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PROTECTION BY SCHISANHENOL AGAINST ADRIAMYCIN TOXICITY IN RAT HEART MITOCHONDRIA

TONG-JUN LIN, GENG-TAO LIU,* YAN PAN, YANG LIU† and GUANG ZHI XU†
Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing 100050; and †Institute of Chemistry, Chinese Academy of Sciences, Beijing 100080, P. R. China

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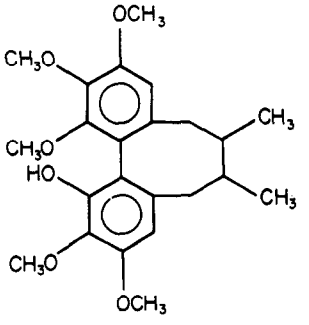
Abstract—The effects of schisanhenol (Sal) on Adriamycin® (ADM)-induced rat heart mitochondrial toxicity *in vitro* were investigated. Malondialdehyde formation, lysis, disintegration and membrane rigidification in mitochondria treated with ADM were reduced significantly by Sal. In the electron spin resonance studies, Sal did not affect significantly the formation of ADM semiquinone radicals ($AQ^{\cdot}$), whereas hydroxyl radicals generated by electron transfer from $AQ^{\cdot}$ to H_2O_2 were scavenged by Sal dose dependently. These results indicate that Sal could protect against ADM-induced rat heart mitochondrial toxicity.

Adriamycin® (ADM), a widely used antineoplastic agent, is limited in clinical use by its unique cardiotoxicity. A prominent site of injury induced by ADM is the cardiac mitochondria [1, 2]. Although the mechanism of cardiotoxicity is not understood fully, there is mounting evidence that reduction of ADM to the semiquinone ($AQ^{\cdot}$) and subsequent redox cycling with O_2 are involved [1, 3]. ADM activated by the heart specific exogenous NADH-oxidoreductase in mitochondria is reduced to $AQ^{\cdot}$ [4, 5]. The redox cycling of the semiquinone radical leads to the formation of superoxide anion, hydrogen peroxide and hydroxyl radicals [2, 6], which are responsible for major injury of cardiac mitochondria, such as the peroxidation of membrane lipids [7–10], subsequent membrane rigidification [11, 12] and inactivation of enzymatic complexes of the respiratory chain [12, 13]. Because cardiac tissue has a less developed antioxidant defense system, it is sensitive to free radical damage [3]. One of the approaches aimed at minimizing ADM cardiotoxicity is the use of free radical scavengers [12]. *Fructus schizandrae* has been used for centuries as an astringent and a tonic in traditional Chinese medicine. Schizandrins isolated from *F. schizandrae* have been shown to exhibit many pharmacological effects such as antioxidant activity [14–17]. Of the schizandrins studied, schisanhenol (Sal) displays the most potent antioxidant activity [16]. The protective effect of Sal against ADM-induced cardiac mitochondrial toxicity in rats was investigated in the present study.



Schisanhenol
Sal

MATERIALS AND METHODS

Chemicals. Sal was supplied by Professor Chen of the Institute of Materia Medica, Chinese Academy of Medical Sciences. It is a pure compound and lipid soluble. The compound was dissolved in dimethyl sulfoxide (DMSO). Adriamycin was purchased from Farmitalia, Carlo Erba Ltd. (Italy). NADH and ATP came from Boehringer, Mannheim. 5,5-Dimethyl-pyrroline-*N*-oxide (DMPO) was purchased from the Aldrich Chemical Co. and purified by charcoal before use. Diphenylhexatriene (DPH) was obtained from the Sigma Chemical Co., and thiobarbituric acid from Merck. H_2O_2, ferrous sulfate and other reagents were products of the Beijing Chemical Reagent Manufactory. All other chemicals were of analytical grade.

Male Sprague–Dawley rats weighing 270–300 g were used. Mitochondria were prepared from rat heart [18] in a medium of 10 mM Tris–HCl, pH 7.4, 0.25 M sucrose and 0.2 mM EDTA. Electron microscopic observations were performed as described by Pillsbury [19] after a 20-min incubation at 37°. Measurement of malondialdehyde (MDA) formation [20] and of mitochondrial membrane fluidity [13] was performed as described in the respective references after a 60-min incubation at 37°. Fe^{2+} was added to the incubations in the form of $Fe_2SO_4 \cdot 7H_2O$. ESR measurements were conducted with a Bruker ESP-300 spectrometer at room temperature using a quartz flat cell [12]. A

* Correspondence: Dr. Geng-Tao Liu, Institute of Materia Medica, Chinese Academy of Medical Sciences, 1 Xian Nong Tan St., Beijing 1000050, P. R. China.

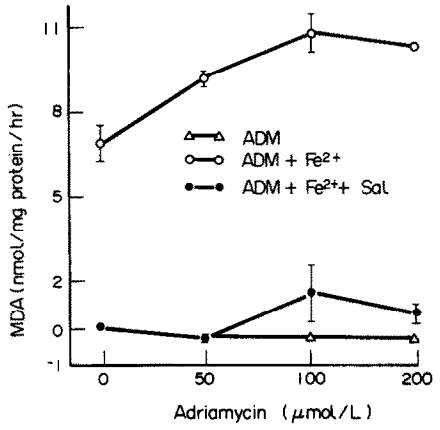


Fig. 1. Inhibitory effect of schisanhenol (Sal) on malondialdehyde (MDA) formation induced by Adriamycin (ADM) and ferrous ion in rat heart mitochondria. MDA was measured after a 1-hr incubation of a mixture containing rat heart mitochondria (1 mg protein), ferrous ion (15 μM) and various concentrations of ADM in the presence and absence of Sal (100 μM). Values are means ± SD, N = 4.

valve lock at the end of the aqueous flat cell prevented further oxygen entry. The double integration method was used for measuring the intensity of the ESR signals, proportional to the free radical concentration. Data, expressed as means ± SD, were evaluated statistically by Student's *t*-test.

RESULTS

Effects of Sal on ADM-induced lipid peroxidation in cardiac mitochondria from rats. Figure 1 shows the effects of Sal on ADM-induced mitochondrial lipid peroxidation. ADM alone at concentrations of 50, 100, and 200 μmol/L did not induce MDA formation in heart mitochondria from rats. However, lipid peroxidation in the mitochondria was stimulated by ADM in the presence of ferrous ion. These results are in agreement with previous reports [3, 7]. Addition of Sal (100 μM) to the above system significantly inhibited ADM-induced MDA formation in rat heart mitochondria.

Effects of Sal on ADM-induced damage in mitochondrial ultrastructures. Using electron microscopy we observed that treatment of rat heart mitochondria with ADM plus iron resulted in severe damage, i.e. swelling, disintegration and lysis (Fig. 2B). These effects induced by ADM were reduced markedly by Sal (Fig. 2C).

Effects of Sal on ADM-induced mitochondrial membrane rigidification. A lipid probe, DPH, was used to evaluate mitochondrial membrane fluidity. Fluorescence polarization (*P*) depends on the mobility of a fluorescent marker (DPH) embedded in the lipid bilayer, i.e. on the fluidity of the membrane being studied. A higher *P* value indicates a decrease of membrane fluidity. Sal did not affect normal mitochondrial membrane fluidity. However, as shown in Table 1, the mitochondria incubated

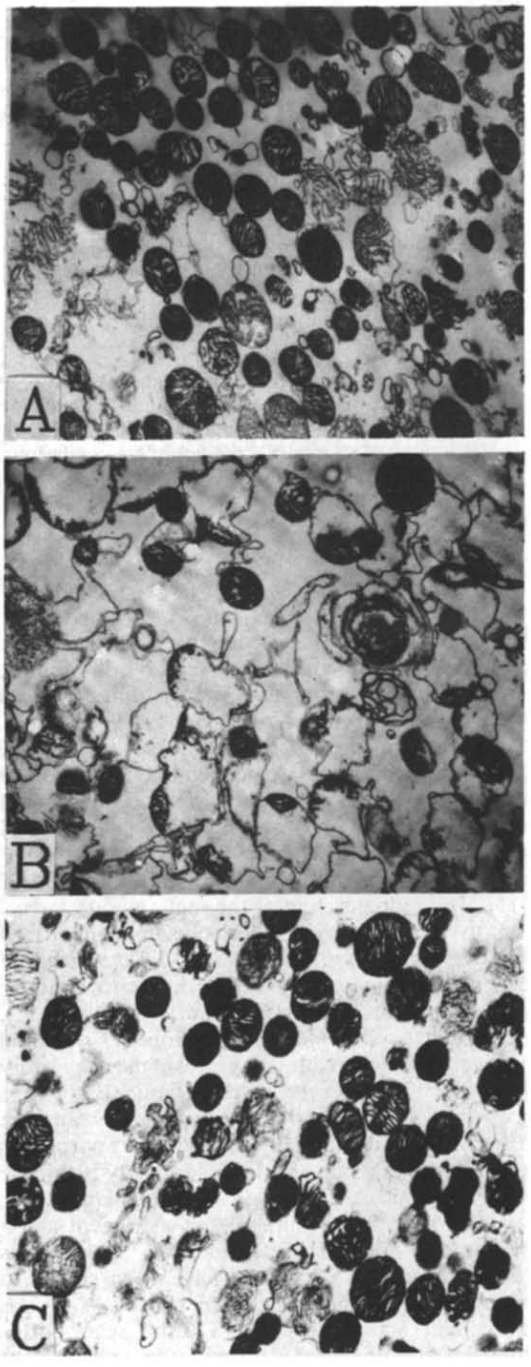


Fig. 2. Electron micrograph of rat heart mitochondria (× 7000). (A) Normal mitochondria. (B) Mitochondria treated with 100 μM ADM + 15 μM ferrous sulfate. (C) Mitochondria treated as in panel B with the addition of 1 mM Sal.

with ADM displayed membrane rigidification, as indicated by a significant increase in the degree of polarization. This result is in agreement with other reports [11–13]. Sal significantly protected against this ADM-induced rigidification of mitochondrial membrane (Table 1).

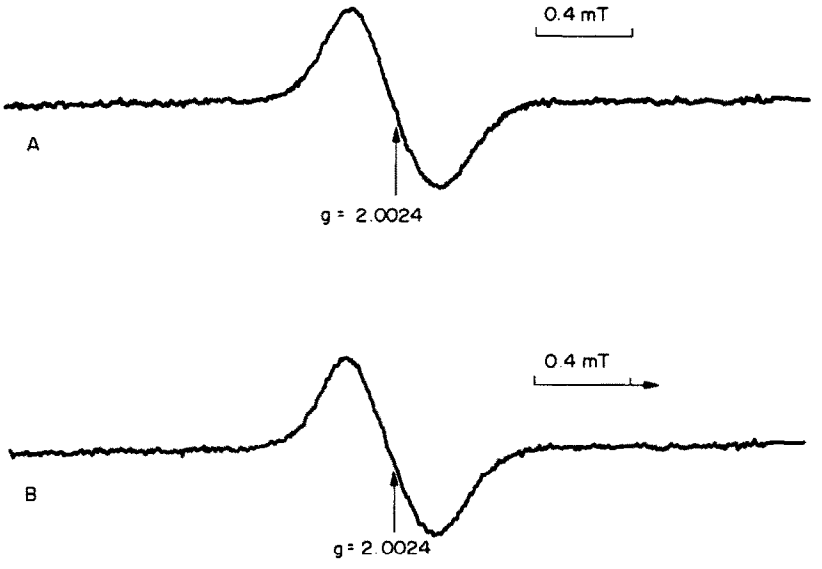


Fig. 3. ESR spectra of ADM semiquinone radicals formed when rat heart mitochondria (2 mg/mL) were incubated with NADH (5 mmol/L) and ADM (0.2 mmol/L) in the absence (A) and presence (B) of Sal (4 mmol/L).

Table 1. Effect of Sal on Adriamycin (ADM)-induced changes in mitochondrial membrane fluidity

	Fluorescence polarization (P)
Control	0.1730 ± 0.002
ADM	0.2075 ± 0.009
Sal + ADM	0.1306 ± 0.006*

The reaction mixture, containing rat heart mitochondria (1 mg protein), NADH (2.5 mmol/L) and DPH (2 μmol/L) was incubated for 60 min at 37°. Adriamycin and Sal concentrations were 0.1 and 1 mmol/L, respectively. Values are means ± SD, N = 4.

* $P < 0.01$ vs ADM.

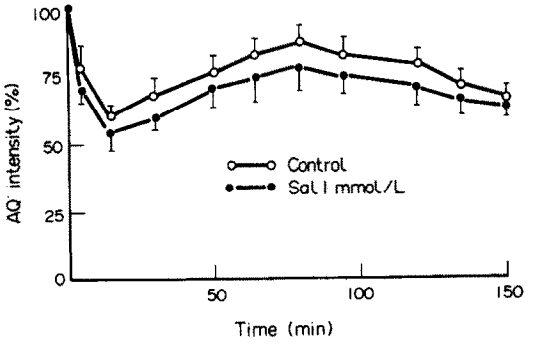


Fig. 4. Effects of Sal on ADM semiquinone radical concentration as detected by electron spin resonance. Values are means ± SD, N = 4.

Effects of Sal on ADM semiquinone radical. After mixing the isolated rat heart mitochondria with ADM and NADH, the semiquinone radical was detected by ESR and identified by its characteristic g-value at 2.0024 (Fig. 3), as reported by Nohl [4], and by a similar unknown ESR signal ($g = 2.0038$; data not shown). The production rate of the semiquinone radical was not decreased significantly by the addition of Sal (final concentration 1 mmol/L) to the reaction mixture (Fig. 4).

Effects of Sal on hydroxyl radicals generated from AQ·. Figure 5 shows the influence of H_2O_2 upon ESR spectra of ADM in the presence of the spin trap DMPO. Figure 5A shows the ESR spectrum of AQ· ($g = 2.0024$) in the same reaction medium as used in the experiment described in Fig. 3A and DMPO. When H_2O_2 was added to the reaction medium, the AQ· signal ($g = 2.0024$) was no longer present and a hydroxyl radical appeared (Fig. 5B). This result strongly confirmed the suggestion that the electron transfer from AQ· to H_2O_2 results in the production of highly reactive hydroxyl radicals. Addition of Sal to the above system scavenged hydroxyl radicals produced by ADM dose dependently (Figs. 6 and 7).

DISCUSSION

Mitochondria are highly susceptible to lipid peroxidation and are prominent sites of injury induced by ADM [1, 21]. It was suggested that ADM may have at least two mechanisms of action that cause tissue damage. One, which involves lipid peroxidation, is blocked by free radical scavengers, such as tocopherol, and appears to play a major role in the development of cardiomyopathy. The other,

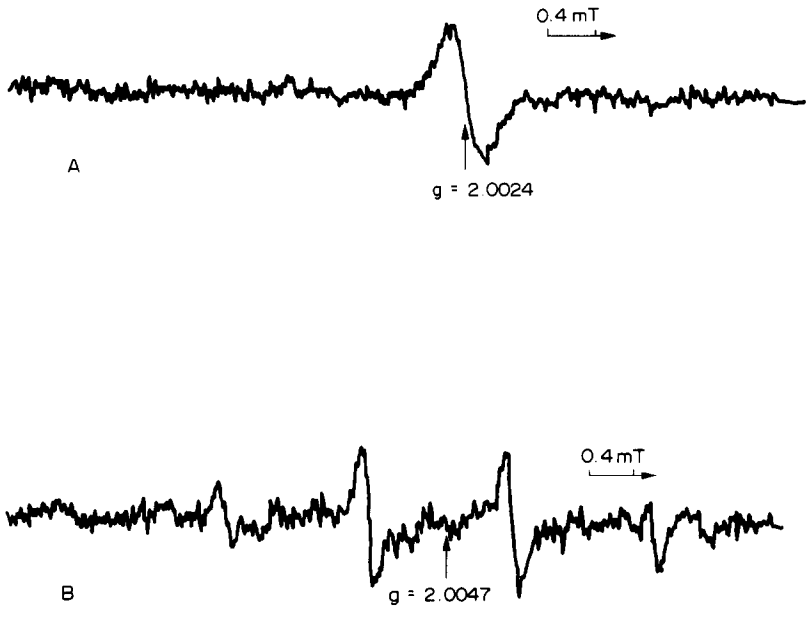


Fig. 5. Electron transfer from ADM semiquinone radical to H_2O_2. (A) ESR spectrum of ADM semiquinone radical formed when rat heart mitochondria (2 mg protein) were incubated with NADH (5 mM) and ADM (0.2 mM) in the presence of DMPO (75 mM). (B) ESR spectrum of DMPO-OH obtained 2 min later after addition of 2 mM H_2O_2 to A. Microwave power, 12.9 mW; modulation amplitude, 0.1 mT.

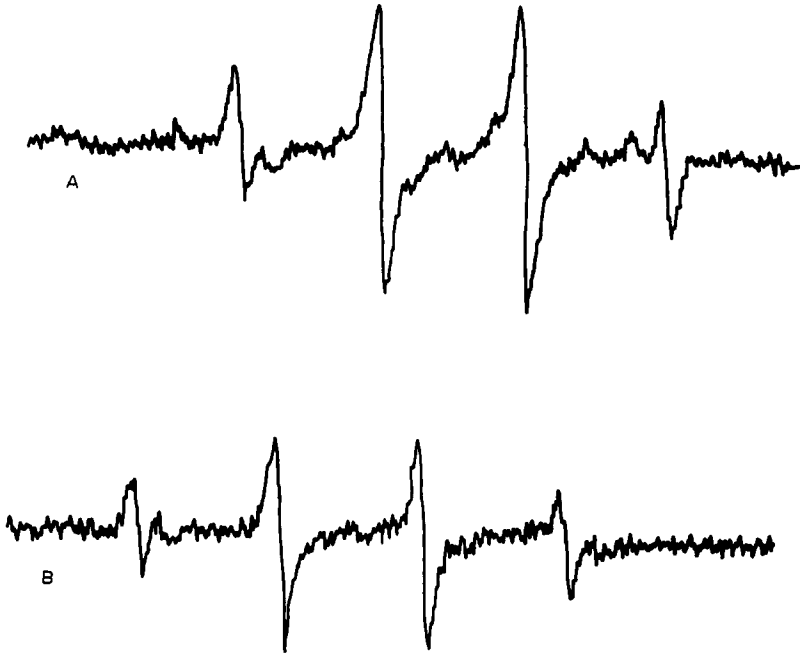


Fig. 6. ESR spectra of $OH^{\cdot}$ spin adduct of DMPO generated from ADM in a reaction mixture containing rat heart mitochondria (2 mg protein), NADH (5 mM), ADM (0.2 mM), H_2O_2 (2 mM) and DMPO (75 mM) in the absence (A) and presence (B) of Sal.

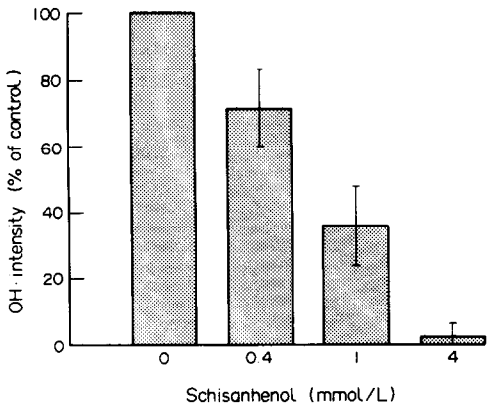


Fig. 7. Scavenging effects of Sal on ADM-induced hydroxyl radicals. The reaction mixture contained: DMPO (75 mM), rat heart mitochondria (2 mg protein), NADH (5 mM), ADM (0.2 mM), H_2O_2 (2 mM) and various concentration of Sal (0.4, 1, and 4 mmol/L). Values are means ± SD, N = 4.

which may involve the binding of ADM to DNA, is unaffected by tocopherol and appears to be the major determinant of ADM toxicity for tumor cells [8]. Hence, the antitumor efficiency of ADM may be dissociated from its side-effect of cardiotoxicity. ADM cardiotoxicity can be minimized by free radical scavengers. In our previous studies [16, 17], compounds isolated from *F. schizandrae* displayed oxygen radical scavenging properties and showed potent and anti-lipid peroxidation activities. Sal was proposed, therefore, as a new compound potentially able to overcome ADM cardiotoxicity which is related to the mitochondrial toxicity of ADM. Our results show that the amount of ADM semiquinone radical detected by ESR was not diminished significantly upon addition of Sal to the reaction mixture of mitochondria and NADH, while the electron transfer from ADM semiquinone radical to H_2O_2, leading to the production of hydroxyl radicals, was confirmed (Fig. 5). Hydroxyl radicals produced by ADM were scavenged dose dependently by Sal. It was suggested that the specific cardiotoxic effects of ADM were due to the formation of hydroxyl radicals via the reaction of ADM semiquinone radical with H_2O_2 [6]. This was expected from the fact that heart cells do not seem to have a significant amount of catalase [6, 22]. Therefore, relatively high steady-state concentrations of H_2O_2 exist in the heart as compared to other organs. Our results show that no MDA formation was detected in mitochondria in the presence of ADM alone. But MDA formation could be stimulated by ADM in combination with low levels of iron. These results are in agreement with the suggestion that iron is necessary for the manifestation of the peroxidative capability of ADM [7]. The role of iron in ADM-induced lipid peroxidation is considered to be its participation in the Fenton reaction [7], which leads to the production of hydroxyl radicals. Sal is able to block this reaction via its hydroxyl radical scavenging property. Thus, the lysis, disintegration and membrane rigidification induced by ADM in mitochondria were reduced significantly by Sal, indicating that Sal could minimize ADM-induced mitochondrial toxicity. Since the data described in the present paper were obtained from *in vitro* experiments, it is very important to further study the protective effect of Sal on ADM cardiotoxicity *in vivo*.

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