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Effect of Sodium Copper Chlorophyllin on Lipid Peroxidation. VI.[1] Effect of Its Administration on Mitochondrial and Microsomal Lipid Peroxidation in Rat Liver

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When sodium copper chlorophyllin (Cu–Chl–Na) was given intraperitoneally to rats (two doses of 50 or 100 mg/kg at 18 and 2 h prior to sacrifice), the ascorbic acid-dependent lipid peroxidation in both mitochondria and microsomes of the liver markedly decreased. The microsomal lipid peroxidation induced by reduced nicotinamide adenine dinucleotide phosphate (NADPH) was also depressed by the treatment with Cu–Chl–Na. In addition, the soluble fraction of liver in injected animals showed an inhibition of the ascorbic acid- and NADPH-stimulated lipid peroxidation in hepatic microsomes from untreated rats. The absorption spectrum of each subcellular fraction in liver from Cu–Chl–Na-treated rats showed a red absorption band with a peak at *ca.* 633 nm, which is characteristic of the copper complexes of chlorophyll derivatives. These findings suggest that the administered Cu–Chl–Na or substance(s) derived from Cu–Chl–Na is taken into the liver and distributed among the mitochondria, microsomes and soluble fraction in an active form functioning as an antioxidant. Subsequently, a single injection of Cu–Chl–Na was observed to prevent effectively the impairment of hepatic microsomal functions (as indicated by the depression of glucose-6-phosphatase and drug-metabolizing enzyme system) resulting from ascorbic acid-induced lipid peroxidation.

Keywords——sodium copper chlorophyllin; lipid peroxidation; antioxidant activity; rat liver; mitochondria; microsome; peroxidative damage

In our previous papers,[2] it has been reported that sodium copper chlorophyllin (Cu–Chl–Na), a mixture of copper chelates of chlorophyll derivatives, has inhibitory activity on the peroxidation of lipids in rat liver homogenate and on the peroxidation of a mixture of linolenic and linoleic acids. Its antioxidative effect was suggested to be attributable to the action of Cu–Chl–Na as a radical scavenger, since Cu–Chl–Na was as effective as α-tocopherol in reducing 1,1-diphenyl-2-picrylhydrazyl, a model compound of free radicals. In addition, we have recently shown that Cu–Chl–Na protects the lysosomal membranes of rat liver from peroxidative damage *in vitro* by preventing lipid peroxidation.[1]

It is well known that in biomembranes such as mitochondria or microsomes, the peroxidation of unsaturated lipid components leads not only to the destruction of membrane structure but also to the depression of its functions, *e.g.*, membrane-bound enzyme activities.[3] Furthermore, lipid peroxidation *in vivo* has been considered to be involved in basic deteriorative mechanisms which are responsible for various events, such as the aging process,[4] some phases of atherosclerosis,[5] some types of liver injury,[6] oxygen toxicity,[7] *etc.*

The present study was designed to confirm the usefulness of Cu–Chl–Na as an *in vivo* lipid antioxidant. Thus, we describe the protective effect of administration of Cu–Chl–Na against liver mitochondrial and microsomal lipid peroxidation in rats.

Experimental

Materials——Cu–Chl–Na and 2-thiobarbituric acid (TBA) were purchased from Wako Pure Chemical Ind.,

Ltd., Tokyo, Japan. Nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate disodium salt and glucose-6-phosphate dehydrogenase were obtained from Boehringer Mannheim Yamanouchi Co., Ltd., Tokyo, Japan. Other chemicals of reagent grade were purchased from commercial sources and used without further purification.

Animal Treatments——Male Wistar rats, weighing approximately 200 g, were used throughout the experiments. They were housed in an air-conditioned room with free access to a commercial chow and tap water. Cu–Chl–Na was dissolved in 0.9% saline, and the resulting solution (1 ml/100 g body weight) was administered intraperitoneally to rats. The dose of Cu–Chl–Na was as noted in the legends to figures and tables. The control animals received an equivalent volume of the saline vehicle. All animals were fasted for about 18 h before sacrifice but were given tap water *ad libitum*. They were killed by decapitation.

Preparation of Hepatic Mitochondria——The livers were perfused *in situ* with ice-cold 0.25 M sucrose solution, quickly excised, minced and then homogenized with 9 volumes of ice-cold 0.25 M sucrose containing 5 mM Tris-HCl buffer (pH 7.4) in a Potter–Elvehjem-type glass-Teflon homogenizer. Mitochondria were isolated from the homogenate according to the method described by Hogeboom.[8] Mitochondria were washed once with 150 mM KCl–10 mM Tris-HCl buffer (pH 7.4) and suspended in the same KCl-buffer solution. Mitochondrial protein was determined by the method of Lowry *et al.*[9] using bovine serum albumin as a standard.

Preparation of Hepatic Microsomal and Soluble Fractions——The livers were removed quickly after perfusion *in situ* with ice-cold 1.15% KCl solution, rinsed with the same solution, and homogenized in 4 volumes of an ice-cold solution of 150 mM KCl–10 mM Tris-HCl buffer (pH 7.4) by use of the above homogenizer. Microsomes and soluble fraction ($105000 \times g$ supernatant) were prepared according to the procedures described by Kamataki and Kitagawa.[10] The microsomal pellet was suspended in the KCl-Tris buffer. The protein contents of the two fractions were estimated by Lowry's method.[9]

Assay of Lipid Peroxidation——Ascorbic acid (AsA) and NADPH were employed as stimulators of lipid peroxidation. A typical reaction mixture contained an appropriate amount of mitochondria or microsomes, 90 mM KCl and 50 mM Tris-HCl buffer (pH 7.4) to make a final volume of 1.0 or 2.0 ml. When necessary, AsA at 0.5 mM or an NADPH-generating system (G.S.) (0.3 mM NADP, 5 mM glucose-6-phosphate, 5 mM $MgCl_2$ and 0.2 unit/ml of glucose-6-phosphate dehydrogenase) was added to the reaction mixture. Incubation was carried out aerobically at 37 °C for a specified period. Lipid peroxides were determined by means of the TBA reaction, which has been widely adopted as a typical sensitive assay method for lipid peroxidation in biological samples,[3b,11] as described previously.[12] The formation of lipid peroxides was expressed in terms of TBA values (absorbance at 532 nm due to malondialdehyde formation/ml of reaction mixture).

Microsomal Enzyme Assays after Preincubation with AsA——The preincubation mixture consisted of microsomes (20 mg of protein), 120 mM KCl, 0.5 mM AsA and 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 8.0 ml. After preincubation at 37 °C for 30 min, 0.1 ml of 15 mM ethylenediamine tetraacetic acid, a potent inhibitor of lipid peroxidation,[10,13] was added to prevent further peroxidative reaction. Then, the formation of lipid peroxides was determined as described above, and the three microsomal enzymes were assayed by the following methods: glucose-6-phosphatase according to Wills,[14] aminopyrine *N*-demethylase according to Kitada *et al.*,[15] and cytochrome P-450 according to Omura and Sato.[16]

Determination of Cu–Chl–Na or Substance(s) Derived from Cu–Chl–Na in Hepatic Subcellular Fractions——The three subcellular fractions in the liver of Cu–Chl–Na-treated and control rats were diluted with 150 mM KCl–10 mM Tris-HCl buffer (pH 7.4) to the following protein concentrations: 2 mg/ml for mitochondria and microsomes, and 8 mg/ml for soluble fraction. Then, the same volume (3.0 ml) of the diluted subcellular fraction from Cu–Chl–Na-treated rats and that from control rats was placed in the sample and reference cuvettes, respectively, for the purpose of cancelling out turbidity effects. The measurements of absorption spectra were performed with a Hitachi model 556 spectrophotometer. The content of Cu–Chl–Na or substance(s) derived from Cu–Chl–Na was determined from the absorption difference between the peak position (at *ca.* 633 nm) and 610 nm using a calibration curve constructed by adding Cu–Chl–Na to the diluted subcellular fraction from the liver of control rats. The content was expressed in terms of μg eq to Cu–Chl–Na/mg protein.

When necessary, the statistical analysis of data was carried out by using Student's *t*-test.

Results and Discussion

Prior to the administration experiments, the *in vitro* effect of Cu–Chl–Na on mitochondrial and microsomal lipid peroxidation in rat liver was investigated in the presence of AsA or NADPH–G.S., a potent stimulator of both reactions[17] or of the latter reaction,[17b,d,18] respectively. As shown in Table I, Cu–Chl–Na at 1×10^{-4}% inhibited the AsA-induced lipid peroxidation in mitochondria and microsomes by about 90 and 50%, respectively, and at concentrations of 5×10^{-4}% or above it showed an about 93% inhibition of the latter

Table I. Effect of Cu–Chl–Na on Mitochondrial and Microsomal Lipid Peroxidation in Rat Liver *in vitro*

Cu–Chl–Na concn. (%, w/v)	Mitochondria		Microsomes			
	0.5 mM AsA		0.5 mM AsA		NADPH-G.S.	
	TBA value	Dec. (%)	TBA value	Dec. (%)	TBA value	Dec. (%)
Nil	0.679±0.047		0.658±0.033		0.941±0.028	
1×10^{-4}	0.072±0.006[a)]	89.4	0.325±0.021[a)]	50.6	0.456±0.029[a)]	51.5
2×10^{-4}	0.029±0.004[a)]	95.7	0.140±0.028[a)]	78.7	0.200±0.005[a)]	78.7
5×10^{-4}	0.027±0.003[a)]	96.0	0.047±0.005[a)]	92.9	0.066±0.007[a)]	93.0
10×10^{-4}	0.022±0.003[a)]	96.8	0.044±0.004[a)]	93.3	0.054±0.006[a)]	94.3

The reaction mixture, consisting of 3.0 mg protein of mitochondria or microsomes, 90 mM KCl, 0.5 mM AsA or NADPH-G.S., and 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 2.0 ml, was incubated at 37 °C for 30 min. Each value is the mean±S.E. of 4 separate experiments.

a) Significantly different from the control value in the absence of Cu–Chl–Na at a p value of 0.01 or less.

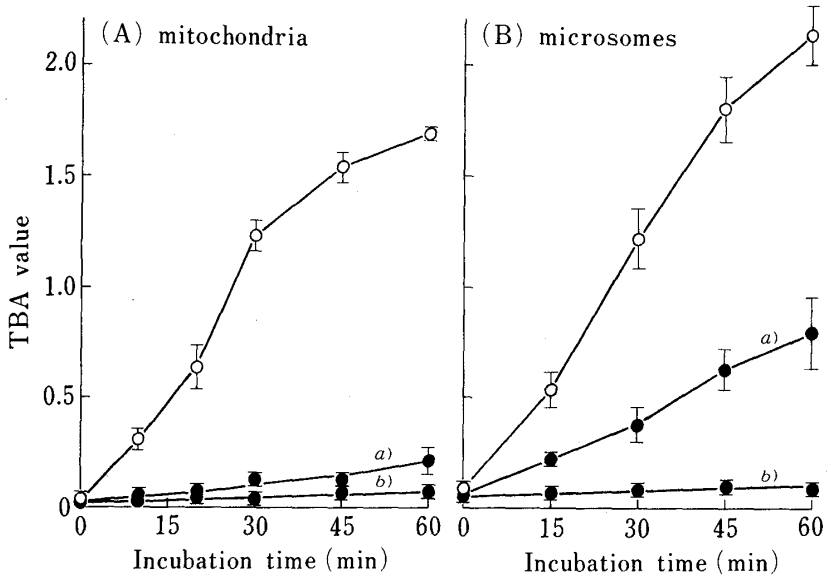

Fig. 1. Effect of Cu–Chl–Na Administration on AsA-stimulated Lipid Peroxidation in Liver Mitochondria and Microsomes of Rats

Rats were injected intraperitoneally with Cu–Chl–Na (50 and 100 mg/kg) twice at 16 h intervals and sacrificed 2 h after the second injection. The reaction mixture, consisting of mitochondria (5 mg prot.) or microsomes (2.5 mg prot.), 90 mM KCl, 0.5 mM AsA and 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 2.0 ml (A) or 1.0 ml (B), was incubated at 37 °C. Each point represents the mean±S.E. (vertical bars) for 4—7 animals.

—○—, control; —●—, Cu–Chl–Na-treated. *a*) 50 mg/kg×2; *b*) 100 mg/kg×2.

reaction. In the microsomal system, the formation of lipid peroxides stimulated by NADPH–G.S. was also decreased by the addition of Cu–Chl–Na; the degree of decrease was dependent on Cu–Chl–Na concentration and was almost the same as that obtained with AsA. These results demonstrate that Cu–Chl–Na possesses an antioxidative effect not only on the non-enzymatic but also on the enzymatic formation of lipid peroxides.

The intraperitoneal injection of Cu–Chl–Na at a dose of 50 or 100 mg/kg was carried out twice, at 18 and 2 h prior to sacrifice of the rats. The changes in AsA-induced lipid peroxidation in the hepatic mitochondria and microsomes after these treatments are shown in Fig. 1. In both subcellular fractions, the progressive elevations of TBA values during the

Table II. Effect of Cu–Chl–Na Administration on NADPH-G.S.-stimulated Lipid Peroxidation in Rat Liver Microsomes

Cu–Chl–Na treatment (mg/kg, *i.p.*)	TBA value	
	No addition	NADPH-G.S.
Control	0.068±0.006	1.285±0.045
50×2	0.058±0.004	0.536±0.076[a]
100×2	0.055±0.003	0.307±0.051[a]

Treatment of rats was carried out as described in the legend to Fig. 1. The reaction mixture consisted of the same components as described in the legend to Fig. 1 except that NADPH-G.S. was added instead of AsA. Incubation was carried out at 37 °C for 30 min. Each value represents the mean±S.E. for 4—8 animals.

a) Significantly different from the control, $p<0.001$.

Table III. Effect of Liver Soluble Fraction from Cu–Chl–Na-treated Rats on Microsomal Lipid Peroxidation

Cu–Chl–Na treatment (mg/kg, *i.p.*)	Soluble fraction (mg protein)	0.5 mM AsA		NADPH-G.S.	
		TBA value	%	TBA value	%
Control	2	0.718±0.039	100	0.849±0.026	100
50×2	2	0.418±0.070[a]	58.2	0.712±0.047[a]	83.9
100×2	2	0.223±0.043[a]	31.1	0.561±0.041[a]	66.1
Control	4	0.730±0.050	100	0.657±0.024	100
50×2	4	0.228±0.105[a]	31.2	0.321±0.045[a]	48.9
100×2	4	0.057±0.010[a]	7.8	0.168±0.033[a]	25.6

Treatment of rats was carried out as described in the legend to Fig. 1. The reaction mixture, consisting of 1 mg protein of hepatic microsomes from control rats, 90 mM KCl, 0.5 mM AsA or NADPH-G.S., and 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 1.0 ml, was incubated at 37 °C for 30 min. Results are expressed as the mean±S.E. for 3—4 animals.

a) Significantly different from the control at a p value of 0.05 or less.

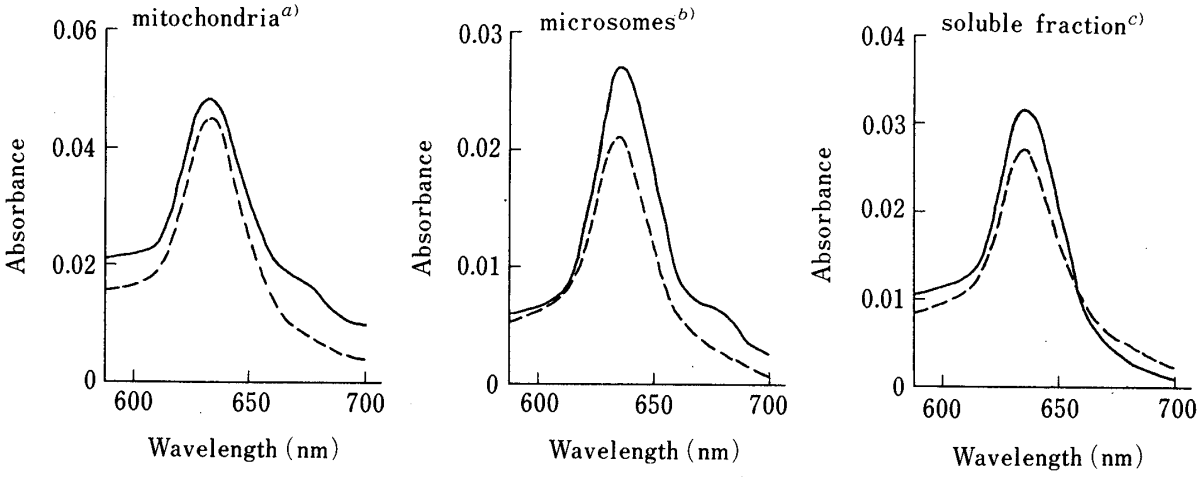

Fig. 2. Absorption Spectra of Liver Subcellular Fractions from Rats Treated with Cu–Chl–Na

Rats received a single intraperitoneal injection of Cu–Chl–Na at a dose of 100 mg/kg and were sacrificed 1 h after the injection.

——, subcellular fractions from Cu–Chl–Na-treated rats.

– – –, addition of Cu–Chl–Na to subcellular fractions of control rats.

Cu–Chl–Na concn. ($\times 10^{-4}$%), *a*) 1.66; *b*) 1.33; *c*) 1.49.

incubation period of 60 min were clearly inhibited in rats treated with Cu–Chl–Na. At a dose of 50 mg/kg, Cu–Chl–Na exhibited a more effective inhibition of the lipid peroxidation in mitochondria than of that in microsomes. When it was given at a dose of 100 mg/kg, an almost complete inhibition was observed in both fractions.

The results in Table II show that Cu–Chl–Na administration did not change the TBA value in microsomes after incubation for 30 min without a stimulator, while the NADPH–G.S.-elevated TBA value was significantly reduced by either dose of Cu–Chl–Na, the extents of decrease being 58% for 50 mg/kg and 76% for 100 mg/kg.

The effect of liver soluble fraction prepared from Cu–Chl–Na-treated rats on lipid peroxidation was examined using an incubation system containing the hepatic microsomes from control rats (Table III). The soluble fraction of rats dosed twice with Cu–Chl–Na exerted a significant depression on both AsA– and NADPH–G.S.-induced lipid peroxidation, and the extent of the depression increased with increasing dose of Cu–Chl–Na and with increasing amount of added soluble fraction.

On the basis of the above-mentioned results, it is assumed that the administered Cu–Chl–Na or substance(s) derived from Cu–Chl–Na is taken into the liver and exists in the mitochondrial, microsomal and soluble fractions in an active form functioning as an antioxidant.

To examine whether the intraperitoneally administered Cu–Chl–Na is incorporated into the three subcellular fractions of liver, the absorption spectrum of each fraction from rats 1 h after a single injection of 100 mg/kg was determined. As shown in Fig. 2, the red absorption band with a peak at *ca.* 633 nm was observed in all three fractions, and each spectral profile was nearly the same as that of the authentic Cu–Chl–Na added to the subcellular fractions of control rats. These results indicate that the administered Cu–Chl–Na or substance(s) derived from Cu–Chl–Na is distributed among the mitochondria, microsomes and soluble fraction of hepatic cells. Since the observed spectral absorption in the red-band region is assumed to be attributable to the chemical structure of copper chlorins, which are constituents of Cu–Chl–Na[19] and may play an important role in the antioxidative action of Cu–Chl–Na,[20] it seems likely that mainly copper chlorin compounds are present in these fractions.

Then, we determined the content of Cu–Chl–Na or substance(s) derived from Cu–Chl–Na in the above subcellular fractions of rats treated with Cu–Chl–Na as described in the legend to Fig. 1. As shown in Table IV, increases in its content were seen with increasing dose of Cu–Chl–Na. Of the three fractions, mitochondria had the highest content of Cu–Chl–Na or substance(s) derived from Cu–Chl–Na; at either dose the content was about 4-fold greater than the content in microsomes. This may account for the result shown in Fig. 1 that the dose of 50 mg/kg of Cu–Chl–Na had a more potent inhibitory effect on the lipid peroxidation in

TABLE IV. Content of Cu–Chl–Na or Substance(s) Derived from Cu–Chl–Na in Subcellular Fractions of Liver after the Injection of Cu–Chl–Na into Rats

Cu–Chl–Na treatment (mg/kg, *i.p.*)	Content (μg eq to Cu–Chl–Na/mg protein)		
	Mitochondria	Microsomes	Soluble fr.
50 × 2	2.50 ± 0.17	0.56 ± 0.06	0.21 ± 0.01
100 × 2	4.43 ± 0.32	1.23 ± 0.05	0.34 ± 0.01

Treatment of rats was carried out as described in the legend to Fig. 1. Each value represents the mean ± S.E. for 5 animals.

TABLE V. Effects of Cu–Chl–Na Administration on AsA-induced Decrease in Activities of Glucose-6-phosphatase and Aminopyrine *N*-Demethylase, and Content of Cytochrome P-450 in Liver Microsomes of Rats

Cu–Chl–Na treatment	Content[a]	Preincubation condition	TBA value/ 30 min	Glucose-6-phosphatase[b]	Aminopyrine *N*-demethylase[c]	Cytochrome P-450[d]
Control	—	−AsA	0.090±0.001	3.73±0.15	84.9±1.9	0.953±0.019
		+AsA (0.5 mM)	1.721±0.086[e]	1.36±0.23[e]	17.7±0.8[e]	0.433±0.027[e]
50 mg/kg	0.49±0.03	−AsA	0.061±0.002	3.85±0.13	88.2±1.7	0.983±0.003
		+AsA (0.5 mM)	0.546±0.149[e]	3.18±0.28	52.0±6.7[e]	0.810±0.053[e]
100 mg/kg	0.93±0.04	−AsA	0.054±0.001	3.84±0.10	84.7±3.7	0.979±0.017
		+AsA (0.5 mM)	0.116±0.013[e]	3.69±0.11	74.6±3.4	0.970±0.022

Rats were given an intraperitoneal injection of Cu–Chl–Na (50 and 100 mg/kg) 2 h prior to sacrifice. Microsomes were preincubated at 37 °C for 30 min in the absence or presence of AsA, then TBA value, glucose-6-phosphatase and aminopyrine *N*-demethylase activities, and cytochrome P-450 content were assayed. Each value is the mean ± S.E. for 5—7 animals.

a) μg eq to Cu–Chl–Na/mg prot. *b*) μmol Pi/mg prot./15 min.
c) nmol HCHO/mg prot./20 min. *d*) nmol/mg prot.
e) Significantly different from the corresponding value in the absence of AsA at a p value of 0.05 or less.

mitochondria than on that in microsomes.

Lipid peroxidation of liver microsomes *in vitro* has been reported to impair the integrity of membrane structure and membrane-bound enzymes such as glucose-6-phosphatase and cytochrome P-450-dependent enzymes.[10,14,21] Thus, we examined the effect of Cu–Chl–Na administration on the peroxidative damage to hepatic microsomes caused by the addition of AsA. The activities of glucose-6-phosphatase and aminopyrine *N*-demethylase, and the content of cytochrome P-450 were assayed as indicators of the alterations in microsomal functions. Cu–Chl–Na was intraperitoneally given at a single dose of 50 or 100 mg/kg to rats 2 h prior to sacrifice. The results are given in Table V.

In the microsomes of animals dosed with Cu–Chl–Na, the stimulation of lipid peroxidation by AsA was increasingly inhibited with increase in content of Cu–Chl–Na or substance(s) derived from Cu–Chl–Na, and the AsA-induced depression of glucose-6-phosphatase activity was prevented almost completely by both doses of Cu–Chl–Na. Preincubation of the microsomes from control rats with AsA produced decreases in aminopyrine *N*-demethylase activity and cytochrome P-450 content amounting to 79 and 55%, respectively. In the 50 mg/kg-treated rat microsomes, these decreases were 41% for the former and 18% for the latter, namely, the extents of decrease were lowered by Cu–Chl–Na treatment. Moreover, Cu–Chl–Na at a dose of 100 mg/kg showed an almost complete inhibition of the decreases in both aminopyrine *N*-demethylase activity and cytochrome P-450 content. These findings indicate that the treatment of rats with Cu–Chl–Na effectively inhibits the AsA-induced impairment of hepatic microsomal functions. This inhibitory effect of Cu–Chl–Na is considered to be a reflection of its antioxidative activity on the peroxidation reaction of membrane lipids.

Finally, the results obtained from the present study suggest the possibility that Cu–Chl–Na functions not only *in vitro* but also *in vivo* as an antioxidant of hepatic lipid peroxidation and affords protection against the peroxidative disintegration of membrane structure of the subcellular organelles and the resulting depression of their functions.

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