

# Yo Jyo Hen Shi Ko (YHK) Modulates the Expression of Proteins Involved in *de novo* Lipogenesis and Lipid Exportation in Experimental Nonalcoholic Steatohepatitis (NASH)

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**Abstract:** Previous study by our group showed the protective effect of Yo Jyo Hen Shi Ko (YHK) a natural compound in experimental nonalcoholic steatohepatitis (NASH). The aim of this study was to evaluate whether YHK modulates lipid metabolism.

NASH was induced in male ob/ob mice by methionine/choline-deficient (MCD) diet for 4 weeks. YHK-treated animals (YHK) received YHK solution orally (20 mg/kg/day) by gavage while MCD (n=6) group received only vehicle. The control animals (CTRL; n=6) received standard diet. Liver fragments were collected for mRNA and protein isolation. The analysis of gene expression and protein was performed by RT-qPCR and western blot, respectively.

A significant decrease in *srebp1c* mRNA and protein expression and *fasn* mRNA expression was observed in MCD+YHK group. A significant increase in MTP protein expression was observed in the MCD+YHK vs MCD group while a decreased expression was observed in the MCD vs CTRL group. The expression of the *scd1* in the MCD group was diminished. The Perilipin protein expression was augmented in the MCD group in comparison with MCD+YHK and CTRL groups.

YHK modulated genes involved in the synthesis and exportation of hepatic lipids, probably limiting hepatocyte lipid accumulation, reducing lipogenesis and upregulating lipid exportation suggesting that the YHK can be a promising drug for treat non-alcoholic fatty liver disease (NAFLD).

**Keywords:** Nonalcoholic fatty liver disease (NAFLD), Nonalcoholic steatohepatitis (NASH), Mice, ob / ob, Yo Jyo Hen Shi Ko (YHK).

## 1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of liver disease and it has been estimated that, with the increasing prevalence of obesity, 20 million patients are affected in the USA [1]. NAFLD represents a spectrum of diseases ranging from simple fatty liver (steatosis) to steatosis with inflammation and necrosis to cirrhosis. Non-alcoholic steatohepatitis (NASH) represents the most severe end of this spectrum and is associated with progressive liver disease, fibrosis, cirrhosis, liver failure and hepatocellular carcinoma [2].

NASH is clearly associated with the so-called metabolic syndrome and can be considered the liver event in this disorder. The mechanism that mediates the development of NASH is multiple such as, changes in lipid metabolism, insulin resistance, oxidative stress and others [3]. Insulin resistance plays a major role in

hepatic fat accumulation through increased influx of free fatty acids (FFA) from peripheral fat stores due to enhanced lipolysis, increased *de novo* lipogenesis (DNL), reduced apoB production and impaired lipoprotein secretion, which diminishes fat export from the liver [4].

The Sterol Regulatory Element Binding Protein-1c (SREBP-1c), a transcription factor, is the major factor that regulates *de novo* lipogenesis which can transcriptional activates most genes required like Fatty Acids Synthase (*fasn*), Stearoyl-CoA desaturase 1 (*scd-1*), Acid Synthase, Acetyl-CoA Carboxylase-1 (*acc*) and Peroxisomal-Proliferator-Activated Receptor- $\gamma$  (*ppar* $\gamma$ ). FASn is the central enzyme in DNL, converting Acetyl-CoA and Malonyl-CoA to palmitate and SCD-1 catalyzes the final step in the synthesis of monounsaturated fatty acids and is required to produce lipoproteins for lipid exportation [5]. SREBP1c and FASn upregulation were observed in experimental NASH [6].

The fatty acids are exported in form of very low density lipoproteins (VLDL), their formation is

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dependent on apoB and mediated by microsomal triglyceride protein (MTP). Some studies suggest that depletion of MTP is sufficient for occurrence of steatosis. In humans, genetic alterations in apoB or MTP lead to the accumulation of hepatic fat [7], as in abetalipoproteinemia [8]. In Japanese and Western populations [9] there is a polymorphism in the promoter of the *MTP* -493 G/T that leads to an increase in hepatic steatosis susceptibility [10].

The fats are stored in compartments called lipid droplets which are surrounded by amphiphilic proteins. The lipid droplets are dynamic cells organelles that control lipolysis and lipogenesis. The amphiphilic proteins are a family named PAT proteins (Perilipin, Adipophilin/Perilipin 2/ADRP and TIP47/Perilipin 3). The role of these proteins in steatosis has not been characterized, although Perilipin seems to be *de novo* expressed in steatotic livers [11-13].

None experimental model can reproduce perfectly NASH in humans, although, certain models with genetic changes like animals knockout for key enzymes in fatty acids metabolism as ACC, SCD-1, Elovl6, GPAT and DGAT can mimic some of the metabolic defects related to DGHNA [14]. The animals ob/ob mice show a spontaneous mutation in the gene encoding leptin and naturally develop steatosis, obesity and insulin resistance, but not develop serious liver injury due to the importance of leptin in the onset of fibrogenesis. The association of a second factor such as fat diet deficient in choline and methionine in these ob / ob mice, plays the NAFLD with biopsy similar to the human form [15].

The diet deficient in choline-methionine (MCD) is a good model described to NASH induction. Produces steatosis due to a decrease in the export of VLDL, as choline and methionine are the precursors of phosphatidylcholine phospholipid lining the VLDL. This diet increases the levels of aminotransferases, generates focal inflammation, necrosis and fibrosis without hepatic insulin resistance present. Animals undergoing MCD generally have reduced weight and visceral fat, although animal db / db, who already have insulin resistance, were reported maintenance of visceral fat, greater tissue damage and a anti-inflammatory profile [16].

Due to the wide spectrum of NASH there is no standard treatment, and it is based on the disease severity as well as their comorbidities. Changes in lifestyle should be indicated in any phase of the disease and include: diet for weight reduction, exercise,

preventing the use of alcohol, tobacco, or also, illicit drugs. Liver transplantation is indicated for patients with decompensated cirrhosis and hepatocellular carcinoma [17].

Traditional Chinese medicine is based on the use of herbal medicines in curing process. Some compounds have been described for the treatment of many disorders of the digestive system. *Magnolol* and *Honokiol* are structural isomers derived from the root of *Barbatimão* and showed results in the treatment of experimental steatohepatitis reducing fat deposition in liver cells due to inhibition of maturation of SREBP1c. Others like *Qushi Huayu decoction* (QHD) and the formula *Yiqi Sanju* can improve the level of aminotransferases, and appear to assist in weight reduction and decrease inflammation in ENA [18-20].

The natural compound called *Yo Jyo Hen Shi Ko* (YHK) (Kyotsujigyo Inc., Japan) has been used in Chinese medicine for many years. It is derived from Henshiko and composed of 4 ingredients: *Panax pseudo ginseng*, *Eucommia ulmoides*, *Polygonati rhizoma* and *Licorice root*. It has pharmaceutical properties and is used in the treatment of various liver diseases [21]. Previous experimental studies performed by our group reported the inhibition of NASH development, reduced markers of oxidative stress, lower score of inflammation, reduced serum triglyceride and cholesterol levels, improvement in levels of aminotransferases, decreased visceral fat and more recently, we also found an increase in *mtp* gene expression and a reduction in the expression of *ppar-γ* [22, 23].

In order to investigate the effect of this compound on lipid metabolism in experimental NASH, the present study aimed to evaluate whether of YHK modulates the hepatic expression of SREBP1c, MTP, Perilipin 1, ADRP, CPT1, FASN and SCD-1 in ob/ob mice receiving a methionine/choline-deficient diet (MCD).

## 2. MATERIALS AND METHODS

*Yo Jyo Hen Shi Ko* (YHK) was provided by Kyotsujigyo Inc., Japan (<http://www.kyotsujigyo.com>). This preparation contains four different botanical derivatives: *Panax pseudoginseng* 40%-60%, *Eucommia ulmoides* 30%-40%, *Polygonatirhizome* 8%-12%, and licoriceroot.

### 2.1. Animals

Ob/ob mice (Jackson Laboratories, Bar Harbor, ME, USA), 8 weeks old and weighing 30–40g, were housed

Table 1: Primers Used for Real-Time RT-qPCR

| Gene                        | Sense                      | Antisense                  |
|-----------------------------|----------------------------|----------------------------|
| SREBP1c - M_011480.3        | GCG CTA CCG GTC TTC TAT CA | GGA TGT AGT CGA TGG CCT TG |
| FASn - NM_007988.3          | TCC ACC TTT AAG TTG CCC TG | TCT GCT CTC GTC ATG TCA CC |
| SCD-1 - NM_009127           | AAA CAC ACG CCG ACC CTC AC | CCG CCC TTC TCT TTG ACA GC |
| ADFP - NM_007408.3          | GGG CAG TCA AGC GAT ATT TT | AGG TTG GCC ACT CTC ATC AC |
| CPT1a - NM_013495.2         | TGC CTC TAT GTG GTG TCC AA | TCA AAC AGT TCC ACC TGC TG |
| MTP - NM_008642             | CCT CTT GGC AGT GCT TTT TC | ATT TTG TAG CCC ACG CTG TC |
| $\beta$ -actina - NM_007393 | TGT TAC CAA CTG GGA CGA CA | GGG GTG TTG AAG GTC TCA AA |

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. 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 soy bean oil; 1% AIN-93M vitamin mix; 3.5% AIN-93M mineral mix; (Rhostrer Ind. Com. Ltd., Sao Paulo, Brazil), 5g/day, for 4 weeks. The ob/ob mice were divided into a control group (n=6) that received a standard diet (Nuvilab; Nutrientes Ltd., Colombo, Brazil) *ad libitum* and two experimental groups which were fed as follows: (1) MCD group (n=6) fed MCD diet plus vehicle (physiologic Ringer's solution); and (2) MCD+YHK group (n=6) fed MCD diet plus YHK solution (20mg/kg) daily by gavage. After 4 weeks of treatment with YHK or vehicle plus MCD diet, ob/ob mice were sacrificed. Livers were collected for histopathologic analysis, mRNA and protein isolations.

## 2.2. Histology

Fragments of liver tissues were fixed in formaldehyde saline (4%) and processed for hematoxylin–eosin (HE) stain. Histological markers of NAFLD activity steatosis (0-3), ballooning (0-2) and lobular inflammation (0-3) were assessed.

## 2.3. RNA Isolation

After liver tissue pulverization (~50mg) with a dismembrator (B. Braun Biotech International, Melsungen, Germany) at liquid nitrogen temperature, total RNA was prepared using TRIzol<sup>®</sup> 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) analysis.

## 2.4. Gene Expression by RT-qPCR

Transcript levels of *srebp1c*, *fasn*, *mtp*, *scd-1*, *adrp* and *cpt1* were determined by quantitative RT-qPCR and the results were normalized according to corresponding values of housekeeping  $\beta$ -actin mRNA. Primers (Table 1) 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](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) [24].

mRNA expressions were carried out in a Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia) using SuperScript<sup>™</sup> III Platinum<sup>®</sup> One-Step Quantitative RT-PCR System (Invitrogen Life Technologies, Carlsbad, EUA), according to the instructions provided by the manufacturer. Reactions lacking reverse transcriptase were also run to generate controls for assessment of genomic DNA contamination. 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.

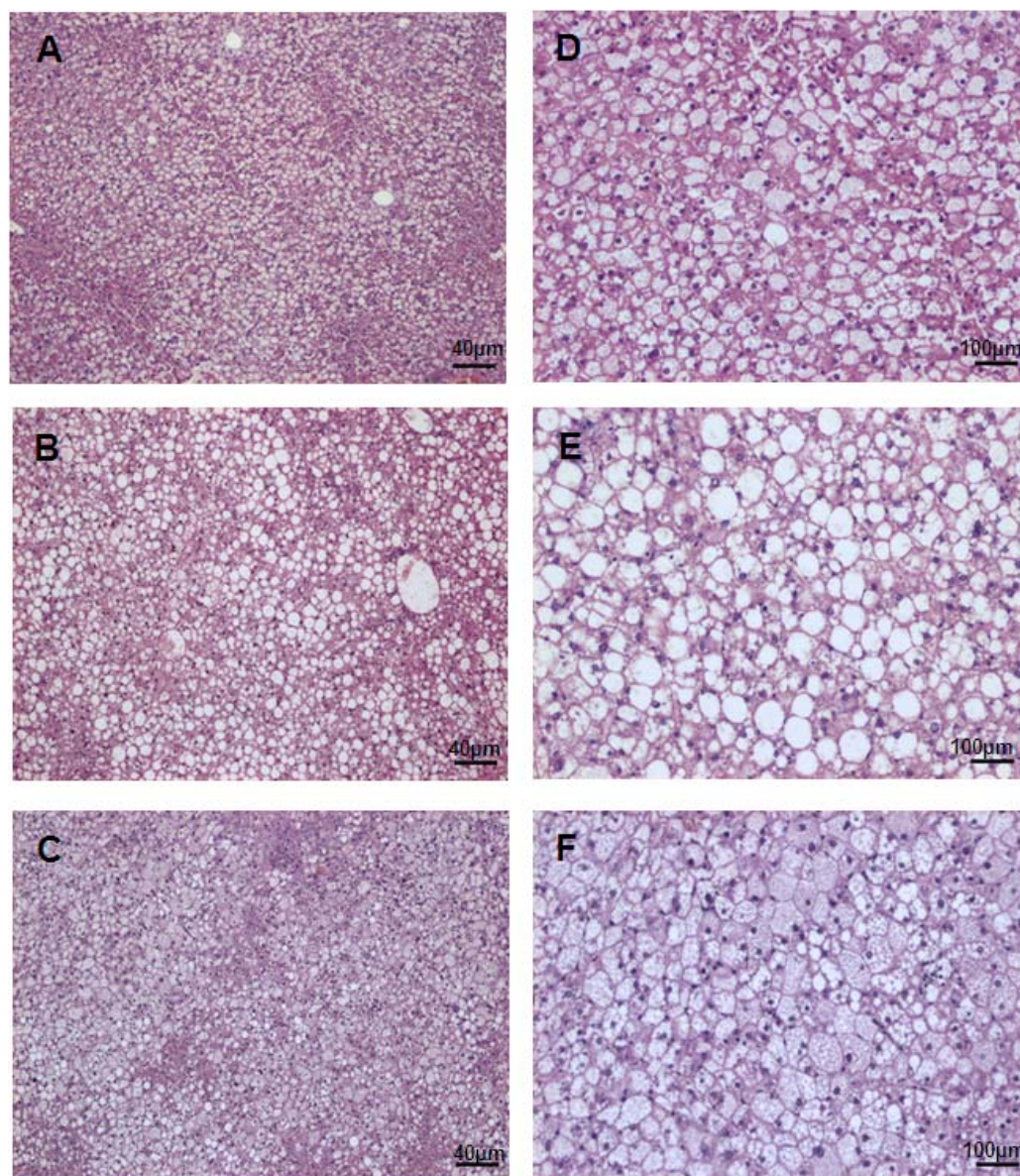
RNA contents of *srebp1c*, *fasn*, *mtp*, *scd-1*, *adrp* and *cpt1* were determined as the number of transcripts relative to those of  $\beta$ -actin and additionally normalized

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/ $\mu$ L). Relative quantification was calculated using the mathematical model described by Pfaffl [25]. Amplifications of all genes were done in duplicate from each sample.

## 2.5. Western Blot

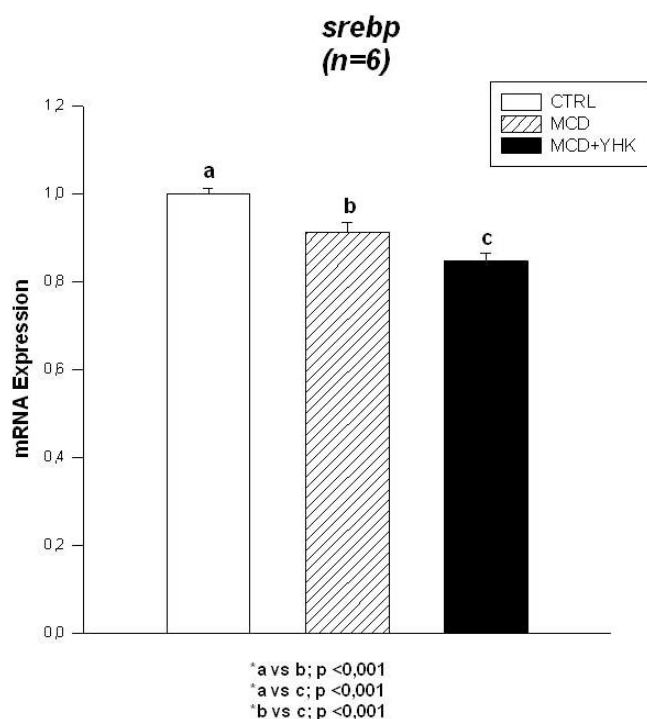
Livers from mice were dissected, washed with ice-cold buffer (5 mM Tris, pH 7.4), and minced into pieces

with a razor blade. The pieces were homogenized in ice-cold homogenization buffer (250 mM sucrose, 8 mM Tris, pH 7.4). The homogenate was centrifuged at  $60 \times g$  for 10 min. The supernatant fluid was removed. The pellet, representing the total liver protein, was resuspended in fresh lysis buffer (20 mM Hepes, 150 mM NaCl, 10% Glycerol, 1% Triton, 1 mM EDTA, 1.5 mM  $MgCl_2$ , 1  $\mu$ g/mL aprotinin, 1  $\mu$ g/mL leupeptin, 1 mM PMSF). The proteins were size-fractionated on a 10% polyacrylamide/SDS gel (50  $\mu$ g of protein per lane); the separated proteins were then electrophoretically transferred to a nitrocellulose membrane. The Western blot was probed with a rabbit antiserum against MTP



**Figure 1:** Representative histological section of liver tissue of ob/ob mice. HE stain. **A** and **D** - Control group: micro steatosis, ballooning and minimal inflammation (40x, 100x), **B** and **E** - Hepatic histological features of ob/ob mice fed a methionine/choline-deficient (MCD) diet: moderate diffuse macro- and microvacuolar steatosis, hepatocellular ballooning, and diffuse inflammatory infiltrate. (40x, 100x), **C** and **F** - Hepatic histological features of ob/ob mice fed a MCD diet plus Yo Jyo Hen Shi Ko (MCD+YHK): micro steatosis, ballooning and minimal inflammation (40 x, 100x).





**Figure 2:** YHK decreases SREBP-1c mRNA expression in treated ob/ob mice. Graphical representation of SREBP1c mRNA content in liver tissue from ob/ob mice in CTRL group (1,000±0,012), MCD group (0,911±0,024), and MCD+YHK group (0,847±0,0174).

(Bristol-Myers Squibb, USA), a rabbit polyclonal antibody against SREBP1c-H160 (Santa Cruz

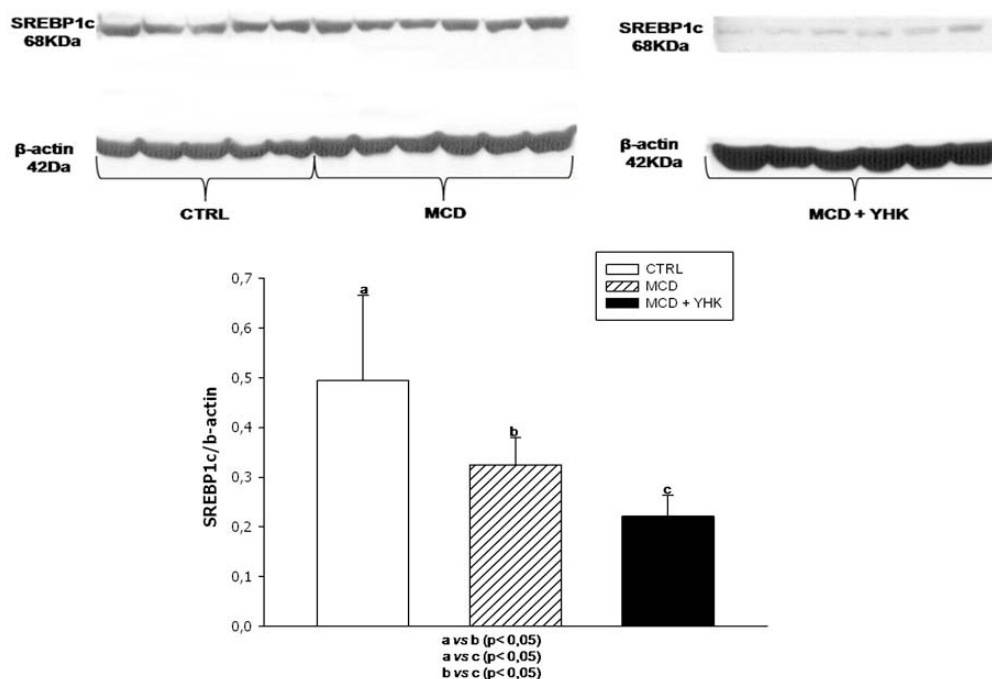
Biotechnology, Santa Cruz, CA) and a rabbit polyclonal antibody anti-Perilipin (Abcam, Cambridge, Inglaterra). The membranes were blocked in blocking buffer (TBS-T 0.1% supplemented with 5% nonfat milk) at room temperature for 2 h. After the primary antibody incubation (1:1000 dilution) overnight at 4 °C, the blots were washed with TBS-T 0.1% and incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Calbiochem, CA, USA). Bands were visualized using enhanced chemiluminescence (Calbiochem) and exposed to X-ray film; membranes were then stripped and reblotted with  $\beta$ -actin to check for equal loading.

## 2.6. Statistical Analysis

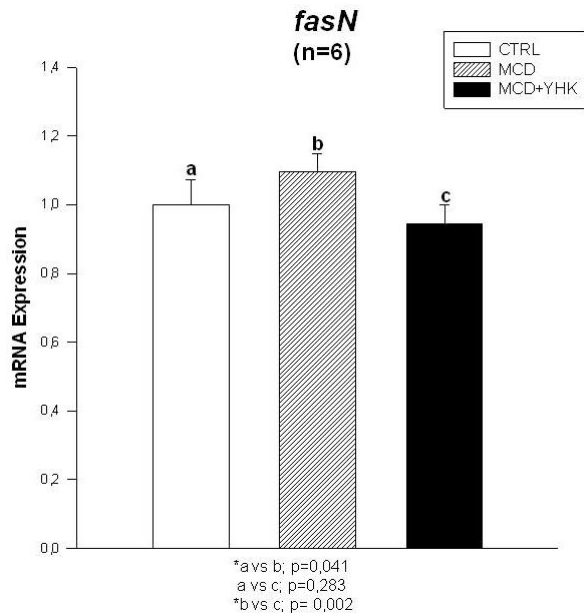
The Shapiro-Wilk normality test and Levene's variance homogeneity test were applied with pos-hoc test of Tukey for normal distribution; or Kruskal – Wallis test for non normal data. Significant differences were evaluated using the ( $p < 0.05$ ). These tests were performed by SPSS 11.0.

## 3. RESULTS

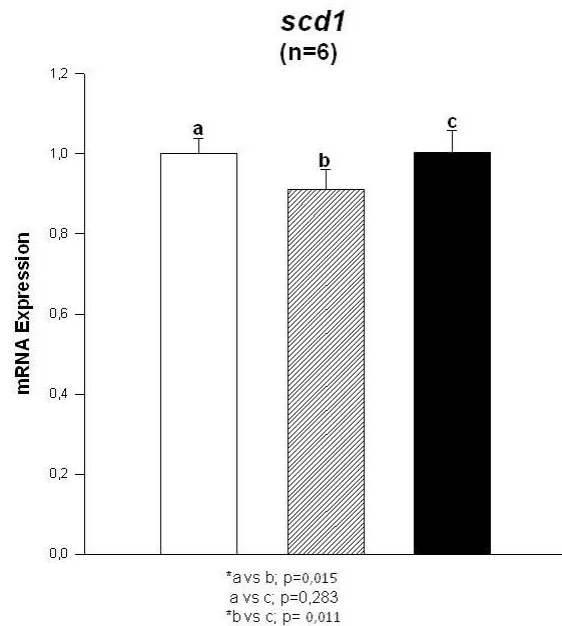
As previously shown, the MCD+YHK group demonstrated a marked decrease in liver steatosis and in parenchymal inflammation (Figure 1C and F) on histopathological analysis while the MCD group



**Figure 3:** Hepatic Sterol regulatory element binding protein-1c (SREBP-1c) expression in CTRL, MCD and MCD+YHK groups. Total liver protein was isolated from mouse livers and used for determination of SREBP-1c protein expression by Western blot as described in methods. Bands were visualized by chemiluminescence and exposed to X-ray film. SREBP-1c expression was decreased in YHK-treated ob/ob mice. YHK decreases SREBP-1c protein expression in treated ob/ob mice. Graphical representation of SREBP1c protein expression in liver tissue from ob/ob mice in CTRL, MCD and MCD+YHK group.



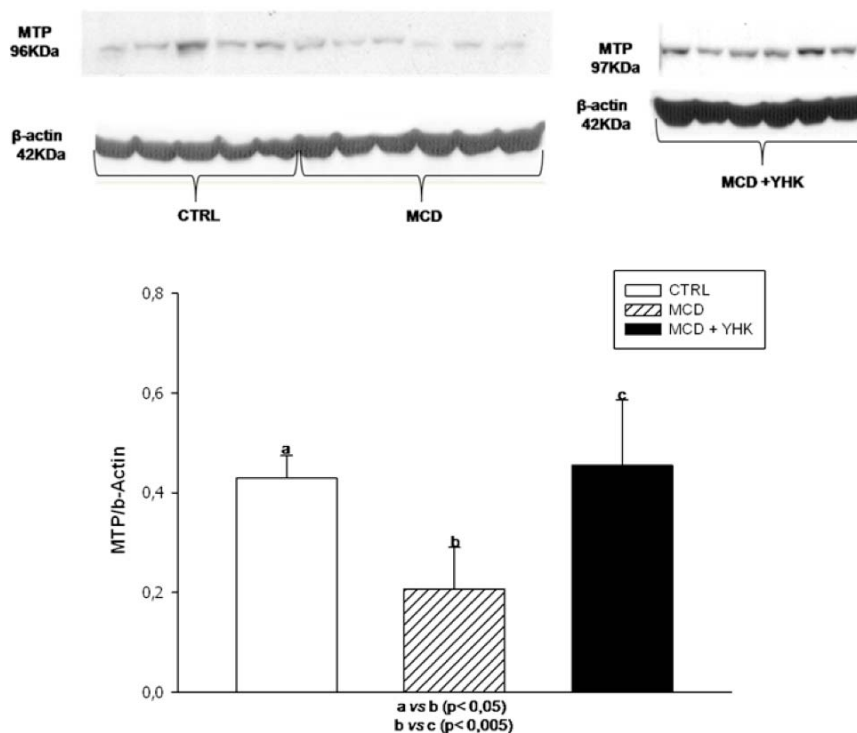
**Figure 4:** YHK decreases FASn mRNA expression in treated ob/ob mice. Graphical representation of FASn mRNA content in liver tissue from ob/ob mice in CTRL group ( $1,000 \pm 0,073$ ), MCD group ( $1,095 \pm 0,053$ ) and MCD+YHK group ( $0,944 \pm 0,055$ ).



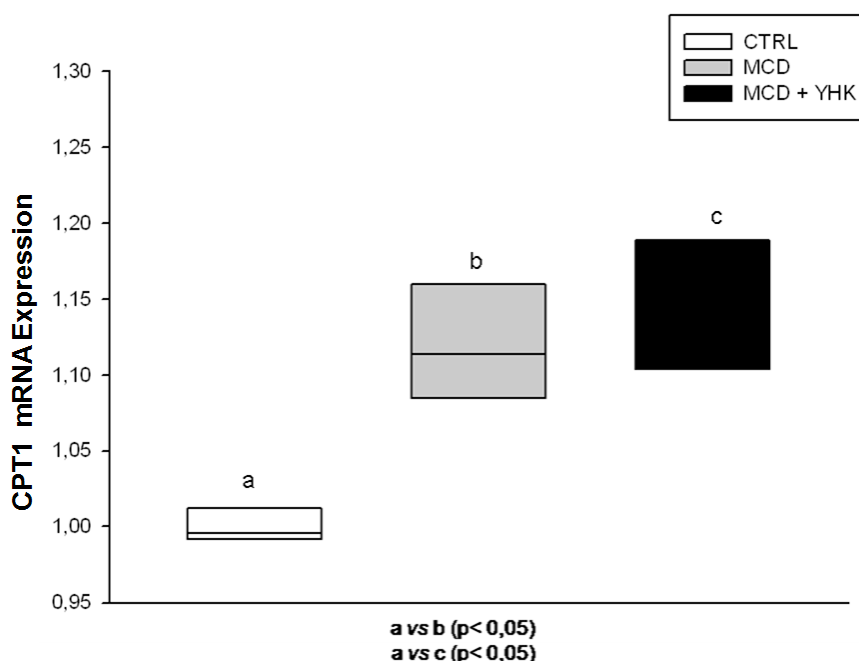
**Figure 5:** YHK increases SCD1 mRNA expression in treated ob/ob mice. Graphical representation of SCD1 mRNA content in liver tissue from ob/ob mice in CTRL group ( $1,000 \pm 0,039$ ), MCD group ( $0,911 \pm 0,050$ ) and MCD+YHK group ( $1,004 \pm 0,053$ ).

developed diffuse macro- and microvesicular steatosis, moderate hepatocellular ballooning and a moderate lobular mixed inflammatory infiltrate (Figure 1B and E).

The control group (standard diet) (Figure 1A and D) had only mild ballooning and minimal inflammation.



**Figure 6:** Hepatic Microsomal triglyceride transfer protein (MTP) expression in CTRL, MCD and MCD+YHK groups. Total liver protein was isolated from mouse livers and used for determination of MTP protein expression by Western blot as described in methods. Bands were visualized by chemiluminescence and exposed to X-ray film. MTP expression was increased in YHK-treated ob/ob mice. YHK increases MTP protein expression in treated ob/ob mice. Graphical representation of MTP protein expression in liver tissue from ob/ob mice in CTRL, MCD group and MCD+YHK group.



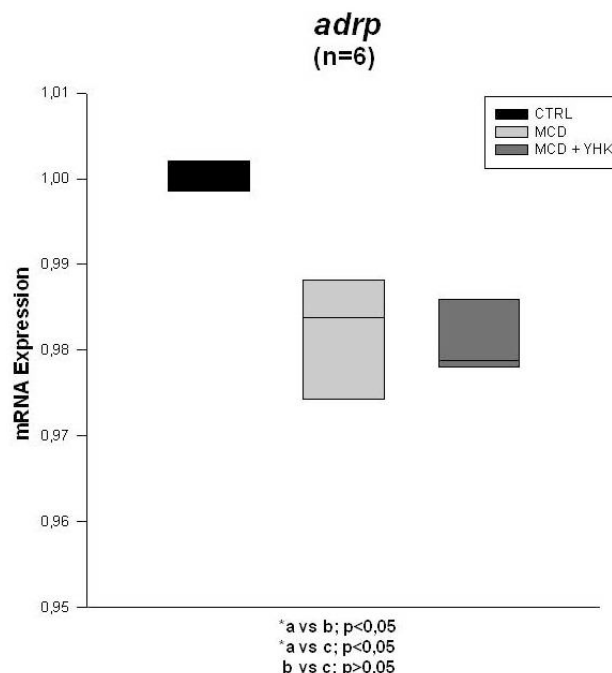
**Figure 7:** MCD increases CPT1A mRNA expression in ob/ob mice submitted to this diet. Graphical representation of CPT1a mRNA content in liver tissue from ob/ob mice in CTRL, MCD and MCD+YHK group.

YHK treatment promoted a significant decrease in *srebp1c* mRNA content in comparison to the MCD and Control groups (Figure 2). The same effect was observed in the protein expression (Figure 3). Also, YHK treatment promoted reduction of *FASn* mRNA expression when comparing with the MCD group ( $p < 0.05$ ) (Figure 4). The expression of the *scd1* showed significant differences in their averages when comparing the MCD+YHK vs. MCD groups and the MCD vs. CTRL groups ( $p < 0.05$ ) (Figure 5). Otherwise, a significant increase in MTP protein expression was observed in the MCD+YHK in comparison to the MCD group while a decreased expression in the MCD compared with the control group was detected (Figure 6). The medians of *cpt1a* gene expression were statistically different when comparing the MCD+YHK and MCD group with the CTRL group ( $p < 0.05$ ) (Figure 7). The medians of *adrp* were decreased in the MCD group in comparison with MCD+YHK and CTRL group ( $p < 0.05$ ) (Figure 8). The Perilipin protein expression was augmented in the MCD group in comparison to the CTRL group. Treatment with YHK decreased Perilipin expression to the level observed in the CTRL group (Figure 9).

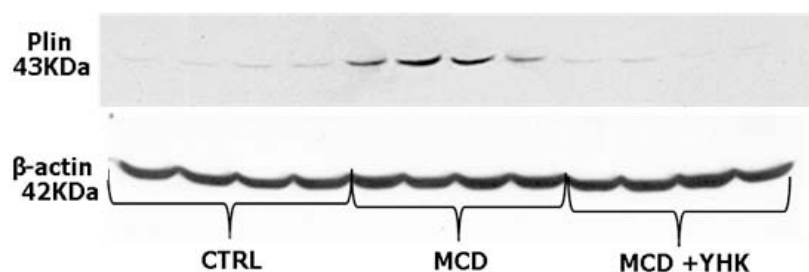
#### 4. DISCUSSION

In this study, we show that administration of YHK, an Asiatic herbal medicine, leads to a reduction of steatohepatitis in ob/ob mice receiving a MCD diet due to the modulation of the expression of proteins involved

in *de novo* lipogenesis, lipid exportation and storage limiting hepatocyte lipid accumulation (Figure 10). We have also demonstrated that mice fed a MCD diet increase the *cpt1a* and decrease *adrp* gene expression



**Figure 8:** MCD decreases ADRP mRNA expression in ob/ob mice submitted to this diet. Graphical representation of medians of ADRP mRNA content in liver tissue from ob/ob mice in CTRL group (0,999), MCD group (0,984) and MCD+YHK group (0,979). The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference.  $H = 11,556$  with 2 degrees of freedom.



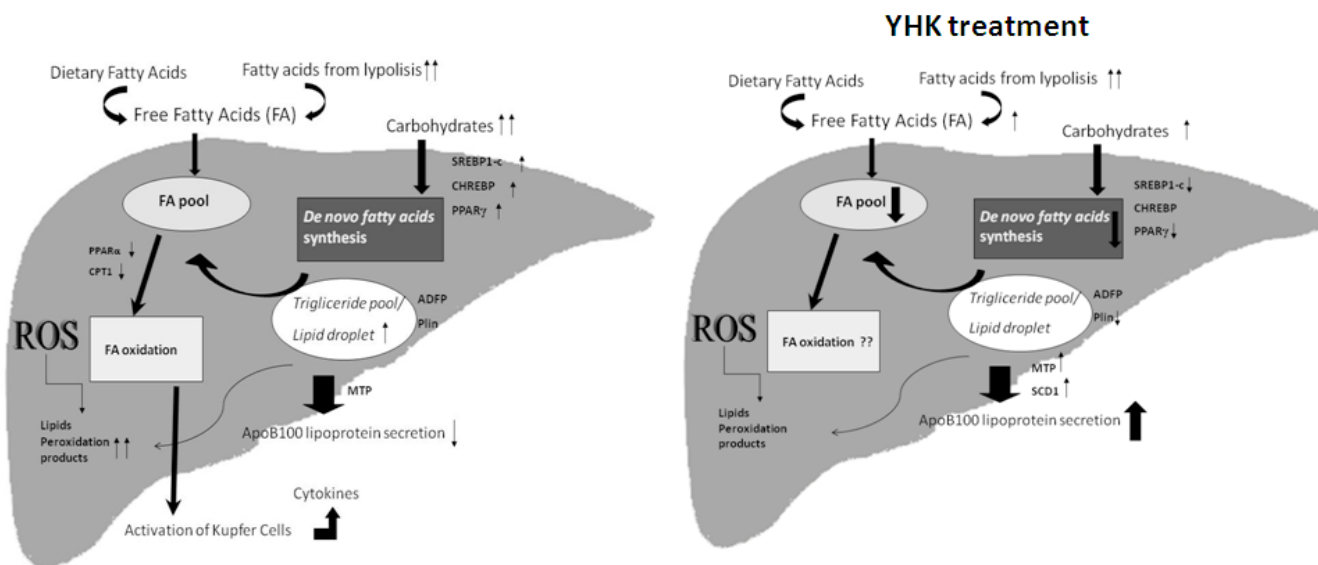
**Figure 9:** Hepatic expression in CTRL, MCD and MCD+YHK groups. Total liver protein was isolated from mouse livers and used for determination of Perilipin protein expression by Western blot as described in methods. Bands were visualized by chemiluminescence and exposed to X-ray film. Perilipin expression was decreased in YHK-treated ob/ob mice.

while augment Perilipin protein expression. The YHK compounds were extensively described in the literature. *Panax ginseng* has been described as composed antidiabetics and antioxidant [26], as well as *Eucommia* which also has anti-inflammatory effects [27] and *Polygonati* and *Licorice* are known antioxidants [28]. Early studies evaluating the therapeutic potential of YHK report its activity as an antifibrotic agent limiting the volume of hepatic tumors [29, 30] and its protective effects in induced hepatic ischemia reperfusion injury [31]. Our group previously showed the inhibition of NASH development in YHK treated mice [22, 23]. Recently, a randomized pilot study demonstrated an important improvement in transferases levels of NASH patients treated with YHK [32].

The ob/ob mice develop spontaneous liver steatosis, but not NASH [33]. The administration of MCD diet increases liver injury by mitochondrial dysfunction and deficient lipid exportation leading to

inflammation and larger lipid accumulation [4]. This diet increases the levels of transaminases, produces inflammation, focal liver necrosis and fibrosis without producing insulin resistance. Animals submitted to MCD generally have reduced weight and visceral fat; although in murine models which already have insulin resistance was reported the maintenance of visceral fat, greater tissue damage and a proinflammatory profile [16]. As we previous showed the group that received MCD+YHK presented relative weight loss in the MCD group in comparison to the group that received only MCD diet.. Comparing each YHK-treated diet group with others group, food intake was not appreciably different by daily inspection of the residual food [22].

The regulation of hepatic lipogenesis involves a complex network of nuclear factors that modulate enzymes involved in the process. The SREBP1c is a nuclear factor responsible for the activation of genes related to lipogenesis, their expression is dependent on



**Figure 10:** The mechanism that mediates the development of NASH: modifications in lipid metabolism, insulin resistance, oxidative stress. YHK treatment showed reduction of steatohepatitis due to a modulation of *de novo* lipogenesis, lipid exportation and storage target genes limiting hepatocyte lipid accumulation.



the action of insulin [34]. Ob/ob mice have a high expression of SREBP1c, even in the presence of severe insulin resistance. This resistance is due to a decrease in expression of IRS-2, but the mechanism by which insulin regulates SREBP1c is dependent on IRS-1 that is not altered in these animals [6]. The inactivation of SREBP1c leads to a significant reduction in hepatic expression of several insulin-dependent lipogenic genes, preventing steatosis in ob/ob animals. In the present investigation, we showed for the first time that the YHK significantly reduces the mRNA and protein expression of SREBP1c in the liver tissue. These data added to previous findings that reported a decrease in the expression of PPAR $\gamma$  [23], a factor regulated by SREBP1c, suggest that the mechanism of action of YHK is centered in decreased expression of SREBP1c and its attendant effects. Yahagi *et al.* demonstrated that the disruption of SREBP-1 leads to a significant reduction in hepatic expression of several genes lipogenic genes, preventing steatosis in ob/ob animals [35]. Because of the central control of SREBP1c in accumulation of intrahepatic triglycerides, Ahmed *et al.* suggests that pharmacological manipulation of SREBPs may be beneficial in the treatment of NASH [36].

The *fasn* catalyzes the last step of DNL, which is crucial in the ability of the liver to metabolize fatty acids, and is regulated by *srebp1c*. In healthy subjects DNL has a small contribution (5%) in Triacylglycerol (TG) content in the fasted state, but in obese subjects DNL is augmented (26%). During the postprandial state there is an elevation in DNL in healthy subjects and it is also elevated in patients with the metabolic syndrome [37]. Animals Knockout for FASn have a phenotype similar to the control when receiving a standard diet, but when fed a diet rich in carbohydrates and low in fat, they develop steatosis. It was proposed that this is due in part to the decrease in  $\beta$ -oxidation, because FASn products regulate the expression of nuclear receptors. The RNA content of *fasn* accompanied the reduction of SREBP1c in the treated group, suggesting an anti-steatotic effect of reduced DNL [38]. These data suggests that the reduced lipid accumulation in YHK supplied animals can cause a diminution of *de novo* lipid synthesis.

Another important enzyme in the lipid metabolism modulated by SREBP is the SCD-1 that regulates the partitioning of saturated fatty acids (SFA), which are cytotoxic, in monosaturated fatty acids (MUFA). The expression of *scd-1* is decreased in animals receiving MCD, due to a state of hypermetabolism with an

increase in FA oxidation and mitochondrial uncoupling [39]. The mRNA *scd-1* expression does not follow the expression of *SREBP1c* protein, suggesting that other mechanisms are also involved in its regulation. In a knockout model for *scd-1* submitted to MCD it was observed a lower index of desaturation, increased apoptosis and cellular injury when compared to the wild animal [36]. Björkegren *et al.* demonstrated liver injury in this model caused by the suppression of MTP and concomitant reduced expression of *scd-1* [40]. In the present study, we observed that YHK promoted an increased of SCD1 mRNA expression, probably promoting an adaptation in metabolism caused by higher levels of MUFA, protecting the liver from hepatic injury and normalizing the lipoproteins promotion. In our previous study we found YHK promoting an upregulation in *MTP* gene expression [23], in this study the same effect was described for protein expression. Increased MTP expression and with regulation of *scd-1* suggest that lipid exportation in MCD+YHK group was reestablished to normal level.

Suppression of liver SCD-1 is probably a compensatory mechanism designed to reduce the synthesis of lipids and increase lipid oxidation in conditions that the export of triglycerides are inactive. The MCD formula is rich in sucrose which contributes to increased DNL aggravating the hepatic lipids accumulation initially cause by the blockade of VLDL secretion. The hepatic  $\beta$ -oxidation is increased in animals fed with MCD, which in turn increases the energy expenditure and promotes mitochondrial uncoupling.

CPT1a has a central role in the traffic of long chain fatty acid into mitochondria acid and its impairment can be one of the targets of progression from steatosis to NASH [38]. Previous studies demonstrate an increase in mRNA expression and diminished protein expression showing the impairment in  $\beta$ -oxidation. Mitochondrial deficiency is proposed as a common mechanism in the development of NASH, the increase in CPT1 activity and consequent increase in transport of long chain fatty acid  $\beta$ -oxidation to also be considered. In our study, the YHK did not cause changes in the CPT1 gene expression, but the analysis of enzyme activity would be important for further conclusions.

The storage of intrahepatic FA is done in micro and macrovesicular droplets recently described as dynamics organelles due to proteins that surrounding these droplets [41]. The PAT proteins are responsible for stabilization and control of lipolysis. The distribution

of these proteins is dependent on the size and the location in the liver. Initially, Perilipin was reported as being exclusive to hepatocytes and steroidogenic cells and TIP47 and to Adipofilina (ADRP) are co-expressed in all types of cells [13].

The over expression of both Perilipin 1 and 2 proteins is associated with increased fat accumulation and lipid droplet formation. Both proteins exerted a trigger role in lipolysis dependent of hormone sensitive lipase [42]. ADRP and TIP47 are ubiquitously expressed in normal livers although Perilipin 1 is *de novo* expressed in steatotic livers. We showed that the group that received YHK has a diminished expression of Perilipin 1 protein and an augment in ADRP mRNA expression. These results are consistent with the hypothesis that the proportion of ADRP/Perilipin 1 expression is dependent of the size and localization of the lipid droplets which is confirmed by the histological data [11, 12, 43].

In conclusion, the current data shows that YHK modulated genes involved in the synthesis and exportation of hepatic lipids, limiting hepatocyte lipid accumulation, reducing lipogenesis and upregulating lipid exportation suggesting that the YHK can be a promising drug for the treatment of NAFLD. Although this finding is promising, there are limitations inherent to the absence of a formal analysis of the compounds of the YHK, a common problem in the study of potentially active herbals. 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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