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ORIGINAL PAPER

Nonalcoholic Steatohepatitis (NASH) in OB/OB Mice Treated with Yo Jyo Hen Shi Ko (YHK): Effects on Peroxisome Proliferator-Activated Receptors (PPARs) and Microsomal Triglyceride Transfer Protein (MTP)

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Abstract YHK has antioxidant properties, has a hypoglycemic effect, and may reduce plasma lipid levels. In this study, we examined the hepatic expression of PPAR-α and -γ and MTP in ob/ob mice receiving or not receiving YHK. Ob/ob mice were assigned to receive oral YHK (20 mg/kg/day) fed solution (methionine/choline-deficient [MCD] diet + YHK group) or vehicle (MCD group) by gavage for 4 weeks. Liver fragments were collected for histologic examination and mRNA isolation. PPAR-α and -γ and MTP gene expression was examined by RT-qPCR. YHK treatment was associated with NASH prevention, weight loss, and reduction of visceral fat and of serum concentrations of aminotransferases in comparison to the MCD group. YHK promoted an increment in PPAR-α and MTP and a decrement in PPAR-γ mRNA contents. These findings suggest that modulation of PPAR-α and -γ and MTP RNA expression may be implicated in the protective effect of YHK in experimental NASH, limiting hepatocyte lipid accumulation.

Keywords Nonalcoholic steatohepatitis · Methionine/choline-deficient diet · Peroxisome proliferator-activated receptors · Microsomal triglyceride transfer protein · Yo Jyo Hen Shi Ko · Reverse transcription-quantitative polymerase chain reaction

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Introduction

NASH is an important form of liver disease that may progress to cirrhosis and liver failure [1–3]. The mechanisms that mediate the transition from steatosis to NASH remain unknown and the "two-hit" hypothesis has been proposed [4]. Although the pathophysiology of steatosis is multifactorial, several data suggest the relationship between PPARs and the occurrence or progression of NASH [5, 6].

PPARs are a family of ligand-activated transcription factors that bind to fatty-derived ligands and activate the transcription of genes which regulate lipid metabolism. PPAR-γ, found predominantly in adipocytes, has a very low expression in the normal liver [7], but its upregulation observed in experimental models of NAFLD induces steatosis through regulation of de novo lipid synthesis [8–10]. PPAR-α is the most abundant subtype found in the liver, where it increases catabolism of fatty acids by upregulating the expression of several genes involved in peroxisomal and mitochondrial β-oxidation and fatty acid transport [11]. Nevertheless, there are controversies regarding hepatic expression of PPAR-α in nonalcoholic fatty liver disease (NAFLD).

Microsomal triglyceride transfer protein (MTP), a target gene of PPAR-α, may play a pivotal role in lipoprotein assembly in the endoplasmatic reticulum. It has been demonstrated that, in vitro, MTP mobilizes neutral lipids from donor vesicles to acceptor vesicles [12, 13]. Downregulation of MTP gene expression and upregulation of VLDL degradation facilitate intracellular fat accumulation in hepatocytes, increasing the susceptibility to hepatic steatosis [14, 15].

Although the role of PPAR in the pathogenesis of NASH remains uncertain, recent studies have suggested the therapeutic value of PPAR-α and -γ agonists in the treatment of this condition [16–18].

YHK (Kyotsujigyo Inc., Japan), derived from Henshiko, has four major ingredients (*Panax* pseudo ginseng, *Eucommia ulmoides*, *Polygonati rhizoma*, and licorice root) that are reported to react with reactive oxygen species (ROS), blocking the propagation of radical reactions [19–21]. Most studies evaluating the potential therapeutic of YHK reports its activity as an antifibrotic agent [22, 23] and its protective effects in induced hepatic injury [19, 20]. Recently, a randomized pilot study demonstrated a significant improvement in aminotransferase levels of NASH patients treated with YHK [24]. Also, we have previously shown that YHK prevents NASH in ob/ob mice fed a high-fat or methionine/choline-deficient (MCD) diet [25].

In order to better investigate the protective effect of this compound in experimental NASH, the objective of this study was to evaluate the role of YHK in the hepatic expression of PPAR-α, PPAR-γ, and MTP, a target gene of PPAR-α, in ob/ob mice receiving a MCD diet.

Materials and methods

Materials

YHK was provided by Kyotsujigyo Inc., Japan (http://www.kyotsujigyo.com). This preparation contains four different botanical derivatives (*Panax* pseudo ginseng [40%–60%], *Eucommia ulmoides* [30%–40%], *Polygonati rhizome* [8%–12%], and licorice root [8–12]).

Animals

Male ob/ob mice (Jackson Laboratories, Bar Harbor, ME, USA), 8 weeks old and weighing 30–40 g, were housed in temperature- and humidity-controlled rooms, kept on a 12-hr light/dark cycle, and provided unrestricted amounts of food and water. All procedures for animal experimentation were in accordance with the Helsinki Declaration of 1975 (NIH Publication No. 85–23; revised 1996) and the Guidelines of Animal Experimentation from the University of Sao Paulo School of Medicine. The ob/ob mice (control group; $n = 5$) received a standard diet (Nuvilab; Nutrientes Ltd., Colombo, Brazil) ad libitum. NASH was induced in ob/ob mice by a MCD diet (62.5% carbohydrate, with starch and sucrose; 17% protein, with casein without methionine/choline; 7% lipid, with soybean oil; 1% AIN-93M vitamin mix; 3.5% AIN-93M mineral mix; (Rhoster Ind. Com. Ltd., Sao Paulo, Brazil), 5 g/day, for 4 weeks. The ob/ob mice were divided into a control group fed a standard diet and two experimental groups which were fed as follows: (1) MCD group ($n = 5$)—fed MCD diet plus vehicle (physiologic Ringer's solution); and (2) MCD + YHK group ($n = 5$)—fed MCD diet plus YHK solution (20 mg/kg) daily by gavage. After 4 weeks of treatment with YHK or vehicle plus MCD diet, ob/ob mice were sacrificed (at 12 weeks). Plasma samples and livers were collected for biochemical analysis, mRNA isolation, and histologic examination. During the study period, food intake was estimated by daily observation of residual food at the time of feeding.

Biochemical analysis

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), cholesterol and triglyceride levels were measured by standard methods using automated techniques (Modular P800; Roche Diagnostics; Indianapolis, IN, USA).

Histological analysis

Liver tissue was fixed in 4% formaldehyde and processed for hematoxylin/eosin and Masson Trichrome stains for histological analysis. Histological variables were blindly scored by an experienced hepatopathologist using a scoring system adapted from the NASH activity score [26]: macrosteatosis (0–3), lobular inflammatory changes (0–3), and hepatocyte ballooning (0–2). Fibrosis was minimal in all of the samples and therefore was not scored.

RNA isolation

After liver tissue pulverization ($\sim$50 mg) with a dismembrator (B. Braun Biotech International, Melsungen, Germany) at liquid nitrogen temperature, total RNA was prepared using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. Total RNA was dissolved in RNase-free water and RNA concentration was determined spectrophotometrically. RNA integrity was judged appropriate at a 260/280-nm ratio >1.8 and without signs of degradation on agarose gel. Samples were kept at -80°C until processing by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses.

RT-qPCR analyses

Transcript levels of PPAR-α and -γ and MTP were determined by quantitative RT-qPCR and the results were normalized according to corresponding values of housekeeping β-actin mRNA. Gene-specific primer pairs were located on two adjacent exons to achieve a high level of specificity and to avoid detection of genomic DNA. Primers were designed to have similar GC contents and annealing temperatures using the Primer3 Program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) [27].

β-Actin (165-bp product): NM-007393
Sense: 5′-TGTTACCAACTGGGACGACA-3′
Antisense: 5′-GGGGTGTTGAAGGTCTCAAA-3′

PPAR-α (168-bp product): NM-011144
Sense: 5′-ATGCCAGTACTGCCGTTTTC-3′
Antisense: 5′-TTGCCCAGAGATTTGAGGTC-3′

PPAR-γ (197-bp product): NM-011146
Sense: 5′-CATAAAGTCCTTCCCGCTGA-3′
Antisense: 5′-GAAACTGGCACCCTTGAAAA-3′

MTP (171-bp product): NM-008642
Sense: 5′-CCT CTT GGC AGT GCT TTT TC-3′
Antisense: 5′-ATT TTG TAG CCC ACG CTG TC-3′

mRNA expressions were carried out in a Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia) using Quantitect SYBR Green RT-PCR for quantitative, real-time, one-step RT-PCR (Qiagen GmbH, Hilden, Germany), according to the instructions provided by the manufacturer. Reactions lacking reverse transcriptase were also run to generate controls for assessment of genomic DNA contamination. The reaction mixture consisted of 12.5 μl of SYBR RT-PCR Master Mix, 0.25 μl of QuantiTect RT Mix, 0.2 mM sense/antisense primers, and 5 μl (20 ng/μl) of total RNA template. The reaction was incubated under the following cycling conditions: 50°C for 30 min for RT, heating to 95°C for 15 min, and then 35 cycles at 94°C for 20 sec, 56°C for 30 sec, and 72°C for 30 sec. Fluorescence changes were monitored after each cycle, and melting curve analysis was performed at the end of cycles to verify PCR product identity (72°C, ramping to 99°C at 0.2°C/sec, with continuous fluorescence readings). Specificity of amplicons was also ensured by agarose gel electrophoresis to visualize a unique product fragment of the appropriate size.

RNA contents of PPAR-α and -γ and MTP were determined as the number of transcripts relative to those of β-actin and additionally normalized to the mean value of control liver. To evaluate the amplification efficiency of each target and housekeeping gene, standard curves were constructed from a control liver RNA sample using duplicate serial dilutions with five different RNA concentrations (500, 100, 20, 4, and 0.8 ng/μl). Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method [28] for PPAR-γ gene based on their equivalent amplification efficiency with β-actin. The mathematical model described by Pfaffl [29] was used to evaluate the relative expression ratio of PPAR-α and MTP genes compared with β-actin. Amplification of PPARs, MTP, and housekeeping control genes were done in duplicate from each sample in two different experimental runs performed on different days.

Statistical analysis

An unpaired two-tailed Student's *t*-test was used to evaluate statistical significance of PPAR-α and-γ and MTP gene expression among the three groups. For biochemical analysis, the results of the groups were compared using univariate analysis of variance. All statistical analyses were performed using JMP Release 5.1.1 software (SAS Institute Inc., Cary, NC, USA). Values are provided as means $\pm$ SD and findings were considered statistically significant at probability levels of $P < 0.05$.

Results

Biochemical analysis

Animals in the MCD group presented significant weight loss in comparison to those in the control group, and YHK treatment also promoted a significant weight loss in comparison to animals receiving MCD diet alone (Table 1), although the food intake was not different among the groups. A notable decrease in visceral fat was observed in the MCD + YHK group in comparison to the MCD group (Fig. 1). Serum concentrations of AST, ALT, cholesterol, and triglycerides were significantly lower in the MCD + YHK group compared to the group that received MCD diet alone (Table 1).

Histopathological analysis

Figure 2 shows micrographs of liver tissues from ob/ob mice submitted to the MCD diet and receiving vehicle (MCD group) or YHK solutions (MCD + YHK group) for 4 weeks. In the MCD group, there was moderate diffuse macro- and microvacuolar steatosis, hepatocelular ballooning, and diffuse inflammatory infiltrate (Fig. 2A). In the MCD + YHK group, there was no liver steatosis, only mild ballooning and minimal inflammation (Fig. 2B).

Gene expression by RT-qPCR

A significant increase was observed in PPAR-α mRNA content in the MCD and MCD + YHK groups in comparison to the control group. YHK treatment promoted a signifi-

Table 1 Serum levels of alanine amininotransferase (ALT), aspartate aminotransferase (AST), triglycerides, and cholesterol, and weight change of ob/ob mice fed a methionine/choline-deficient diet treated (MCD + YHK) or not treated (MCD) with YHK

Group	*N*	AST (U/L)	ALT (U/L)	Triglycerides (mg/dl)	Cholesterol (mg/dl)	Weight change (%)
Control	5	146 ± 63	25 ± 2	75 ± 35	89 ± 31	13.6
MCD	5	623.7 ± 460.8**	230.0 ± 165.2**	93.0 ± 27.0**	105.0 ± 48.6**	4.6**
MCD + YHK	5	217.3 ± 97.9*	38.7 ± 9.1*	65.7 ± 25.5*	80.0 ± 15.1*	−9.6*

Note. Data expressed as mean ± SD. Reference values: AST, 10–34 U/L; ALT, 10–44 U/L; triglycerides and cholesterol, 45–89 mg/dl. *$P < 0.05$ versus MCD group; **$P < 0.05$ versus control group.

cant increase in PPAR-α mRNA content in relation to MCD diet alone (Fig. 3A). As shown in Fig. 3B, there was no relevant modification in PPAR-γ mRNA content in the MCD group compared to the control group, however, a statistically significant decrease in PPAR-γ mRNA content was detected in the MCD + YHK group in comparison to the MCD group. There was no significant change in MTP mRNA content in the MCD group compared with the control group, while a statistically significant increase was observed in the MCD + YHK group in comparison to the MCD and control groups (Fig. 3C).

Discussion

In this study, we show that administration of YHK (Asiatic herbal medicine) leads to a reduction of steatohepatitis in ob/ob mice receiving a MCD diet and that modulation of PPAR-α, PPAR-γ, and MTP RNA expression may be implicated in the protective effect of YHK in experimental NASH, limiting hepatocyte lipid accumulation.

Although ob/ob mice develop spontaneous liver steatosis, the development of a more significant injury requires the administration of a so-called "second metabolic hit." In the MCD model, mitochondrial dysfunction occurs concomitantly with lipid peroxidation and increased mitochondrial ROS formation [30–34]. We previously hypothesized that the antioxidant effect of YHK results in abrogation of the cytotoxic effects of ROS and lipid peroxides, preventing mitochondrial injury and, consequently, NASH development in animals receiving MCD diet [25]. The development of steatohepatitis in mice is dependent on the activation of pathways for hepatic lipid turnover, which, in rodents, are largely modulated by some genes such as PPARs, CPT1, ChREBP, SREBP1, and MTP [35]. In the present study, we further investigated YHK's protective effect in experimental NASH induced by a MCD diet comparing the hepatic mRNA expression of PPAR-α, PPAR-γ, and MTP in ob/ob mice receiving or not receiving YHK treatment. An increase in the mRNA content of PPAR-α and its target gene MTP and downregulation of PPAR-γ mRNA were observed in animals treated with YHK.

The role of PPAR-α in hepatic lipid turnover, upregulating several genes involved, among other things, in mitochondrial β-oxidation, explains its increased expression in situations where there is a predisposition to accumulate hepatic fat, such as in the ob/ob mouse [36]. The significant upregulation of PPAR-α mRNA content in ob/ob mice receiving a MCD diet in relation to the ob/ob mice observed in this study probably reflects the exacerbation of triglyceride accumulation in hepatocytes promoted by phosphatidylcholine deficiency resulting from a dietary lack of methionine and choline [37]. Treatment with YHK elicited an additional upregulation of PPAR-α mRNA, suggesting that, in addition to its antioxidant effects, this compound might prevent NASH development by protecting from lipid overload. Ip et al. [38] demonstrated that the activation of PPAR-α by an agonist in

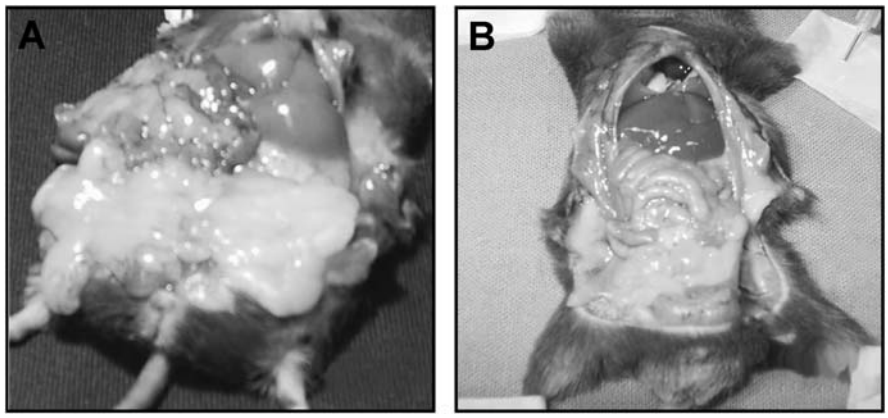

Fig. 1 Ob/ob mice after 4 weeks of (A) treatment with vehicle plus a methionine/choline-deficient (MCD) diet or (B) treatment with YHK plus a MCD diet. A marked decrease in visceral fat was observed

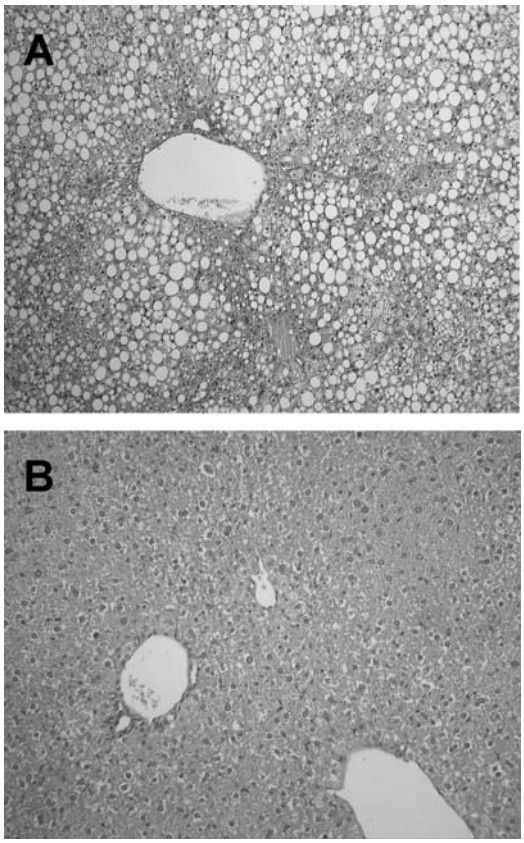

Fig. 2 (A) Hepatic histological features of ob/ob mice fed a methionine/choline-deficient (MCD) diet: moderate diffuse macro- and microvacuolar steatosis, hepatocelular ballooning, and diffuse inflammatory infiltrate. (B) Hepatic histological features of ob/ob mice fed a MCD diet plus Yo Jyo Hen Shi Ko (MCD + YHK): no liver steatosis, only mild ballooning and minimal inflammation

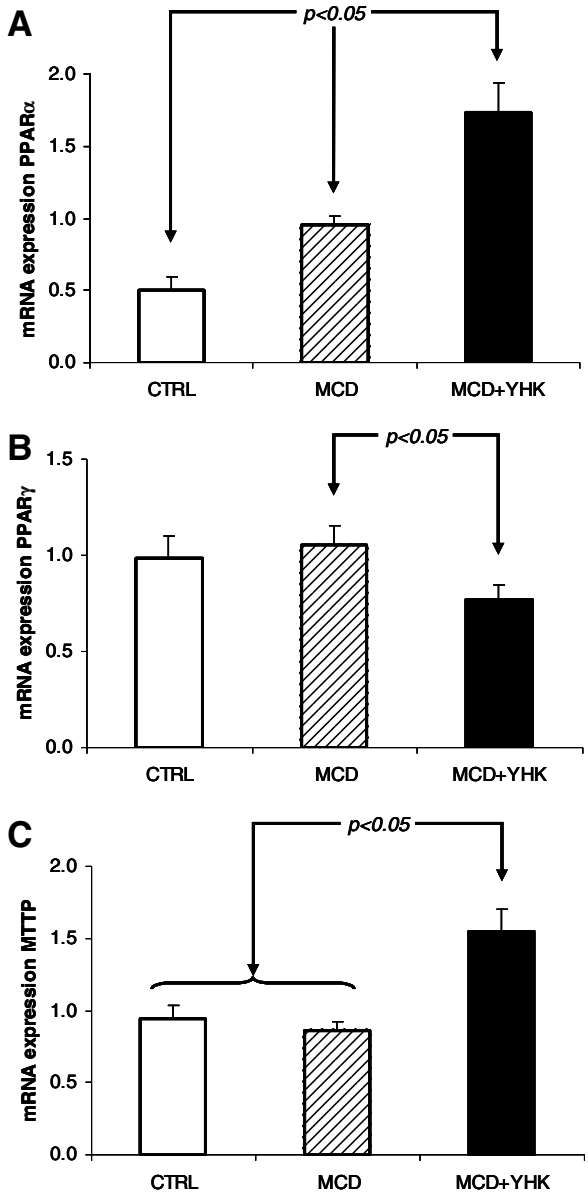

Fig. 3 Effect of a methionine/choline-deficient (MCD) diet with YHK (MCD + YHK) or without YHK (MCD) on hepatic mRNA expression of PPAR-α (A), PPAR-γ (B), and MTP (C)

mice fed a MCD diet reduced the triglyceride content in the liver by stimulating hepatic fatty acid disposal, eventually depleting the liver of substrate for lipid peroxidation. The activation of PPAR-α was not evaluated in this study, and although the expression level is not necessarily parallel to PPAR activity, the increased expression of MTP mRNA, a target gene of PPAR-α [39], after YHK treatment suggests that the increment in PPAR-α mRNA results in functional consequences.

Recent studies have demonstrated that the abundance of mRNA and the activity of MTP, which lipidates apolipoprotein B into triglyceride-rich very low-density lipoprotein (VLDL) particles, are increased in ob/ob mice and hamsters [40, 41]. Phillips et al. [42] showed a significant increase in MTP mRNA in liver of fat rats and a correlation between MTP mRNA and MTP activity. This augmented MTP expression might contribute to the protective effect of YHK, promoting lipidation of apolipoprotein B into triglyceride-rich VLDL particles and subsequent hepatic secretion, however, serum concentrations of triglycerides were significantly lower following YHK treatment, speaking against increased VLDL secretion. Since treated animals presented a significant weight loss, it is possible that the positive effects of this weight change on the lipid profile could be prevailing over the increased MTP activity. The lack of parallelism between PPAR-α and MTP mRNA contents after the MCD diet might result from the already very high MTP expression in ob/ob mice, but a discrepancy between expression level and PPAR-α activity cannot be ruled out.

Regarding PPAR-γ, several studies have demonstrated that its hepatic overexpression is closely associated with the development of NAFLD [7, 43], playing a key role in hepatocyte lipid accumulation through stimulation of various

adipogenic and lipogenic genes [8–10, 44]. In the present study, no relevant modifications in the mRNA content of this gene were observed in ob/ob mice receiving the MCD diet in comparison to ob/ob mice, known to present a marked hepatic upregulation of PPAR-γ mRNA [36]. The significant reduction in the hepatic abundance of PPAR-γ following YHK treatment suggests that another mechanism underlying the protective effect of this agent might be a diminution of de novo lipid synthesis.

Given the known effects of some of the botanical derivatives present in the YHK formulation on carbohydrate and lipid metabolism [45–49], we cannot rule out that extrahepatic actions which indirectly improve hepatic metabolism are taking place, especially considering the weight loss and the decreased visceral adiposity associated with YHK treatment. Based on the existing literature, its contents are likely to be active in a number of respects. For example, ginseng [45, 46] and derivatives of *Eucommia* [47, 48] are known to have hypoglycemic effects, while derivatives of *Polygonatum*, such as steroidal glycosides, increase insulin sensitivity in skeletal muscle [49]. Regarding licorice derivatives, the most active compound, glycyrrhizic acid, possesses mineralocorticoid, glucocorticoid, antiandrogenic, and estrogenic activity [50]. The most relevant property, however, may be mediated by carbenoxolone antagonism of 11β-hydroxy steroid dehydrogenase, which appears to improve hepatic insulin sensitivity [51]. However, we believe that the modifications in PPAR-α and -γ mRNA content in the liver of treated mice do not result from an improvement in insulin sensitivity, because if this were the case, the changes in PPAR mRNA abundance would have been the opposite of those observed, since insulin increases PPAR-γ mRNA expression [52] and decreases PPAR-α mRNA expression in hepatocytes [53].

In conclusion, modulation of PPAR-α and -γ RNA expression may be implicated in the protective effect of YHK in experimental NASH, limiting hepatocyte lipid accumulation. Our study has inherent limitations, such as the absence of a formal analysis of the composition of the YHK, a common problem in the study of potentially active herbal medications, as well as the lack of investigation of potential extrahepatic effects of YHK, especially those related to decreased visceral adiposity. Since no difference in food intake among groups was observed, further studies are needed to evaluate possible effects of YHK on caloric expenditure and to provide insights about the clinical implications of these findings, in an attempt to develop novel therapeutic strategies for the treatment of NAFLD.

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