AN IN VITRO STUDY ON THE BENEFICIAL EFFECT OF YHK, A NATURAL HERBAL COMPOUND, ON METAL-INDUCED OXIDATIVE DAMAGE OF HEPATOCYTES AND LYSOSOMAL FRACTION

7th International ISSX Meeting
Vancouver, Canada. 29th August ~ 2nd September 2004

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Short title: Protective effect of YHK on metal-induced oxidative damage

Key words: metal-induced, lipid peroxidation, cultured hepatocytes, lysosomal fraction

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Summary
Hepatocytes were isolated from Wistar rats by collagenase perfusion method and cultured as such and also with α-linolenic acid (LNA)-bovine serum albumin (BSA). Hepatocytes were then cultured in with graded dilution of YHK (Kyotsu, Yokyo, Japan) sample (100μg/ml and 200μg/ml) or sylbin (100μg/ml) dissolved in dimethyl sulfoxide 10min before the addition of metallic salts (iron, copper and vanadium). Lysosomal fractions were prepared to carry out lysosome fragility test by measuring β-galactosidase activity and lactate dehydrogenase leakage and oxidative damage tests in the presence of hydrophilic and lipophilic free radical generators. Quenching activity by DPPH was also assessed. Either YHK and sylbin showed a significant protective effect against all challenge metal ions, as expressed by the half inhibition concentration (IC₅₀) of against lipid peroxidation and MDA formation. However, YHK seemed to be more effective than sylbin in Fe-induced peroxidative damage (p<0.05). Both test compounds, irrespective of the concentration, significantly reduced the LDH and β-galactosidase concentration in lysosomal fractions. As compared to untreated lysosomal fractions challenged with the two peroxide radicals generators, either YHK and sylbin exerted a significant protection. Both compounds showed a comparably significant DPPH radical-scavenging activity. These data support the potential clinical application of this novel natural compound in clinical practice.

Introduction
Iron performs a central role in cellular and organismal physiology because it is a cofactor for a large number of proteins required for cell function. However, Iron may accumulate in biological system and, in particular, in the liver for a number of pathological conditions such as due to a consequence of genetic defects in the gut absorption or following repeated parenteral administration. Such abundant free iron acts as a strongly noxious hepatotoxin as well as pro-fibrogenetic factor especially in the presence of chronic alcohol consumption, viral hepatitis or hepatotoxic xenobiotics (1). In the course of such conditions associated to iron overload (2,3) oxidative stress is a common finding since this metal catalyst fulfills all characteristics of a potent generator of a number of free radicals species while also being an inducer of lipid peroxidation. Similarly, copper represents another strong catalyst of oxidative stress (4) as it occurs in copper-storage diseases such as Wilson’s disease. Under physiological and pathological processes the homeostasis of free radicals balance is a complicated system where endogenous and exogenous antioxidant protecting cells and tissues interplay with the generation of reactive oxygen species which may bring about damaging effects. In particular, in the case of hepatic iron overload there is strong evidence that that this condition is associated with hepatocellular injury, activation of inflammatory cascade, fibrosis and also hepatocellular carcinoma (2). Indeed, it has been suggested that either copper and iron, both important transition metals in the body, may participate in the induction of DNA damage and oncogenesis (5), being mutagenic in bacteria (6,7) and in a transgenic strain of Chinese hamster lung cells (8). Overall, on the clinical ground it has been shown a direct correlation between increased body iron stores and an increased risk of cancer of all organs and tissues in individuals even not suffering from iron overload diseases (9-11). This is not surprising when considering that iron- or copper-mediated catalysis leads to the generation of reactive oxygen species that can avidly attack biomolecules, with the consequent lipid peroxidation of cellular membrane, protein oxidation and DNA damage which involves site-specific Fenton-type chemistry. We have previously shown either in vitro and in vivo experimental studies that YHK exerts a potent protective effect on hepatocellular damage and on liver microcirculation in an ischemia-reperfusion model (12,13). Thus, the aim of the present study was to further investigate this compound in vitro testing of hepatocytes oxidative damage by iron, copper and vanadium which is also known to trigger oxidative damage to cellular membranes and nuclear DNA (14,15).

Materials and methods

Isolation and culture of hepatocytes. Male Wistar rats weighing 180-210g were fed with standard chow and water ad libitum. Hepatocytes were isolated by collagenase perfusion method as described by Wolkoff et al. (16). Briefly, the liver was perfused with collagenase type IV (Sigma Chemical, St. Louis, MO, USA) and isolated hepatocytes were suspended in culture medium consisting of Waymouth’s 752/1 (Gibco, Grand Island, NY, USA) containing 5% heat-inactivated fetal bovine serum, 2.5mM additional CaCl2, 5μg/mL bovine insulin (Sigma), 100U/mL penicillin and 0.1mg/mL streptomycin. The isolated cells were further fractionated on Percoll density gradients to obtain a viability higher than 98%, as ascertained by trypan blue. Approximately 1.5 x 10⁶ cells in 3mL or 5.0 x 10⁶ cells in 10mL were plated in individual 60- or 100-mm diameter Lux culture dishes and placed in an incubator in an atmosphere of 5% CO-95% air at 37°C. After a 9hr incubation, the monolayer of hepatocytes were cultures for an additional 12hr in the medium containing 1.0mM α-linolenic acid (LNA)-bovine serum albumin (BSA). More than 70% of added LNA was adsorbed by cultured cells after incubation. The control hepatocytes were maintained in culture in the medium without LNA and the amount of cell protein was determined by the method of Lowry et al. (17).
**Hepatocyte culture test.** Hepatocytes were washed twice with Hanks’ medium and further cultured in 60-mm (1.5 x 10^6 cells/dish) with graded dilution of YHK (panax pseudo-ginseng, Eucommia Ulmoides, polygonati rhizome, glycyrrhiza licorice, panax ginseng, Kyotsu, Tokyo, Japan) sample (100μg/ml and 200μg/ml) or silybin (100μg/ml) dissolved in dimethyl sulfoxide 10min before the addition of metallic salts dissolved in saline a concentration of 100μM each. After incubation for 6hr, the medium was separated. Malonildialdehyde (MDA) in the medium was assessed by a slight modification of the Uchiyama and Mihara method (18). Briefly, to 0.1ml of the medium in a 12ml glass tube, 3ml of 1% phosphatic acid and 1ml of 0.67% thiobarbituric acid was added and heated at 100°C for 45min. After cooling in ice water, 4ml of n-butanol was added and the resulting mixture was then shaken and centrifuged to separate the organic layer. The fluorescence intensity in the butanol layer was assayed at the excitation and emission wavelengths of 515 and 553 nm, respectively. The auto-oxidation products of fatty acid in the medium during the assay procedure were within 0.3nmol and were used as blank. Dimethyl sulfoxide (20μl) was diluted in 2000μl of the culture medium, including control cultures which were exposed to metal ions without the test compounds and the final concentration of 1% dimethyl sulfoxide had no measurable effect on lipid peroxidation in basal cultured hepatocytes.

**Preparation of LNA-BSA complex.** LNA was adsorbed to bovine serum albumin as described previously (19). One mml of LNA was dissolved in 10ml of 0.1 N NaOH solution. This solution was serially added to 240ml of complete Williams’ medium E 1mM BSA which had a fatty acid/albumin molar ratio of 4. The resulting fatty acid-BSA complex was sterilized by filter-passage through a 0.2μm Millipore filter.

**Preparation of lysosomal fractions.** After liver homogenization in 9 volumes of 0.3 M sucrose and centrifuged at 450×g for 10 min. The supernatants were again centrifuged at 3500×g for 10 min, the pellet discarded, and the lysosome-containing supernatant centrifuged at 10000×g for 10 min. The pellets were washed and re-centrifuged at 10000×g for 10 min, and resuspended in the sucrose buffer to a protein concentration of ~15 mg/ml. The resultant lysosome enriched fraction was found to be stable in the homogenization buffer at 4°C for up to 6 h.

**Lysosome fragility test.** The fraction was incubated with the test compound and each metal ions and β-galactosidase activity was assessed as described by Olsson et al. (20), using 4-methylumbelliferyl-β-galactosidase as a substrate. The results were expressed as percentage of total β-galactosidase released. Lactate dehydrogenase leakage was also measured in the culture medium as described elsewhere (21).

**Oxidative damage tests of lysosomes.** Assays for the release of acid phosphatase and β-N-acetylglucosaminidase from lysosomes were carried out by incubating lysosomal suspensions with test compounds in the presence of 50mM 2,2’-azobis(2-aminopropane)dihydrochloride (AAPH) or 1mM 2,2’-azobis(2,4-dimethylvaleronitrite (AMVN) which are azo-compounds that generate peroxide radicals after thermal hemolysis in aqueous phase and lipid phase, respectively. The effect of the test compounds on lysis was calculated as a percentage of control. Further, quenching activity of either YHK and silybin against 1,1-diphenyl-2-picrylhydrayzyl (DPPH) radicals was assessed by spectrophotometry. One ml sample solutions and lysosomal suspensions preloaded with the compounds, were incubated with 2ml ethanol solution of 0.25mM DPPH radicals and 2ml 0.1M acetate buffer (pH 5.5) for 45min at 37°C and then absorbance was measured at 517nm. For this experiment, lysosomal suspensions were preincubated with 1mM of the compounds for 30min and centrifuged at 12000 x g for 10min. Then the pellets were washed in 0.15 M KCl-5mM Tris buffer (pH 7.4), centrifuged and re-suspended in 0.1M acetate buffer (pH 5.5).
Research Data of YHK Tablets 250mg

Statistical analysis
All experiments were repeated three times. Significance was established by analysis of variance and the level of significance was determined by employing a Duncan’s multiple-range test. Data were expressed in the text as means (SD) and a probability value of <0.05 was set as indicating that a statistically significant difference existed between experimental groups.

Results

Metal-induced lipid peroxidation. MDA accumulation in the medium showed a direct time-course increase with the incubation time up to 6hr after the addition of metal catalysts. The amount of MDA concentration for Fe, Cu and V ions was 2.8, 2.7 and 2.4 nmol/mg protein/6hr in normal hepatocytes and 8.8, 6.2 and 10.7nmol/mg protein/6hr in LNA-loaded hepatocytes, respectively. These data are in agreement with the findings of Furuno et al. (22). Both silybin and YHK significantly decreased at the same extent MDA generation in the medium (p<0.05) (data not shown). As shown in table 1 and 2, either YHK and silybin showed a significant protective effect against all challenge metal ions, as expressed by the half inhibition concentration (IC$_{50}$) of against lipid peroxidation. Fe-induced lipid peroxidation either in normal hepatocytes and in LNA-loaded hepatocytes was suppressed by both test compounds at a significantly lesser extent than in Cu- and V-induced challenge-test (p<0.05). Both compounds, irrespective of the concentration, were significantly effective in suppressing Cu- and V-induced lipid peroxidation in normal and LNA-loaded cells at a comparable level. On a molar ratio, the protective effect of YHK against Fe-induced peroxidative damage, either on normal hepatocytes and in LNA-loaded cell, was comparable to silybin. However, YHK at higher concentration was significantly more effective (p<0.05). Higher concentration of silybin didn’t further improve the effect of lower concentration (data not shown).

Lysosomal fragility test. When challenged with metal ions, lysosomal fractions expressed a significant increase of LDH leakage and β-galactosidase release (p<0.01), as shown in fig 1 and 2. Both test compounds, irrespective of the concentration, significantly reduced the LDH concentration recovered in the medium of lysosomal fractions (p<0.05). Silybin and only the higher concentration of YHK significantly decreased the β-galactosidase release from lysosomes (p<0.05, fig. 2).

Tests of lysosomal oxidative stress. As compared to untreated lysosomal fractions challenged with the two peroxide radicals generators, either YHK and silybin exerted a significant protection (p<0.01, table 3 ). In particular, such protection was comparably effective between hydrophilic and lipophilic generated free radicals. However, YHK showed a significantly more protective effect than silybin against lipophilic generators (p<0.05). Both compounds showed a comparably significant DPPH radical-scavenging activity (p<0.01, fig. 3).

Discussion
It has been shown that metals, including iron, copper, and vanadium undergo redox cycling, while cadmium, mercury, nickel and lead deplete glutathione and protein-bound sulfhydryl groups, resulting in the production of reactive oxygen species as superoxide ion, hydrogen peroxide, and hydroxyl radical (23). Indeed, the most important mechanism of oxidative damage to proteins is metal-catalyzed oxidation (24, 25) which may end up in the loss of enzymatic activity and alteration of protein structure (26, 27). This process involves generation of H$_2$O$_2$ and reduction of
Fe or Cu by a suitable electron donor like NADH, NADPH, ascorbate and others. Fe and Cu ions bind to specific metal binding sites on proteins and react with $\text{H}_2\text{O}_2$ to generate $\cdot\text{OH}$ and the resulting highly reactive oxygen species attacks amino acid residues. There is a growing body of evidences suggesting the role of free radical generation and oxidant injury in the pathogenesis of liver injury and fibrosis in metal storage diseases (28-30). Although several antioxidants may decrease oxidative stress-related tissue damage, there are concerns over toxicity of some synthetic analogues such as phenolic compounds and to date there are only scanty clinical reports (31, 32). We have recently shown that this compound exerted potent protective effect in an experimental severe liver toxicity model as well as in a ischemia-reperfusion liver injury (12, 13). It is likely that the antioxidative (Panax pseudo-ginseng, eucommia ulmoides, glycyrrhiza glabra linn, gallic acid)(33-36) and immunomodulative (gallic acid, glycyrrhiza) (37-38) properties of the components of this compound have to be advocated for to explain its propective role in the above studies. In the present in vitro study, such compound showed to significantly protect hepatocytes from metal ions-induced lipid peroxidation at even better extent than sylbibin and it is also conceivable that eucommia ulmoides, among others, might have further contributed to such effect, given its potent antioxidant property (39). This is an interesting findings considering that, to the contrary of many herbal remedies experimentally tested, the present phytotherapeutic compound has shown to significantly lower within three weeks the ALT level in the majority of HCV-related chronic liver disease patients (40) and, moreover, to decrease Maruyama score in an awarded pilot clinical study done on the same subjects (41). It has been proved that free radicals-modified membrane lipids and proteins in hepatic iron overload bring about a derangement of hepatic microsomal enzyme activity, electron transport, respiration and lysosomal function (42). AAPH and AMVN are azo-compounds which generate radicals after thermal homolysis in aqueous phase and lipid phase, respectively, and our findings show that YHK significantly protects lysosomal integrity with a mitigated LDH and $\beta$-galactosidase release. This is likely to be the result of its effective DPPH radical-scavenging activity and its activity against lipophilic-generators of free radicals which was stronger than sylbibin. Indeed, during metal-induced injury the oxidant stress damage is preferentially targeted to the lysosomal compartment which is particularly rich in low molecular weight redox-active iron and the rupture of lysosomes, followed by relocation of labile iron to the nucleus, could be an important intermediary step in the generation of oxidative DNA damage, as it has been very recently demonstrated (43). These latter findings are of interest in view of recent data suggesting that metal-induced lysosome alterations are advocated among the mechanisms of liver carcinogenesis (44). Herbal therapy is known and practiced since long time, especially in far eastern countries. However, one of the most lacking points in complementary and alternative medicine is that it is mainly based on empirical experience rather than on scientifically-controlled experimental studies. Taken overall, these experimental data support the potentiality of the clinical application of this compound while well-designed clinical studies are ongoing.

References


41. Harada M, Marotta F, Sha SH, Minelli E. YHK, a novel herbal remedy with effective antifibrotic action, in chronic liver disease: a pilot clinical study aiming to a successful


1)

### Table 1

Inhibiting activity of YHK and sylibin on FeSO\(_4\), Cu SO\(_4\) and VCl\(_3\)-induced lipid peroxidation in normal hepatocytes (mean ± SD)

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>YHK</th>
<th>Silybin</th>
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<tbody>
<tr>
<td></td>
<td>100μM</td>
<td>200μM</td>
</tr>
<tr>
<td>FeSO(_4)</td>
<td>15.6 ± 4.6(^\ddagger)</td>
<td>12.2 ± 4.4(^\ddagger)*</td>
</tr>
<tr>
<td>Cu SO(_4)</td>
<td>7.9 ± 0.3</td>
<td>6.7 ± 0.7</td>
</tr>
<tr>
<td>VCl(_3)</td>
<td>8.7 ± 0.99</td>
<td>9.4 ± 0.85</td>
</tr>
</tbody>
</table>

Values represent the concentrations that inhibit lipid peroxidation by 50% (IC\(_{50}\), μM). IC\(_{50}\) is calculated from the concentration-activity curves.

\(^\ddagger\)p<0.05 vs Cu SO\(_4\) and VCl\(_3\).

\(^\ast\)p<0.05 vs Silybin

### Table 2

Inhibiting activity of YHK and sylibin on FeSO\(_4\), VCl\(_3\) and Cu SO\(_4\)-induced lipid peroxidation in LNA-loaded cells

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>YHK</th>
<th>Silybin</th>
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<tbody>
<tr>
<td></td>
<td>100μM</td>
<td>200μM</td>
</tr>
<tr>
<td>FeSO(_4)</td>
<td>73.4 ± 7.4(^\ddagger)</td>
<td>59.2 ± 9.2(^\ddagger)*</td>
</tr>
<tr>
<td>Cu SO(_4)</td>
<td>15.9 ± 2.2</td>
<td>19.8 ± 1.7</td>
</tr>
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</table>
Values represent the concentrations that inhibit lipid peroxidation by 50% (IC$_{50}$, μM). IC$_{50}$ is calculated from the concentration-activity curves.

$p<0.05$ vs Cu SO$_4$ and VCl$_3$.

*p$<0.05 vs silybin

Table 3
Effect of K-17.22 on the release of lysosomal enzymes in the presence of hydrophilic or lipophilic radical generators: enzyme activity (% of control ± SE)

<table>
<thead>
<tr>
<th></th>
<th>Acid phosphatase</th>
<th>$\beta$-N-acetylglucosaminidase</th>
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<tbody>
<tr>
<td><strong>AAPH-induced release</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YHK 10$^{-4}$ M</td>
<td>52.4 ± 6.1*</td>
<td>47.7 ± 4.2*</td>
</tr>
<tr>
<td>Sylbin 10$^{-4}$ M</td>
<td>51.9 ± 5.6*</td>
<td>54.6 ± 4.7*</td>
</tr>
<tr>
<td><strong>AMVN-induced release</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YHK 10$^{-4}$ M</td>
<td>64.4 ± 7.9*§</td>
<td>61.3 ± 8.7*§</td>
</tr>
<tr>
<td>Sylbin 10$^{-4}$ M</td>
<td>83.9 ± 10.4*</td>
<td>77.3 ± 7.4*</td>
</tr>
</tbody>
</table>

* $p<0.01$ vs DMSO which served as a control compound.
§ $p<0.05$ vs. silybin

EFFECT OF YHK AND SYLIBIN ON LDH LEAKAGE DUE TO METAL IONS DAMAGE IN CULTURED HEPATOCYTES
EFFECT OF YHK AND SYLIBIN ON METAL IONS-INDUCED 
\( \beta \)-GALACTOSIDASE RELEASE IN LYSOSOMAL FRACTIONS

DPPH RADICALS-SCAVENGING ACTIVITY OF YHK AND SYLIBIN 
IN LYSOSOMAL FRACTIONS (mean ± SD)