

## BASIC STUDIES

**Yo Jyo Hen Shi Ko, a novel Chinese herbal, prevents nonalcoholic steatohepatitis in ob/ob mice fed a high fat or methionine–choline-deficient diet**

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**Abstract**

**Background:** Oxidative stress plays a role in the pathogenesis of nonalcoholic steatohepatitis (NASH). Yo Jyo Hen Shi Ko (YHK) is a complex compound purported to reduce reactive oxygen species (ROS) by blocking the propagation of radical-induced reactions. The aim of this study was to evaluate the role of the effect of YHK in experimental NASH. **Methods:** NASH was induced in male ob/ob mice by a high-fat (HF) diet or methionine/choline-deficient (MCD) diet for 4 weeks. YHK-treated animals received YHK solution orally (20 mg/kg/day) in both experimental diets ( $n = 6$ ; each group) while control animals received only vehicle. **Results:** The MCD and HF groups developed moderate diffuse macrosteatosis, hepatocellular ballooning, and a diffuse inflammatory infiltrate. With the addition of YHK, there was a marked reduction in macrosteatosis in both groups. This was associated with decreased lipoperoxide and reduced glutathione–GSH concentrations as well as reduced serum aminotransferases and improved histological markers of inflammation. These changes were also associated with weight loss in the MCD+YHK group and diminished weight gain in the HF+YHK group. **Conclusion:** YHK therapy blunts the development of macrosteatosis in these models of NASH and significantly reduces markers of oxidative stress. YHK also diminishes weight gain in this obesity prone model. Our findings warrant further study on the mechanisms involved with these effects.

Nonalcoholic steatohepatitis (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. The ‘two-hit’ hypothesis has been proposed to explain the pathogenesis of NASH, with an initial metabolic disturbance causing steatosis and a second pathogenic stimulus promoting oxidative stress, increased generation of reactive oxygen species (ROS), lipid peroxidation, and resultant NASH (4–6). Thus, oxidative stress appears to play a central role in the pathogenesis of NASH. The increased production of ROS is known to cause lipid peroxidation, followed by an inflammatory response, and activation of stellate cells, leading to fibrosis (7, 8).

Yo Jyo Hen Shi Ko (YHK), derived from Henshiko (Kyotsujigyo Inc., Japan), has four major ingredients

(Panax pseudo ginseng, Eucommia ulmoides, Polygonati rhizoma, and Licorice root) that are reported to react with ROS, thus blocking the propagation of radical reactions in a wide range of oxidative stress situations (9–11). Naturally derived herbal medicines, often imported from Asia, have experienced an increasing degree of scientific scrutiny in the past 20 years in the West. Although sometimes subject to variation in growth patterns and often limited by the lack of availability for technical analysis from the manufacturers, there is an emerging body of literature in this field.

The potential therapeutic efficacy of YHK in NASH has not been extensively explored especially in terms of its possible mechanism although we are aware of one recent small clinical trial showing reduction of the aminotransferases in NASH patients treated with a

short course of this substance (12). The aim of this pilot study was to evaluate the role of YHK in NASH prevention in ob/ob mice submitted to a high-fat (HF) diet or a methionine/choline-deficient (MCD) diet. These animal models provide somewhat different perspectives on the potential activity of YHK: the MCD provokes injury by several mechanisms including altered transmembrane signaling and interference with lipid-cholesterol transport, while the HF diet exacerbates overall liver fat loading of the liver in this obesity-prone animal predisposing to oxidative stress through the accumulation of free fatty acids (13).

## Materials and methods

### Materials

YHK was provided from Kyotsujigyo Inc., Japan (<http://www.kyotsujigyo.com>). This preparation contains four different botanical derivatives (Panax pseudo ginseng, Eucommia ulmoides, Polygonati rhizome, and Licorice root), which may have antioxidant properties. As with many commercially available herbal medications, precise compositional analysis of this proprietary was not available to us. Based on existing literature, its contents are likely to be active in a number of respects. For example, Ginseng contains quinquefolans that may mediate its hypoglycemic effects (14, 15). Similarly, derivatives of Eucommia are also known to have hypoglycemic effects thought to be mediated by phytochemicals including polyphenolics, flavonoids, and triterpenes although the biochemical pathways have not, to our knowledge, been resolved (16). Derivatives of the Polygonatum are also known to have significant physiological activity. This includes reports of antioxidant effects and altered insulin signaling mediated by steroidal glycosides and steroidal saponins (17, 18). Much more is known about licorice derivatives (the Glycyrrhiza) compared with the other major ingredients of YHK. The most active compound, glycyrrhizic acid, possesses mineralocorticoid, glucocorticoid, antiandrogenic, and estrogenic activity (19). The most relevant property, however, may be mediated by carbenoxolone antagonism of 11 $\beta$ -hydroxy-steroid-dehydrogenase, which appears to improve hepatic insulin sensitivity (20).

### Animals

Male ob/ob mice (Jackson Laboratories, Bar Harbor, ME), 8 week old, weighing 30–40 g, were housed in temperature- and humidity-controlled rooms, kept on a 12 h light/dark cycle, and provided unrestricted amounts of food and water. All procedures for animal

**Table 1.** Experimental diets (g/kg): methionine/choline diet (MCD); high-fat diet(HF)

Ingredient	MCD diet (g/kg)	HF diet (g/kg)
Carbohydrates	625	510.5
Casein (79% de protein)	214	214
Lipid	180	
Coconut oil	–	120
Soybean oil	66	47.5
Cholesterol	–	12.5
Fiber	50	50
Vitamins	10	10
Minerals	35	30
L-cysteine	–	3
Methionin	–	–
Choline bitartrate	–	2.5
Tertbutylhydroquinone	0.014	0.014

Composition of diets:

Methionine–choline deficient diet: carbohydrates (62.5% – corn starch and sucrose); protein (17%), lipid (7%), fiber (5%), mix vitamins (1%), mix mineral (3.5%).

High-fat diet: carbohydrates (51.0%), protein (17%), lipid (18%, e coconut oil, soybean oil is cholesterol), fiber (5%); mix vitamins (1%), mix minerals (3.5%).

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 ( $n=6$ ) was provided with a standard diet (Nuvilab<sup>®</sup> Nutrientes Ltda, Colombo, Brazil) *ad libitum*. NASH was induced in the ob/ob mice by MCD group ( $n=6$ ) or HF group ( $n=6$ ) (Rhooster Ltda, São Paulo, Brazil) 5 g/day for each ob/ob mice for 4 weeks (Table 1).

The ob/ob mice were divided into a control group fed a standard diet and four experimental groups that were fed as follows: Group 1: MCD group ( $n=6$ ) fed MCD diet plus vehicle (physiologic Ringer's solution), Group 2: HF group ( $n=6$ ) fed saturated HF plus vehicle (physiologic Ringer's solution), Group 3: YHK+MCD group ( $n=6$ ) fed MCD diet plus YHK solution (20 mg/kg), and Group 4: YHK+HF group ( $n=6$ ) fed HF diet plus YHK solution (20 mg/kg) both daily by gavage. After 4 weeks of treatment with YHK or vehicle associated with both diets, ob/ob mice were killed (12 week old). Plasma samples and the livers were collected for biochemical, histological and oxidative stress examination. During the study period, food intake was estimated by daily observation of residual food at the time of feeding (Table 2).

### Serum biochemical analysis

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), cholesterol, and triglyceride

levels were measured by standard methods using automated techniques.

### Histological analysis

Liver tissue was fixed in 4% formaldehyde and processed for hematoxylin–eosin (HE) and Masson Trichrome staining for histological analysis. Histological variables were blindly scored by an experienced hepatopathologist (V. A. F. A.) using a scoring system adapted from the NASH activity score (21): macrosteatosis (0–3), lobular inflammatory changes (0–3), and hepatocyte ballooning (0–2). Fibrosis was minimal in all of the samples and was therefore not scored.

### Oxidative stress analysis

#### Malondialdehyde (MDA) assay

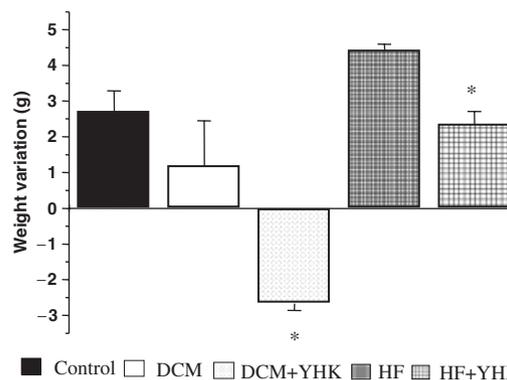
MDA formation was used to quantify the lipid peroxidation in tissues and measured as thiobarbituric acid-reactive material (TBARS). Tissues were homogenized (100 mg/ml) in 1.15% KCl buffer, and centrifuged at 14 000g for 20 min. The supernatant was then stored at  $-70^{\circ}\text{C}$  until assay. An aliquot of supernatant was added to a reaction mixture of 1.5 ml 0.8% thiobarbituric acid, 200  $\mu\text{l}$  8.1% (vol/vol) SDS, 1.5 ml 20% (vol/vol) acetic acid (pH 3.5), and 600  $\mu\text{l}$  distilled  $\text{H}_2\text{O}$  and heated to  $90^{\circ}\text{C}$  for 45 min. After cooling to room temperature, the samples were cleared by centrifugation (10 000  $\times$  g for 10 min), and their absorbance was measured at 532 nm using 1,1,3,3-tetramethoxypropane as an external standard (Genios, Tecan, Switzerland). The quantity of lipid peroxides was expressed as nanomoles MDA per milligram of protein.

#### Reduced glutathione (GSH) assay

Tissues were homogenized (100 mg/ml) in 5% (vol/vol) sulfosalicylic acid. The homogenates were centrifuged at 10 000 3 g for 20 min, and an aliquot of the clear supernatant (20 ml) was combined with 0.3 M  $\text{Na}_2\text{HPO}_4$  (160 ml) and 0.04% 5,59-dithiobis-(2-nitrobenzoic acid) in 1% sodium citrate (20 ml). After a 10 min incubation at room temperature, absorbance was read at 405 nm in a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA). Concentrations of GSH were calculated from a standard curve constructed with known concentrations of GSH and expressed as  $\mu\text{g}$  GSH per mg protein (Genios).

### Statistical analysis

The data were expressed as means  $\pm$  standard deviation (SD). Groups were compared using univariate



**Fig. 1.** Effects of Yo Jyo Hen Shi Ko (YHK) on the body weights in ob/ob mice submitted to standard, methionine–choline deficient (MCD) or high fat (HF) experimental diets for four weeks: Shown is the relative change in weight in the ob/ob control group fed standard chow compared to the study groups: (MCD,  $n=6$ ) fed MCD diet plus vehicle, MCD+YHK (MCD+YHK,  $n=6$ ) fed MCD diet plus YHK solution (20 mg/day), the High Fat diet group fed saturated high-fat diet plus vehicle (HF,  $n=6$ ), and the High Fat diet plus YHK group (HF+YHK,  $n=6$ ) fed saturated high-fat diet plus YHK solution (20 mg/day). Data expressed as mean  $\pm$  SD; \* $P < 0.05$  diets  $\times$  YHK.

analysis (ANOVA) and by the random permutation test for comparison of histological variables (a  $P$  value below 0.05 was considered to be significant).

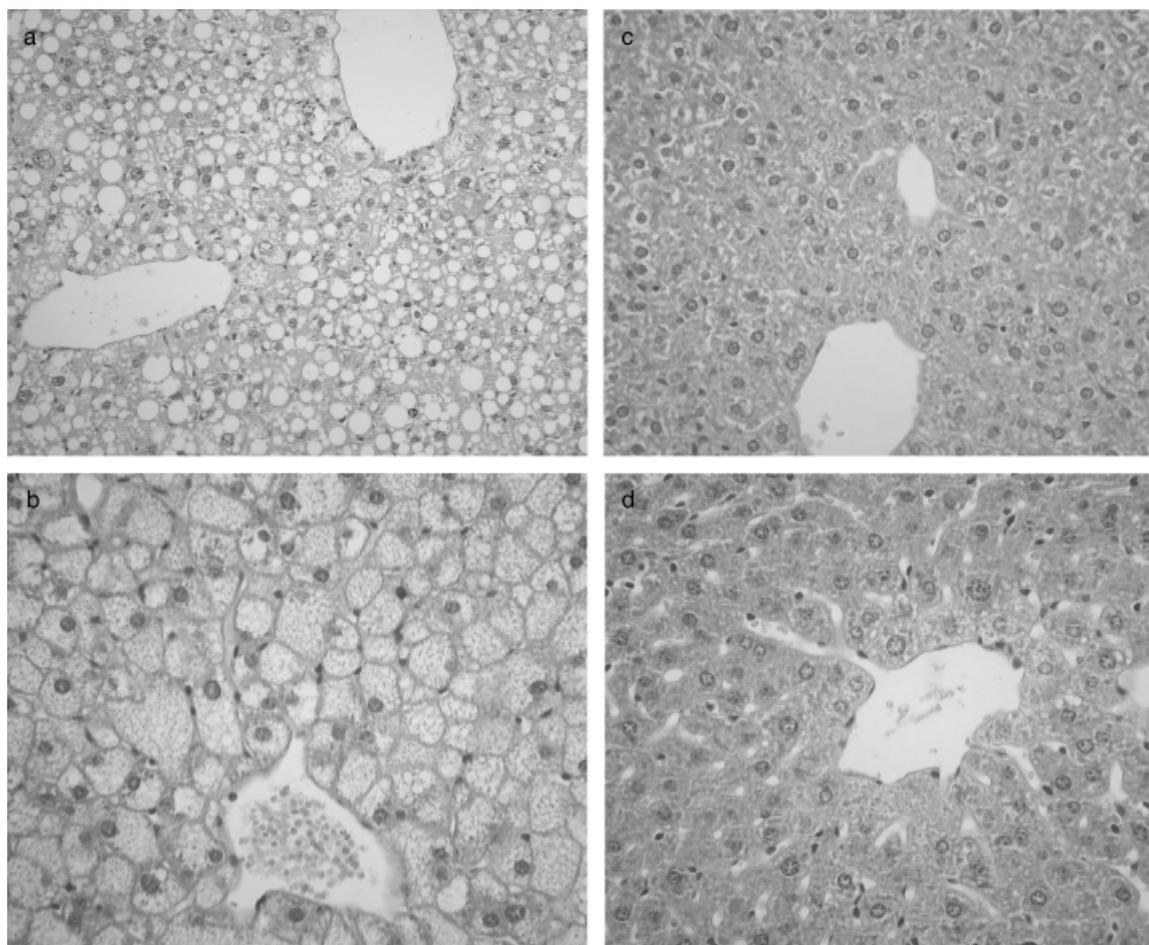
## Results

### Total body weight

YHK therapy was associated with significant favorable changes in total body weight in both treated groups compared with controls on a standard diet and with each of the untreated experimental diet groups. Figure 1 shows the variation of weight in the study animals relative to those fed a standard diet. YHK blunted weight gain in the HF diet group and produced relative weight loss in the MCD group compared with the experimental diet without YHK. Comparing each YHK-treated diet group with its respective diet only group, food intake was not appreciably different between by daily inspection of the residual food.

### Liver histology

These changes in total body weight were associated with significant changes in hepatic histology, especially in the degree of macrosteatosis with YHK therapy. As can be seen in Table 3 and in Fig. 2, both the MCD and HF diets induced substantial steatosis. Although the small N limited statistical comparisons, there was a clear trend in macrosteatosis in both YHK-treated



**Fig. 2.** (a) Histological features on ob/ob livers that were fed a methionine–choline-deficient diet (MCD group) showed diffuse macrosteatosis, hepatocellular ballooning, and lobular inflammation, H&E,  $\times 100$ . (b) Histological features on ob/ob livers that were fed high-fat diet (HF group) showed a more mixed steatosis and slight inflammatory infiltrate, H&E,  $\times 400$ . (c) Histological features on ob/ob livers that were fed the MCD diet plus YHK showing marked reduction in steatosis, H&E,  $\times 200$ . (d) Histological features on ob/ob livers that were fed the high fat diet plus YHK showing marked reduction in steatosis, H&E,  $\times 400$ .

groups comparing the ob/ob plus either MCD or HF diet with YHK therapy. For example, YHK markedly reduced macrosteatosis in both groups ( $P = 0.06–0.07$ , comparison by random permutation test). Lobular inflammation consisting of mixed mononuclear and polymorphonuclear cells was observed with both diets. With YHK, the inflammation scores appeared to improve but the change did not reach statistical significance. Ballooning scores also appeared unchanged. We did not observe more than minimal fibrosis in any of the animals and thus this parameter was not further analyzed (Table 3).

#### Serum aminotransferases and lipids

Measurement of the aminotransferases revealed significant improvement in both treated groups but this

was most striking in the MDC+YHK group where YHK was associated with an eight-fold reduction in ALT. Serum lipid levels were not dramatically abnormal with either diet; however, serum triglyceride and cholesterol levels were significantly reduced in the YHK-treated animals on the MCD diet. In contrast, triglyceride and cholesterol levels were not different with YHK in the HF diet group (Table 2).

#### Markers of oxidative stress

Evidence of oxidative stress was significantly reduced by YHK in both treated groups as measured by the MDA assay. Compared with the untreated animals on each of the experimental diets, hepatic lipoperoxide concentrations MDA were significantly decreased with administration of YHK. Similarly, reduced glutathione

**Table 2.** Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol and triglycerides in the serum of ob/ob mice fed with methionine–choline-deficient diet or high-fat diet with or no YHK

Group	AST (U/l)	ALT (U/l)	Triglycerides (mg/dl)	Cholesterol (mg/dl)
MDC	623.7 ± 460.8*	230.0 ± 165.2*	93.0 ± 27.0*	105.0 ± 48.6*
MDC+YHK	217.3 ± 97.9	38.7 ± 9.1	65.7 ± 25.5	80.0 ± 15.1
HF	3405.5 ± 1334.0*	527.6 ± 121.0*	46.0 ± 6.7	123.3 ± 26.4
HF+YHK	2050.2 ± 642.6	366.6 ± 33.1	43.4 ± 7.2	138.6 ± 16.5

Data expressed mean ± SD.

\* $P < 0.05$  Diets × YHK.

Normal values in U/l for AST: 10–34; ALT: 10–44; mg/dl: cholesterol and triglyceride: 45–89. Groups: MCD, animals fed methionine-choline deficient diet; MCD+YHK, animals fed methionine-choline deficient diet and treated with YHK administration; HF, animals fed high-fat diet; HF+YHK, animals fed high-fat diet and treated with YHK administration.

**Table 3.** Major scored histological variables

	Inflammation (0–3)	Ballooning (0–2)	Steatosis (0–3)
Ob/ Ob+chow	0.2	1.8	0.2
MCD diet	1	2	2.6
HF diet	1.4	1.4	2.6
MCD+YHK	0	2	0
HF+YHK	1	1	0.2

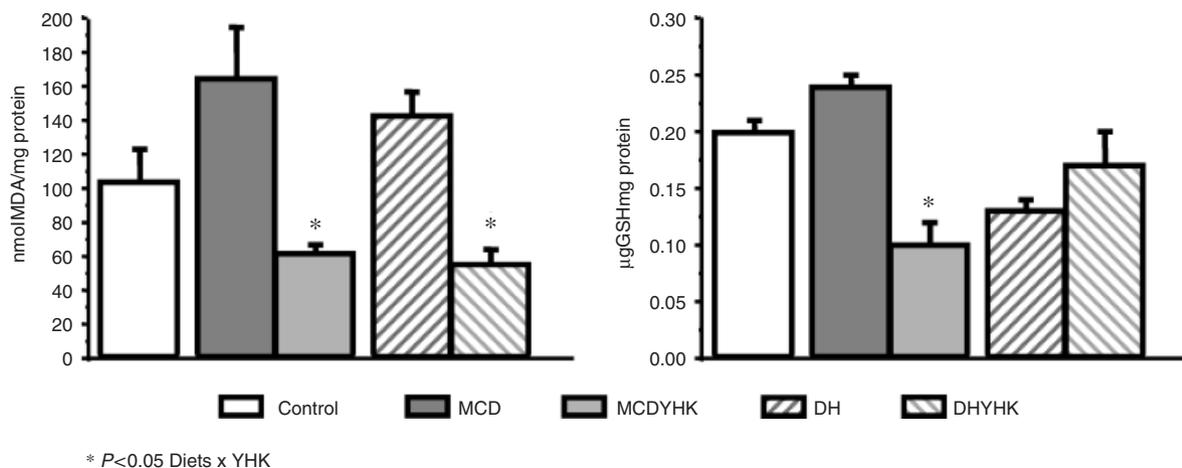
Each histological variable was semi-quantitated according to Kleiner *et al* (21).

was significantly decreased after YHK in the MCD diet animals. However, YHK did not significantly alter reduced glutathione, except in the HF diet group (Fig. 3).

## Discussion

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’ (22). MCD is a classical model of NAFLD, where Cyp2E1 is upregulated (23) and the animals develop steatosis and steatohepatitis without fibrosis. In the present study, ob/ob mice received either a HF diet enriched with lard and egg yolk (saturated fatty acid) or an MCD diet. Both models produced patterns typical of NASH, although different mechanisms are likely to be involved. The MCD diet alters transmembrane signaling and lipid-cholesterol transport, causing increased fat accumulation while the HF diet exacerbates the predisposition of the ob/ob mouse to accumulate hepatic fat. A number of studies have shown that markers of oxidative stress are increased in NASH (24, 25), while levels of endogenous antioxidants (vitamin E and glutathione) are decreased (26). However, the use of antioxidants in the prevention of NAFLD is not yet well established. In previous studies conducted by our group, we observed reduction of liver steatosis and oxidative stress by vitamin C in rats submitted to a choline-deficient diet

**Fig. 3.** Effects of YHK on TBARS–malondialdehyde (MDA) equivalents and glutathione assay in liver samples of ob/ob mice submitted to normal chow, MCD or HF experimental diets for 4 weeks. Lipid peroxides are reported as nmol MDA/mg protein. Glutathione concentrations are reported per µg GSH per mg protein. Data expressed as mean ± SD. \* $P < 0.05$  diets × YHK.

(27). The purpose of the current study was to evaluate the effects of YHK in established models of NASH – the ob/ob mice with NASH induced by a MCD diet or a HF diet.

Our results have shown an inhibitory effect of YHK on the development of NASH in this animal model. Specifically, YHK macrosteatosis declined to nearly zero in both groups of treated animals. This effect was associated with significantly reduced markers of oxidative stress (MDA) and improved markers of inflammation including serum aminotransferases and to a lesser extent on inflammatory scores. The effects are similar to those that we reported for ascorbic acid but much more effective than vitamin E, which we previously found to be ineffective (27). However, we did observe some differences in the effects of this agent in the two different diets. Total body weight in the YHK-treated animals was less than the untreated diet groups although the magnitude of this effect was greater in the MCD model. In addition, we could not detect an effect of YHK on reduced glutathione in the HF diet animals. The exact explanation for these differences remains to be determined. However, because the MCD diet favors accumulation of small droplet fat in the endoplasmic reticulum due to inhibition of VLDL formation, we speculate that the effect of YHK may be at the level of droplet formation or perhaps in the peroxidation that has recently been observed in the phospholipid monolayer surrounding these droplets (28). Although measures of blood lipids were not dramatically changed, the significant reduction in triglyceride levels with YHK in the MCD diet group further suggests the possibility that the effect is related to small droplet metabolism in the liver. Alternatively, this could indicate a change in insulin signaling although we have not yet investigated this possibility.

There may also be other significant but less readily apparent effects of this agent on fatty acid metabolism mediated by PPAR- $\alpha$  receptors as we recently observed in a separate study of this agent (29). Alternatively, we also observed blunted weight gain in the HF diet group and actual weight loss in the MCD group, suggesting that there may be as yet unexplained systemic effects on fat metabolism related to this agent. Additionally, mitochondrial dysfunction occurs concomitantly with lipid peroxidation and increased mitochondrial ROS formation in these animal models (30, 31). It can be conjectured that the antioxidant effect of YHK results from abrogation of the cytotoxic effects of ROS and lipid peroxides, preventing mitochondrial injury.

Our study has several inherent limitations. As with many herbal remedies, we could not provide a formal analysis of composition of the YHK. This is a common

problem in the study of potentially active native or herbal medications and remains to be fully resolved. However, based on known properties of the main ingredients and the significant effects observed in markers of lipid peroxidation, we suspect that the antioxidant properties attributed to this agent were actually being observed. We also cannot fully explain the changes in weight observed in the treated animals. This appears to be associated with decreased visceral adiposity but formal measurements of this unanticipated finding were not included in the prestudy planning. We did not observe a difference in the food intake between the groups based on assessment of residual food at each feeding but further study is needed to assess this possibility and the possible effects of increased activity (increased calorie expenditure) in the treated animals.

In summary, YHK demonstrated antioxidant effects supporting the significance of the antioxidant effect attributed to this agent. It is not clear whether this represents a more systemic or primarily local effect on fat metabolism. A variety of possible mechanisms can be evoked based on existing literature. For example, *Eucommia ulmoides* and *Polygonati rhizome* are reported to have hypoglycemic effects (32, 33) and may have beneficial effects in hypercholesterolemia (34). In addition, licorice extract is reported to reduce plasma lipid levels and systolic blood pressure in hypercholesterolemic patients (35) and licorice flavonoides are reported to suppress visceral fat accumulation and to alter insulin signaling (36). Although these effects cannot yet be fully explained, the effects of YHK on liver fat accumulation and indices of lipid peroxidation are clearly evident. Future studies are needed to elucidate these findings and to determine the possible clinical utility of this novel agent.

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