol. 604, 125–131.
Rosini, T.C., Silva, A.S., Moraes, C., 2012. Diet-induced obesity: rodent model for the study of obesity-related disorders. Rev. Assoc. Med. Bras. 58, 383–387.
Sanches, S.C., Ramalho, L.N., Augusto, M.J., da Silva, D.M., Ramalho, F.S., 2015. Nonalcoholic steatohepatitis: a search for factual animal models. BioMed Res. Int. 2015, 574832.
Spruss, A., Kanuri, G., Stahl, C., Bischoff, S.C., Bergheim, I., 2012. Metformin protects against the development of fructose-induced steatosis in mice: role of the intestinal barrier function. Lab. Investig. J. Tech. Methods Pathol. 92, 1020–1032.
Srividhya, S., Anuradha, C.V., 2002. Metformin improves liver antioxidant potential in rats fed a high-fructose diet. Asia Pac. J. Clin. Nutr. 11, 319–322.
Woo, S.L., Xu, H., Li, H., Zhao, Y., Hu, X., Zhao, J., Guo, X., Guo, T., Botchlett, R., Qi, T., Pei, Y., Zheng, J., Xu, Y., An, X., Chen, L., Chen, L., Li, Q., Xiao, X., Huo, Y., Wu, C., 2014. Metformin ameliorates hepatic steatosis and inflammation without altering adipose phenotype in diet-induced obesity. PLoS One 9, e91111.
Xu, H., Zhou, Y., Liu, Y., Ping, J., Shou, Q., Chen, F., Ruo, R., 2016. Metformin improves hepatic IRS2/PI3K/Akt signaling in insulin-resistant rats of NASH and cirrhosis. J. Endocrinol. 229, 133–144.
Yee, S.W., Lin, L., Merski, M., Keiser, M.J., Gupta, A., Zhang, Y., Chien, H.C., Shoichet, B.K., Giacomini, K.M., 2015. Prediction and validation of enzyme and transporter off-targets for metformin. J. Pharmacokinet. Pharmacodyn. 42, 463–475.
Zhou, S., Wang, Y., Jiang, Y., Zhang, Z., Sun, X., Yu, L.L., 2017. Dietary intake of structured lipids with different contents of medium-chain fatty acids on obesity prevention in C57BL/6J mice. J. Food Sci. 82, 1968–1977.