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Metal-induced oxidative damage in cultured hepatocytes and hepatic lysosomal fraction: beneficial effect of a curcumin/absinthium compound

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OBJECTIVE: Metals undergo redox cycling and there is increasing evidence of free radical generation and oxidative injury in the pathogenesis of liver injury and fibrosis in metal storage diseases. The aim of the present study was to test a natural hepatoprotective compound in metal-induced liver injury.

METHODS: 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 with a graded dilution of PN-M001 (100 μg/mL and 200 μg/mL), which is a curcuma/absinthium-containing compound, or sylibin (100 μg/mL) dissolved in dimethyl sulfoxide for 10 min before the addition of metallic salts (iron, copper and vanadium). Lysosomal fractions were prepared for lysosome fragility tests in which β-galactosidase activity and lactate dehydrogenase (LDH) leakage were measured, as well as oxidative damage tests in the presence of hydrophilic and lipophilic free radical generators. Quenching activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) was also assessed.

RESULTS: Malonildialdehyde accumulation in the medium showed a direct time-course increase with incubation time. Both PN-M001 and sylibin showed a significant protective effect against all challenge metal ions, as expressed by the half inhibition concentration (IC_{50}) against lipid peroxidation. However, on a molar ratio, sylibin seemed to be more effective than PN-M001 in Fe-induced peroxidative damage ($P < 0.05$). Both test compounds, irrespective of the concentration, significantly reduced the LDH and β-galactosidase concentration in the lysosomal fractions. As compared with untreated lysosomal fractions challenged with the two peroxide radicals generators, either PN-M001 or sylibin exerted significant protection However, PN-M001 was significantly better than sylibin in suppressing acid phosphatase enzyme activity. Both compounds showed comparable and significant DPPH radical-scavenging activity.

CONCLUSION: These data support the potential clinical application of curcumin-containing compounds.

KEY WORDS: hepatocytes, lipid peroxidation, lysosomal fraction, metal-induced oxidative damage.

INTRODUCTION

Iron may accumulate in the liver with various pathological conditions, such as a consequence of genetic defects in gut absorption or following repeated parenteral administration. Such excess free iron acts as a strongly noxious hepatotoxin, as well as a pro-fibrogenetic factor, expecially in the presence of chronic alcohol consumption, viral hepatitis or hepatotoxic xenobiotics.[1] In the course of such conditions associated with iron overload,[2,3] oxidative stress is a common finding because this metal catalyst has all the characteristics

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of a potent generator of a number of free radicals species, as well as being an inducer of lipid peroxidation. Similarly, copper represents another strong catalyst of oxidative stress,[4] as in Wilson's disease. Under physiological and pathological processes the homeostasis of free radicals is a complicated system in which endogenous and exogenous antioxidant protective cells and tissues interplay with the generation of the reactive oxygen species (ROS) that can cause damage. In particular, in the case of hepatic iron-overload there is strong evidence that this condition is associated with hepatocellular injury, activation of the inflammatory cascade, fibrosis and also hepatocellular carcinoma.[2] Indeed, it has been suggested that either copper or iron, both of which are 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, in the clinical situation a direct correlation between increased body iron stores and an increased risk of cancer of all organs and tissues has been shown, even in individuals not suffering from Fe-overload diseases.[9–11] This is not surprising when considering that Fe- or Cu-mediated catalysis leads to the generation of ROS that avidly attack biomolecules, with the consequent lipid peroxidation of the cellular membrane, protein oxidation and DNA damage, which involves site-specific Fenton-type chemistry. We have recently shown that a curcumin/absinthium compound exerted potent protective effect *in vitro* and *in vivo* in a carbon tetrachloride liver injury model.[12] Thus, the aim of the present study was to further investigate this compound by *in vitro* testing of hepatocytic oxidative damage by iron, copper and vanadium, all of which is also known to trigger oxidative damage of cellular membranes and nuclear DNA.[13,14]

MATERIALS AND METHODS

Isolation and culture of hepatocytes

Male Wistar rats weighing 180–210 g were fed with standard chow and water ad libitum. Hepatocytes were isolated by the collagenase perfusion method as described by Wolkoff *et al.*[15] 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, 2.5 mmol/L additional $CaCl_2$, 5 μg/mL bovine insulin (Sigma), 100 U/mL penicillin and 0.1 mg/mL streptomycin. The isolated cells were further fractionated on Percoll density gradients to obtain a viability higher than 98%, as ascertained by trypan blue staining. Approximately 1.5×10^6 cells in 3 mL or 5.0×10^6 cells in 10 mL were plated onto individual 60- or 100-mm diameter Lux culture dishes and placed in an incubator in an atmosphere of 5% CO_2–95% air at 37°C. After a 9 h incubation, the monolayer of hepatocytes were culturesdfor an additional 12 h in the medium containing 1.0 mmol/L α-linolenic acid (LNA)-bovine serum albumin (BSA). More than 70% of added LNA was adsorbed by the 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.*[16]

Hepatocyte culture test

Hepatocytes were washed twice with Hanks' medium and further cultured in 60-mm culture dishes (1.5×10^6 cells/dish) with a graded dilution of PN-M001 (a controlled herbal formula containing *Curcumae xanthorrhizae* rhizome 25%, *Menthae piperitae folium* 35% and *Absinthii herba* 40%; Hepaticum-Pascoe, Named srl, Lesmo, Italy; 100 μg/mL and 200 μg/mL) or sylibin (100 μg/mL) dissolved in dimethyl sulfoxide for 10 min before the addition of the metallic salts dissolved in saline at a concentration of 100 μmol/Leach. After incubation for 6 h, the medium was separated. Malonildialdehyde (MDA) in the medium was assessed by a slight modification of the Uchiyama and Mihara method.[17] Briefly, to 0.1 mL of the medium in a 12 mL glass tube, 3 mL of 1% phosphatic acid and 1 mL of 0.67% thiobarbituric acid were added and heated at 100°C for 45 min. After cooling in ice water, 4 mL 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 0.3 nmol and were used as blank. Dimethyl sulfoxide (20 μL) was diluted in 2000 μL of culture medium, including the control cultures, which were exposed to the metal ions without the test compounds, and the final concentration of 1% dimethyl sulfoxide had no measurable effect on lipid peroxidation in the basal cultured hepatocytes.

Preparation of the LNA-BSA complex

α-linolenic acid was adsorbed to BSA as described previously.[18] Briefly, 1 mL LNA was dissolved in 10 mL of 0.1 N NaOH solution and this solution was serially added to 240 mL of complete Williams' medium E

1 mmol/L BSA, which had a fatty acid/albumin molar ratio of 4. The resulting fatty acid–BSA complex was sterilized by filter-passage through a 0.2 μm Millipore filter.

Preparation of the lysosomal fractions

After liver homogenization in 9 volumes of 0.3 mol/L sucrose and centrifuged at 450 *g* for 10 min. The supernatants were again centrifuged at 3500 *g* for 10 min, the pellet was discarded, and the lysosome-containing supernatant was centrifuged at 10 000 *g* for 10 min. The pellets were washed and re-centrifuged at 10 000*g* for 10 min, and resuspended in the sucrose buffer to a protein concentration of approximately 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 lysosomal fraction was incubated with the test compound and each metal ion and then the β-galactosidase activity was assessed as described by Olsson *et al.*[19] 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.[20]

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 50 mmol/L 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) or 1 mmol/L 2,2′-azobis(2,4-dimethylvaleronitrite (AMVN), which are azo-compounds that generate peroxide radicals after thermal hemolysis in the aqueous and lipid phases, respectively. The effect of the test compounds on lysis was calculated as a percentage of the control. Further, the quenching activity of either PN-M001 or sylibin against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was assessed by spectrophotometry. Briefly, 1 mL of the sample solutions and lysosomal suspensions preloaded with the compounds, were incubated with a 2 mL ethanol solution of 0.25 mmol/L DPPH radicals and 2 mL of 0.1 mol/L acetate buffer (pH 5.5) for 45 min at 37°C and then absorbance was measured at 517 nm. For this experiment, lysosomal suspensions were preincubated with 1 mmol/L of the compounds for 30 min and centrifuged at 12 000*g* for 10 min. Then the pellets were washed in 0.15 mol/L KCl-5 mmol/L Tris buffer (pH 7.4), centrifuged and re-suspended in 0.1 mol/L acetate buffer (pH 5.5).

Statistical analysis

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

RESULTS

Metal-induced lipid peroxidation

The MDA accumulation in the medium showed a direct time-course increase with incubation time up to 6 h after the addition of the metal catalysts. The respective amounts of MDA concentration for the iron, copper and vanadium ions were 2.8, 2.7 and 2.4 nmol/mg protein/6 h in normal hepatocytes and 8.8, 6.2 and 10.7 nmol/mg protein/6 h in LNA-loaded hepatocytes (data not shown). These data are in agreement with the findings of Furuno *et al.*[21] As shown in Tables 1 and 2, either PN-M001 or sylibin showed a significant protective effect against all challenge metal ions, as

Table 1. Inhibition of $FeSO_4$, $CuSO_4$ and VCl_3-induced lipid peroxidation in normal hepatocytes by PN-M001 and sylibin

	PN-M001		Sylibin
Metal ion	100 μmol/L	200 μmol/L	100 μmol/L
$FeSO_4$	27.6 ± 2.4†	21.2 ± 2.4$^{\dagger *}$	18.9 ± 3.2$^{\dagger *}$
$CuSO_4$	7.9 ± 0.6	8.4 ± 0.5	7.3 ± 0.3
VCl_3	11.7 ± 0.99	12.4 ± 0.61	10.8 ± 1.2

Cu, copper; Fe, iron; V, vanadium. Values represent the concentrations that inhibit lipid peroxidation by 50% (IC_{50}, μmol/L). IC_{50} is calculated from the concentration-activity curves. $^{\dagger}P < 0.05$ vs $CuSO_4$ and VCl_3, $^{*}P < 0.05$ vs PN-M001 at 100 μmol/L concentration.

Table 2. Inhibition of $FeSO_4$, VCl_3 and $CuSO_4$-induced lipid peroxidation in LNA-loaded cells by PN-M001 and sylibin

	PN-M001		Sylibin
Metal ion	100 μmol/L	200 μmol/L	100 μmol/L
$FeSO_4$	106.6 ± 9.4†	91.6 ± 8.4$^{\dagger *}$	79.9 ± 9.2$^{\dagger *}$
$CuSO_4$	25.9 ± 2.2	19.8 ± 1.7	16.8 ± 1.5
VCl_3	13.7 ± 1.2	14.4 ± 0.87	17.3 ± 1.2

Cu, copper; Fe, iron; V, vanadium; LNA, α-linolenic acid. Values represent the concentrations that inhibit lipid peroxidation by 50% (IC_{50}, μM). IC_{50} is calculated from the concentration-activity curves. $^{\dagger}P < 0.05$ vs $CuSO_4$ and VCl_3, $^{*}P < 0.05$ vs PN-M001 at 100 μmol/L concentration.

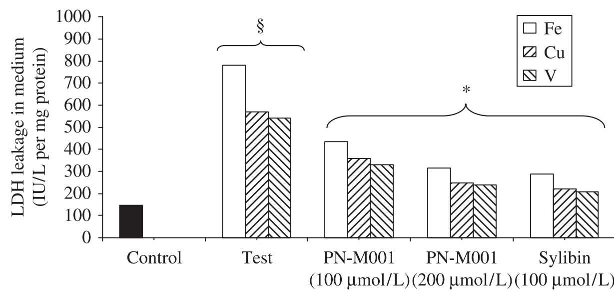

Figure 1. Effect of PN-M001 and sylibin on lactate dehydrogenase leakage caused by metal-ion induced damage to cultured hepatocytes. $*P < 0.05$ metal-tested lysosomal fractions, $^{\S}P < 0.01$ vs control (undamaged lysosomal fractions).

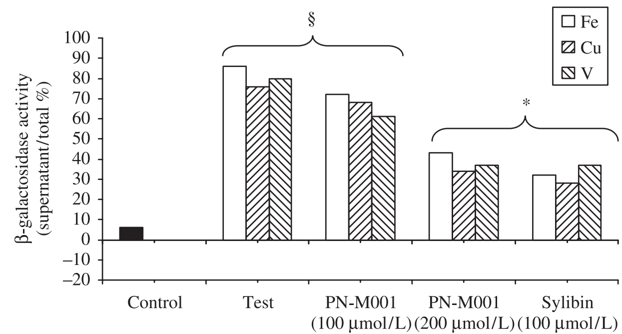

Figure 2. Effect of PN-M001 and sylibin on metal-ion-induced β-galactosidase release in lysosomal fractions $*P < 0.05$ metal-tested lysosomal fractions, $^{\S}P < 0.01$ vs control (undamaged lysosomal fractions).

expressed by the half inhibition concentration (IC_{50}) against lipid peroxidation. Fe-induced lipid peroxidation in either normal hepatocytes or LNA-loaded hepatocytes was suppressed by both test compounds to a significantly lesser extent than in the 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 to a comparable degree. However, on a molar ratio, sylibin seemed to be more effective than PN-M001 against Fe-induced peroxidative damage ($P < 0.05$).

Lysosomal fragility test

When challenged with metal ions, the lysosomal fractions expressed a significant increase in both LDH leakage and β-galactosidase release ($P < 0.01$), as shown in Figures 1 and 2. Both test compounds, irrespective of the concentration, significantly reduced the LDH concentration recovered in the medium of the lysosomal fractions ($P < 0.05$). Sylibin, and only the higher concentration of PN-M001, significantly decreased the β-galactosidase release from lysosomes ($P < 0.05$, Figure 2).

Tests of lysosomal oxidative stress

As compared with untreated lysosomal fractions challenged with the two peroxide radicals generators, either PN-M001 or sylibin exerted significant protection ($P < 0.01$, Table 3). In particular, the protection was comparably effective between hydrophilic- and lipophilic-generated free radicals. However, PN-M001 was significantly better than sylibin in suppressing acid phosphatase enzyme activity ($P < 0.05$). Both compounds showed comparably significant DPPH radical-scavenging activity ($P < 0.01$, Figure 3).

DISCUSSION

Metals such as iron, copper, and vanadium, undergo redox cycling, whereas cadmium, mercury, nickel and lead deplete glutathione and protein-bound sulfhydryl groups, resulting in the production of ROS as superoxide ions, hydrogen peroxide, and hydroxyl radicals.[22] Indeed, the most important mechanism of oxidative damage to proteins is metal-catalyzed oxidation,[23,24] which may result in the loss of enzymatic activity and altered protein structures.[25,26] This process involves generation of H_2O_2 and reduction of iron or copper by

Table 3. Effect of PN-M001 on the release of lysosomal enzymes in the presence of hydrophilic or lipophilic radical generators

	AAPH-induced release		AMVN-induced release	
	Acid phosphatase	β-*N*-acetylglucosaminidase	Acid phosphatase	β-*N*-acetylglucosaminidase
PN-M001 10^{-4} mol/L	62.4 ± 4.2*	45.7 ± 3.5*	49.4 ± 8.3*†	61.3 ± 9.7*
Sylibin 10^{-4} mol/L	51.9 ± 5.6*	54.6 ± 4.7*	83.9 ± 10.4*	77.3 ± 7.4*

Data are enzyme activity (% of control ± SE).
AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride, AMVN, 2,2′-azobis(2,4-dimethylvaleronitrite).
$*P < 0.01$ vs DMSO as the control compound, $^{\dagger}P < 0.05$ vs sylibin.

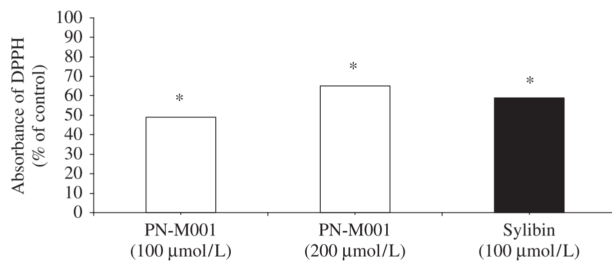

Figure 3. Scavenging activity of PN-M001 and sylibin for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals in lysosomal fractions. $*P < 0.05$ vs DPPH solution.

a suitable electron donor such as NADH, NADPH, ascorbate and others. The iron and copper ions bind to specific metal binding sites on proteins and react with H_2O_2 to generate OH and the resulting highly ROS attack amino acid residues. There is a growing body of evidence of the role of free radical generation and oxidative injury in the pathogenesis of liver injury and fibrosis in metal storage diseases.[27–29] Although phenolic antioxidants may decrease oxidative stress-related tissue damage, there are concerns about the toxicity of synthetic phenolic compounds, which has revived the issue of the clinical usefulness of natural phenolics, but to date there are few clinical reports.[30,31] Indeed, curcumin (the primary active principle in turmeric, *Curcuma longa* Linn.) is a potential antioxidant and anti-inflammatory agent with bioprotective properties[32–37] and we have recently shown that a curcumin-containing natural compound exerted potent protective effect in an experimental severe liver toxicity model.[12] In the present *in vitro* study, such a compound showed significantly protection of hepatocytes from metal ion-induced lipid peroxidation to a comparable extent as sylibin, and it is also conceivable that the absinthium in the compound may have further contributed to this effect, given its mild antioxidant property.[38] This is an interesting finding, considering the increasing awareness of the possible pathogenetic role of iron in metabolic disorders associated with nonalcohol-related liver steatosis[39,40] and given the recent data that curcumin may inhibit hepatic stellate cells *in vitro*.[41] It has been proved that free radical-modified membrane lipids and proteins in hepatic Fe-overload cause deranged hepatic microsomal enzyme activity, electron transport, respiration and lysosomal function.[42] Our findings show that PN-M001 significantly protected lysosomal integrity with mitigated LDH and β-galactosidase release, which is likely to be the result of its effective DPPH radical-scavenging activity and its activity against lipophilic-generators of free radicals, an effect that was stronger than sylibin. Indeed, during metal-induced injury oxidant stress damage preferentially targets the lysosomal compartment, which is particularly rich in low molecular weight redox-active iron, and rupture of lysosomes, followed by relocation of labile iron to the nucleus. This 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 among the mechanisms of liver carcinogenesis.[44] Interestingly, curcumin has been shown *in vitro* to protect also metal-induced DNA damage.[45]

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