Curcumin protects against failure to generate a transmembrane potential and protein aggregation of rat liver mitochondria induced by tert-butylhydroperoxides

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Abstrak

Kurkumin, bahan aktif tanaman kurkuma diduga bermanfaat dalam pengobatan penyakit hati. Dalam penelitian terdahulu, diperlihatkan efek protektif kurkumin terhadap peroksidasi lipid dan swelling mitokondria yang diisolasi dari hati tikus karena pemberian t-butilhidroperoksida (t-BuOOH). Dalam penelitian ini, pemberian t-BuOOH 90 μM menyebabkan mitokondria tidak dapat membentuk potensial transmembran (Ψm). Kegagalan pembentukan potential transmembran diduga berhubungan dengan transisi permeabilitas dan apoptosis. Dari 3 dosis kurkumin yang dicoba (0,5 μM, 2,5 μM dan 5,0 μM), ternyata kurkumin dosis 2,5 μM dapat mencegah kegagalan pembentukan potensial transmembran akibat t-BuOOH (79,13 ± 6,28%). Pemeriksaan elektroforesis protein mitokondria menunjukkan kurkumin 1000 μM dapat mencegah agregasi protein yang terjadi akibat t-BuOOH. Dari penelitian ini diperlihatkan efek proteksi kurkumin terhadap kerusakan sistem pembentukan energi dan protein mitokondria yang disebabkan oleh t-BuOOH. ***(Med J Indones 2007; 16:139-45)***

Abstract

Curcumin, an active ingredient of curcuma plant has been thought to be beneficial in the treatment of liver diseases. In the previous studies, we have shown the protective effects of curcumin against lipid peroxidation and swelling of the rat liver mitochondrial preparation induced by tert-butylhydroperoxide (t-BuOOH). In the present study, the administration of t-BuOOH of 90 μM caused the mitochondria failed to generate a transmembrane potential (Ψm). Of 3 doses of curcumin administered (0.5 μM, 2.5 μM dan 5.0 μM) the maximum protective effect against failure to generate a transmembrane potential caused by t- BuOOH was obtained by 2.5 μM of curcumin (79.13 ± 6.28%). Further, curcumin of 1000 μM could prevent protein aggregation formation caused by t-BuOOH in the electrophoretogram. This study shows the protective effects of curcumin against damaged of energy production system and protein of the mitochondria caused by t-BuOOH. ***(Med J Indones 2007; 16:139-45)***

Keywords: *curcumin, t-BuOOH, mitochondria, transmembrane potential, protein electrophoresis.*

Oxidative stress has been thought to play an important role in the pathogenesis of toxic and infectious liver diseases. Currently, there are no specific medicines that can be used in the treatment of hepatitis. Various efforts have been tried to develop reliable remedies for the diseases. One among those is empirical approach, i.e. to explore the potential use of certain herbs like curcuma rhizomes and their active ingredient, curcumin. In the previous paper, we have described the potent antioxidant activity of curcumin[1] and its inhibitory effect against mitochondrial swelling induced by t-butylhydroperoxide (t-BuOOH).[2]

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In the present study we demonstrate the protective effect of curcumin against failure of transmembrane potential generation and prevention of protein damage of rat liver mitochondria induced by t-BuOOH.

METHODS

Materials

Curcumin, EGTA, Trizma HCl, bovine serum albumin (BSA), t-BuOOH, rotenone, safranin O were purchased from Sigma Chemical Co., USA. Sucrose, Folin Ciocalteus phenol reagent, 2-6 dichlorophenol indophenol, glycine, trisaminomethane, sodium dodecyl sulphate were from E. Merck, Darmstadt, Germany. Ammonium

persulfate, TEMED, acrylamide, bisacrylamide, broad range molecular weight protein standard, Coomassie brilliant blue, dithiothreitol were purchased from Bio Rad Laboratories Inc. USA. Other analytical grade reagents of the highest purity available were purchased from either Sigma Chemical Co. or E. Merck.

Isolation of mitochondria

The method of isolation of rat liver mitochondria was carried out as described in the previous study[3] with minor modification.[2]

Mitochondrial transmembrane potential (Δ Ψm) were evaluated using safranin 0 as the indicating dye in a Perkin-Elmer UV-visible lambda 3B spectrophotometer. The procedure was conducted essentially as described by Mittnacht et al[4], except that the mitochondrial proteins used were 1.2 - 1.4 mg/ml and that Δ Ψm was measured by substracting the absorbance obtained at 516 nm (A 516) with that obtained at 495 nm (A 495).

Gel electrophoresis

Polyarcrylamide gel electrophoresis was carried out in 10% (w/v) resolving gel with a 3% (w/v) acrylamide stacking layer as described by Laemmli.[5]

Prior to electrophoresis an amount of 40-60 μg of mitochondrial protein was incubated for 30 min at 37°C in the presence or absence of t-BuOOH, various concentrations of curcumin or other substances. Thereafter, the samples were dissolved in a solution containing 60 mM Tris HCl pH 6.8, 40 mM, DTT, 10% sucrose, 3% SDS, 0.001% bromophenol blue and incubated for 30 min at 37°C. The samples were applied onto the gel in a vertical mini Protean II Electrophoresis Cell (Bio Rad) system and stained with Coomassie brilliant blue.

Statistical analysis

The degree of normality and variant homogeneity of the data on transmembrane potential were tested by Kolmogorov-Smirnov (K-S test for rankable scores) and F test[16], respectively. Since the data obtained showed a normal and homogeneous distribution, they were analyzed using the related one-way ANOVA followed by Tukey method[7] at a significant level of $\alpha = 0.05$.

RESULTS

Extent of purification of the isolated mitochondrial preparation

The mitochondrial preparation was homogenously purified as shown by the high relative specific activity (RSA) of succinate dehydrogenase of 35.73 ± 2.78; the mitochondrial protein yield was 19.96 ± 1.95 mg/g wet liver.[2]

Mitochondrial transmembrane potential (Ψm)

The ability of the mitochondria to generate trans-membrane potential was influenced by incubation time, incubation temperature and in vitro aging, SET medium, and the protein content of the mitochondrial preparation.

The transmembrane potential was evaluated using safranin O[4]. Mitochondrial energization caused a decrease in the maximum absorption of 25 mM safranin O from 516 nm to 495 nm after addition of 3 mM succinate, which was thought to be the result of the dye accumulation.[8,9] The mitochondria were still able to generate transmembrane potential when the assay was conducted immediately after addition of t-BuOOH. However, the mitochondria failed to generate transmembrane potential when they were incubated with t-BuOOH for 60 min at 37°C. Since the mitochondrial preparation underwent a rapid in vitro aging when they were left at room temperature, they were kept on ice after isolation and the measurement of the transmembrane potential was carried out at room temperature. Suspending the mitochondria in SET-34 medium did not result in the generation of transmembrane potential in control preparation. Dilution of SET-34 with water (25:34) gave an optimal result. Use of 0.25 M sucrose gave similar results, but in vitro aging occurred faster than that after dilution of SET-34 with H_2O. Finally, the results would be optimal when the amount of protein added was 1.2-1.4 mg/ml (1.2-1.4 mg/ml). Table 1, Figures 1, 2 depict the results of study on mitochondrial transmembrane potential.

Table 1. Percentage of protection against failure of the mitochondria to generate a membrane potential

	% of protection								
Group	C	IC	IC_{05}	$IC_{2.5}$	IC_{25}	ID	ID_{05}	$ID_{2.5}$	ID_{25}
Mean ± SD	100	92.20	89.18[1]	87.54[1]	43.35[1]	0	30.97[2]	79.13[2]	39.29[2]
		2.20	6.67	8.33	4.53	0	2.87	6.28	3.52

C : control mitochondria
IC : mitochondria incubated without curcumin
IC0.5-25 : mitochondria incubated with curcumin of 0.5, 2.5 and 25 µM
ID : mitochondria incubated with 90 µM t-BuOOH
ID0.5-25 : mitochondria incubated with 90 µM t-BuOOH and curcumin of 0.5, 2.5 and 25 µM
1 : significantly different from group C ($p < 0.05$)
2 : significantly different from group ID ($p < 0.05$)

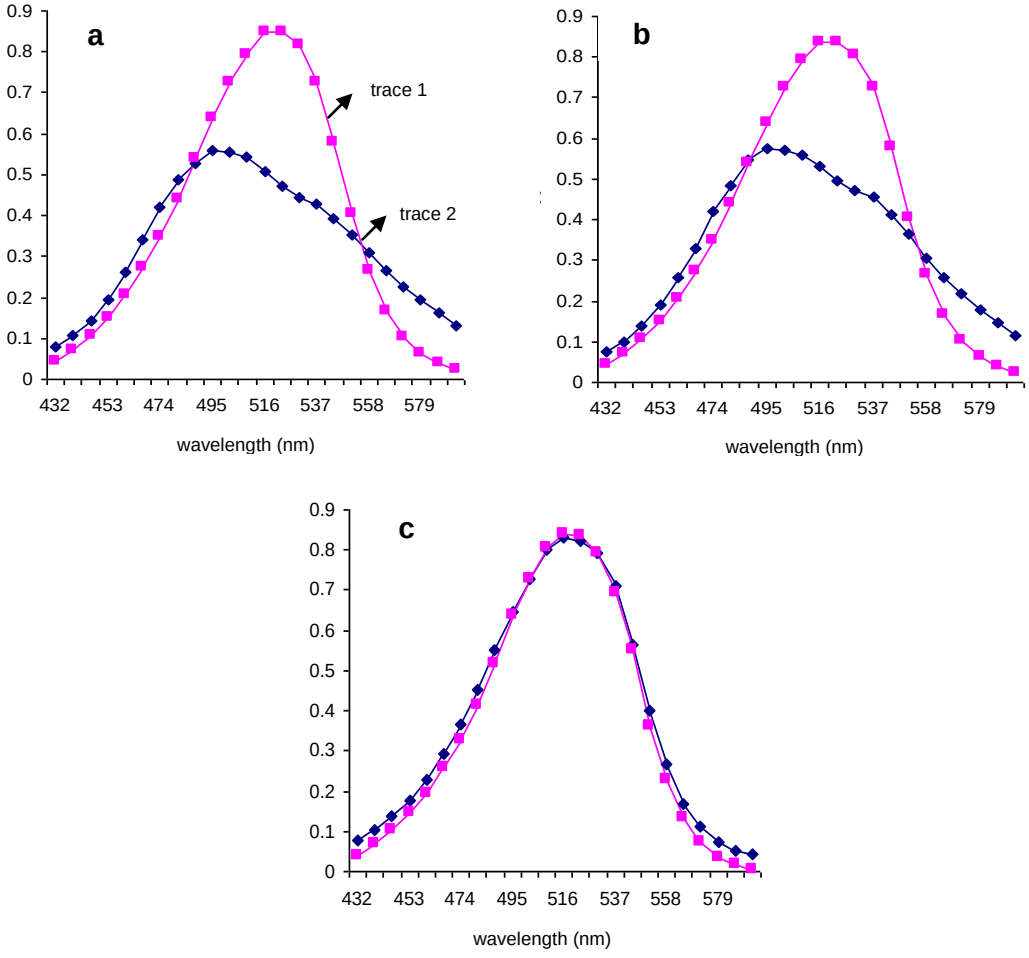


Figure 1. Inability of mitochondria to generate a membrane potential upon addition of t-BuOOH.

a. Control mitochondria incubated with 2.5 µM safranin O before (tracing 1) and after (tracing 2) addition of 3 µM succinate. The maximum absorption of safranin O was decreased from 516 nm (tracing 1) to 495 nm (tracing 2), showing mitochondrial energization and the ability to generate membrane potential

b. Mitochondria incubated with curcumin of 2.5 µM in the absence of t-BuOOH, showing the ability to generate membrane potential.

c. Mitochondria incubated with 90 µM t-BuOOH failed to generate a membrane potential.

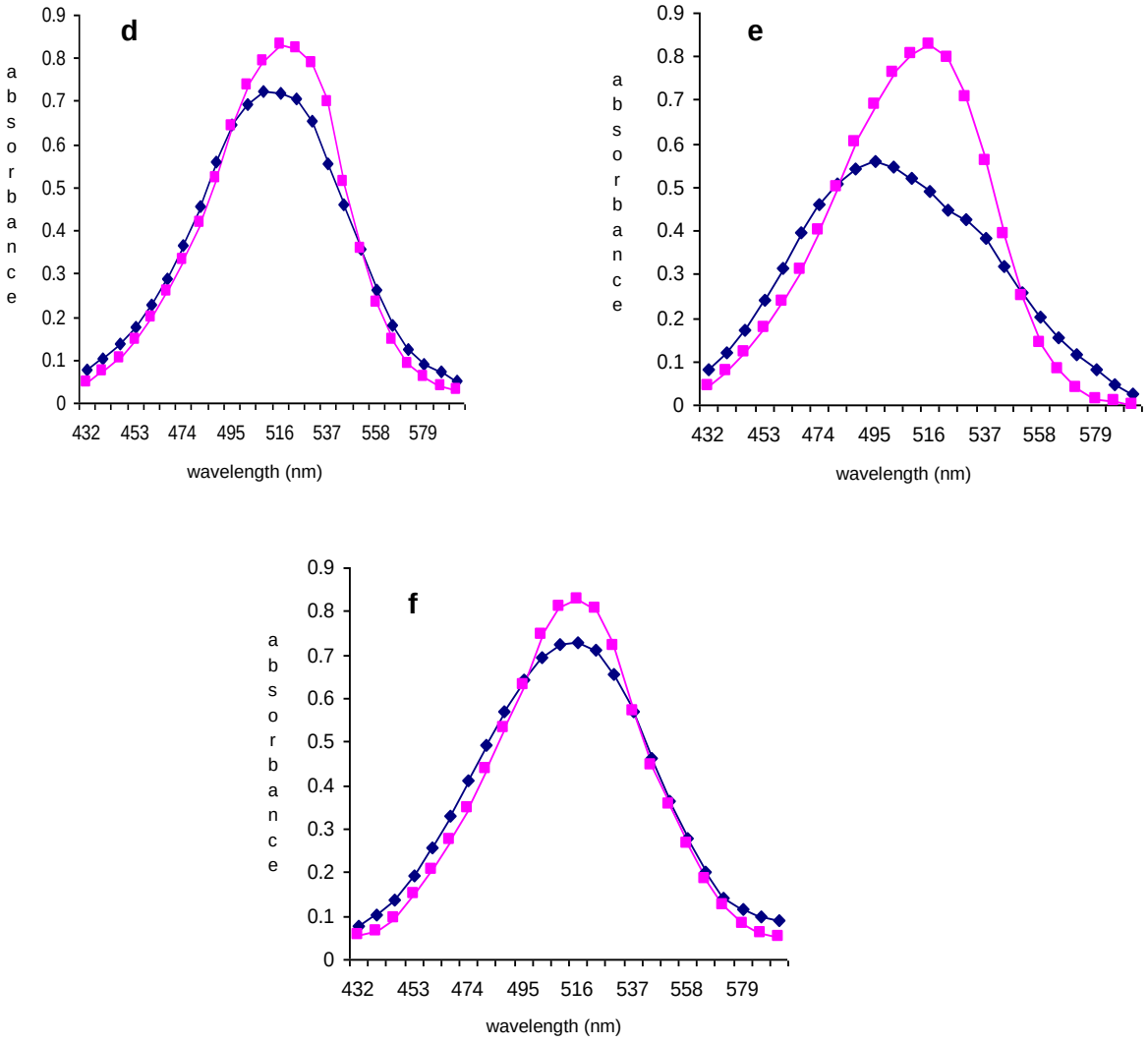


Figure 2. The effects of curcumin of 0.5 μM (d), 2.5 μM (e) and 5.0 μM (f) against failure to generate membrane potential induced by 90 μM t-BuOOH.
The maximum protective effect was obtained after preincubation with curcumin of 2.5 μM (e).

Incubation of mitochondria without curcumin (IC) with curcumin of low dose ($IC_{0.25}$) and intermediate dose ($IC_{2.5}$) in the absence of t-BuOOH did not differ among each other, but differed from control (C) in the ability to generate transmembrane potential. At high dose (IC_{25}), curcumin even caused a significantly less ability of the mitochondria to generate transmembrane potential in comparison with that of the IC group.

Incubation of mitochondria with 90 μM t-BuOOH resulted in a complete inability to generate trans-membrane potential (ID). Curcumin of 2.5 μM (IC_{25}) showed the highest protection against failure to generate tranmembrane potential (79.13 ± 6.28%), whereas at lower (0.25 μM) and higher doses (25 μM), repectively, the protective effects were much lower (30.97 ± 2.87% and 39.29 ± 3.52%)

Polyacrylamide gel electrophoresis (PAGE)

The alteration of polypeptide pattern of mitochondrial preparation caused by t-BuOOH and the protective effects of various doses of curcumin were studied using gel electrophoresis.

When the mitochondrial preparation was incubated with 90 μM of t-BuOOH for 30 min at 37°C and run on PAGE, the electrophoretogram showed protein aggregates at the origin and a decreasing intensity of the protein band of 116 kD (Fig. 3). The alteration of the mitochondrial polypeptide pattern induced by t-BuOOH could be restored to normal by curcumin of 1000 μM added just prior to t-BuOOH. Curcumin of less than 1000 μM and more than 2000 μM did not give protective effect.

DTT of 0.15 mM, 0.45 mM and 1.35 mM were also protective against the mitochondrial polypeptide pattern alteration, whereas DMSO of 3% (v/v) was not.

DISCUSSION

Our previous results showed that t-BuOOH of 90 μM caused swelling of the mitochondria isolated from rat liver and that curcumin of 2.50 μM was protective against the swelling.[2] Swelling proceeded after mitochondrial permeability transition pore (MPTP) opening and might be an initial signal in the cascade process leading to cell death.[10]

The protective effect of curcumin against mitochondrial membrane alteration induced by t-BuOOH was shown by the present study on mitochondrial transmembrane potential (Ψm).

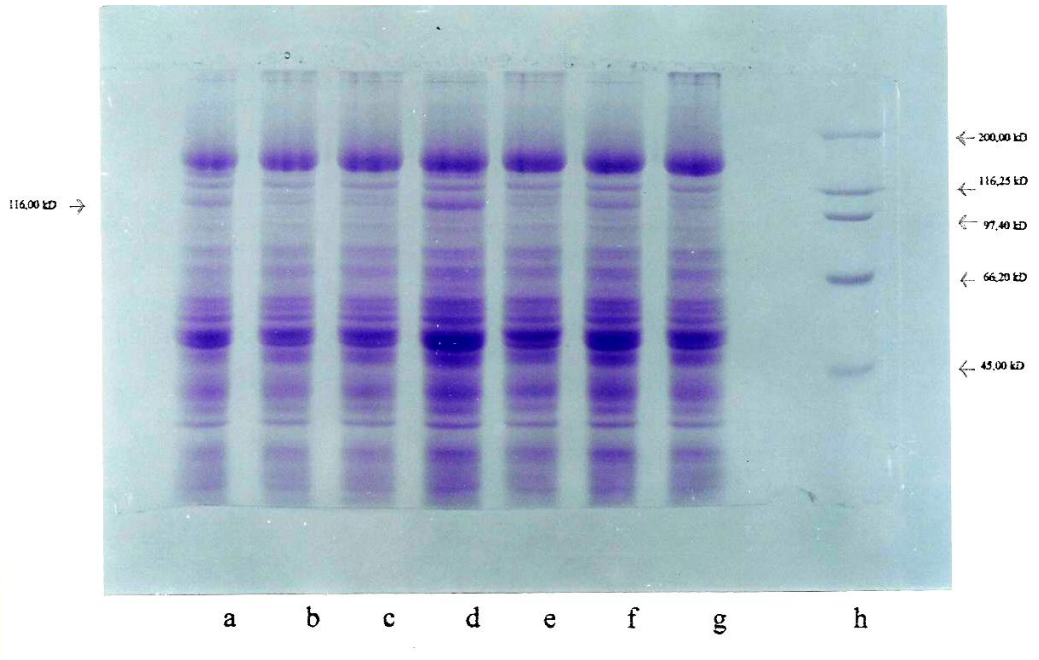
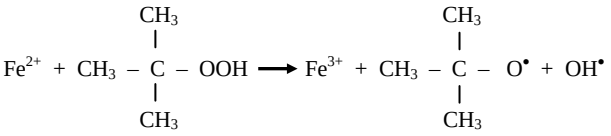


Figure 3. SDS-PAGE electrophoresis of mitochondrial proteins.
Lanes a: control; b: t-BuOOH-treated mitochondria; c-g: t-BuOOH-treated mitochondria in the presence of curcumin of 0.5 μM (c), 2.5 μM (d), 250 μM (e), 500 μM dithiothreitol (f) and 10% dimethylsulfoxide (g). Lane h: low molecular weight standard.
There was a decrease in the intensity of a polypeptide band of 116 kD in the t-BuOOH-treated mitochondria (b), but restored to control upon incubation with curcumin of 2.5 μM (d) and dithiothreitol of 500 μM (f).
The amount of mitochondrial protein run on a 10%
SDS-PAGE was 60 mg.

In this study, the optimal concentration of curcumin that provide optimal protection was 2.5 μM, which was similar to that obtained in the swelling experiment. Tert butylhydroperoxide, a generator of hydroxyl radicals appeared to trigger the formation of MPTP, by peroxidation of lipid or protein cross linking of the mitochondrial membrane. Current opinion proposed the association of failure of transmembrane potential generation with permeability transition. According to Susin et al[11], the formation of MPTP which allowed diffusion of ions and small molecules of less than 1500 daltons eventually resulted in collapse of transmembrane potential. The ability of curcumin of 2.5 μM to prevent the failure of transmembrane potential generation supports this opinion. The mechanism by which curcumin protects against swelling and failure to generate the transmembrane potential induced by t-BuOOH has not been clearly elucidated. Curcumin was thought to conjugate with GSH leading to some reduction of GSH level[12] or it may be metabolized by peroxidases to prooxidant phenoxyl radicals which could oxidize GSH or NADH.[13] Although it may have some reduction of GSH level, curcumin could still be preventive against t-BuOOH induced oxidation of mitochondrial membrane.[1] It might be possible that curcumin spares the GSH content to certain extent by scavenging the hydroxyl radicals generated by t-BuOOH. Tert-butylhydroperoxide in the presence of Fe^{2+}, produces hydroxyl radicals by the reaction:



Curcumin incorporated in the membrane behaves as a chelating agent for Fe^{2+} ion thereby inhibits the formation of hydroxyl radical

Curcumin may also act as a free radical scavenger by means of its phenolic and methoxy group[14,15] and that biophysical calculation concluded that the OH bond is the most important region of curcumin as antioxidant.[16]

The generation of hydroxyl radical by t-BuOOH caused damage of mitochondrial proteins as shown by the formation of protein aggregates and the reducing intensity of a polypeptide band of 116 kD after electrophoresis. Tert-butylhydroperoxide rendered protein cross-linking by the reactions:

$$RSH + OH^{\bullet} \rightarrow RS^{\bullet} + H_2O$$
$$RS^{\bullet} + O_2 \rightarrow RSOO^{\bullet}$$
$$RSOO^{\bullet} + RSOO^{\bullet} \rightarrow RSSR + O_2$$

Protein aggregation formation of high molecular weight due to protein thiol oxidation was also obtained when mitochondrial membranes were incubated with Ca^{2+} and diamide[17] or peroxynitrite.[18] Some of the reduced polypeptide band of 116 kD appeared to be a constituent of the aggregates, since their visibility were inversely correlated.

Structural damage of mitochondrial membrane protein might lead to membrane permeabilization that eventually results in Ψm collapse and swelling. Curcumin and DTT as shown by our electrophoresis study prevented loss of a polypeptide band of 116 kD. For the same amount of protein incubated, curcumin could inhibit swelling with a similar optimal concentration as that used in the study of mitochondrial transmembrane potential in the presence of t-BuOOH.

The stoichiometry of curcumin (mg) vs. t-BuOOH (molarity) in both conditions was 1:4. The amount of curcumin needed to prevent protein cross-linking in the electrophoresis study, however, was much higher (12:1), making it difficult to reconcile with the data obtained from the swelling and transmembrane potential studies.

It might be entirely possible that the affected polypeptide band of 116 kD was not of mitochondrial membrane origin, since at the same concentration of t-BuOOH and amount of protein added, swelling and failure to generate transmembrane potential could be significantly prevented by a much lower concentration of curcumin. Based on the above consideration, we believed that the protein aggregates and the reduced intensity of polypeptide band of 116kD did not reflect subtle changes of mitochondrial membrane proteins. These results suggest that in order to reduce structural damage of mitochondrial proteins caused by t-BuOOH, a higher concentration of curcumin is needed.

The present study showed that curcumin could prevent failure to generate transmembrane potential and structural protein damage of rat mitochondria caused by t-BuOOH.

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