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Scavenging Effects of Dexrazoxane on Free Radicals

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Summary **Dexrazoxane (ICRF-187) has been clinically used to reduce doxorubicin-induced cardiotoxicity for more than 20 years. It has been proposed that dexrazoxane may act through its rings-opened hydrolysis product ADR-925, which can either remove iron from the iron-doxorubicin complex or bind to free iron, thus preventing iron-based oxygen radical formation. However, it is not known whether the antioxidant actions of dexrazoxane are totally dependent on its metabolization to its rings-opened hydrolysis product and whether dexrazoxane has any effect on the iron-independent oxygen free radical production. In this study, we examined the scavenging effect of dexrazoxane on hydroxyl, superoxide, lipid, DPPH and $ABTS^+$ free radicals *in vitro* solution systems. The results demonstrated that dexrazoxane was an antioxidant that could effectively scavenge these free radicals and the scavenging effects of dexrazoxane did not require the enzymatic hydrolysis. In addition, dexrazoxane was capable to inhibit the generation superoxide and hydroxyl radicals in iron free reaction system, indicating that the antioxidant properties of dexrazoxane were not solely dependent on iron chelation. Thus the application of dexrazoxane should not be limited to doxorubicin-induced cardiotoxicity. Instead, as an effective antioxidant that has been clinically proven safe, dexrazoxane may be used in a broader spectrum of diseases that are known to be benefited by antioxidant treatments.**

Key Words**: dexrazoxane, antioxidant, oxygen free radicals, free radical scavenger, ion-chelater**

Introduction

Dexrazoxane (ICRF-187; Fig. 1) has been clinically used to reduce doxorubicin-induced cardiotoxicity for more than 20 years [*1–8*]. Evidence has indicated that the iron-dependent oxygen free radical formation on the relatively unprotected cardiac muscle is a major cause of the cadiotoxicity induced by doxorubicin [*9*, *10*]. It has been proposed that dexrazoxane can be converted by dihydroorotase (DHOase) to its rings-opened hydrolysis product ADR-925 (Fig. 1), which can either remove iron from the iron-doxorubicin complex or bind to free iron, thus preventing iron-based oxygen radical formation [*11–16*]. However, several questions remain unanswered. First, are the antioxidant actions of dexrazoxane totally dependent on its metabolization to its rings-opened hydrolysis product ADR-925? Second, does dexrazoxane have any effect on the iron-independent oxygen free radical production or it only scavenges oxygen free radical generated from iron-dependent process? Elucidation of these questions may help to evaluate the clinical use of dexrazoxane as an antioxidant directly in conditions other than doxorubicin-induced cardiotoxicity.

Here we examined the scavenging effect of dexrazoxane on hydroxyl, superoxide, lipid, diphenylpicrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^+$) free radicals *in vitro* reaction solution systems and found that dexrazoxane effectively scavenged these free radicals. The results also demonstrated that the antioxidant actions of dexrazoxane did not require its enzymatic hydrolysis or solely depend on iron chelation,

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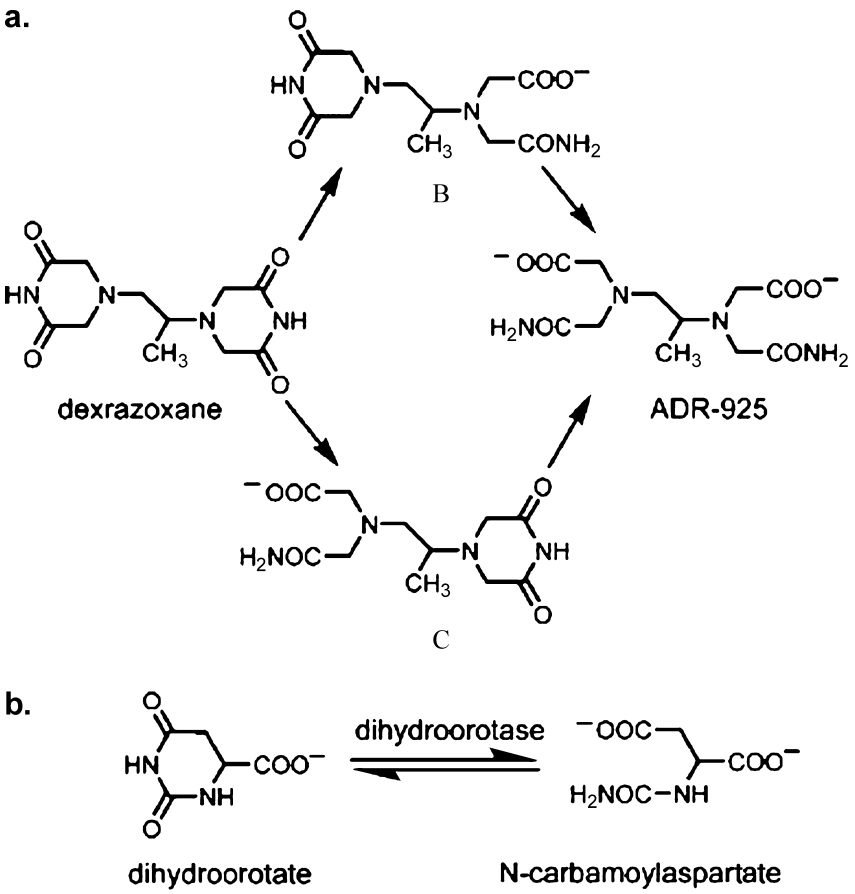

Fig. 1. a. A reaction scheme for the hydrolysis of dexrazoxane to metabolites B and C and its strong metal ion-chelating form ADR-925; b. DHOase-catalyzed reversible conversion of L-dihydroorotate into N-carbamoyl-L-aspartate (carbamyl aspartate).

suggesting the potential applications of dexrazoxane in a broader spectrum of diseases that are known to be benefited by antioxidant treatments in addition to doxorubicin-induced cardiotoxicity.

Materials and Methods

Chemicals

Dexrazoxane was purchased from Najing Aosaikang Medicinal Group Co. (Nanjing, China). 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) diammonium salt, DMPO (5,5-dimethyl-1-pyroline-1-oxide), diphenylpicrylhydrazyl (DPPH), TEMP (2,2,6,6-tetramethul-1-piperide), 2-BA-2-DMHB (2-butylamino-2-demethoxyhypocrellin B), and 4-POBN(-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone) were purchased from Sigma Chem Co. (USA). Other agents were of analytical grade from China.

Scavenging effect of dexrazoxane on $ABTS^+$ free radical measured by spectrophotometer

A stable stock free radical $ABTS^+$ solution was prepared the day before the analysis. In brief, 192 mg of ABTS and 33 mg of $K_2S_2O_8$ were dissolved in a 25 mL volumetric flask and stored overnight (12–16 h) in the dark at room temperature. A working $ABTS^+$ solution was prepared on the day of the analysis, by dilution of the stock $ABTS^+$ solution in ethanol to reach an absorbance of 1.30 ± 0.05 AU at 734 nm in a 1 cm cuvette measured with spectrophotometer [*17*, *18*]. Different concentrations of dexrazoxane were added to the $ABTS^+$ working solution and measured at 734 nm until the absorption was stable for 5 min.

Scavenging effect of dexrazoxane on free radicals measured by ESR

To measure free radicals using Electron Spin Resonance (ESR) spectroscopy, spin trap reagents were added to *in vitro* free radical generation systems in the absence or presence of dexrazoxane following the procedure described previously [*19–21*]. The reaction systems for generation of different free radicals are listed in Table 1. ESR signal intensities in the absence of or presence of dexrazoxane were recorded as Ho and Hx, respectively. The scavenging effect was

Table 1. Reaction systems for free radical scavenging experiments

Free radicals	Reaction systems
DPPH	DPPH (10 μM) in ethanol (85%) + different concentrations of sample (reacted time 1 min)
Superoxide	DMPO (100 μM) + EDTA (5 mM) + riboflavin (0.3 mM) + different concentrations of sample then lighted for 1 min
Hydroxyl (photolysis)	H_2O_2 (0.1%) + photolysis + DMPO + different concentrations of sample
Hydroxyl (Fenton reaction)	DMPO + ferrous sulfate (25 mM) + H_2O_2 (0.1%) + different concentrations of sample
Lipid	Mitochondria (20 mg protein/ml) + 0.1 M 4-POBN + different concentrations of sample

calculated by [(Ho-Hx)/Ho] × 100%. The ESR measurements were performed using a Brucker ER200 D-SRC ESR spectrometer (Brucker Analytischem Messtechjik GMBH, Sibeerstreifen, Germany) with following parameters: X-band, 100 kHz modulation with amplitude 1 G, microwave power 10 mW, central magnetic field 3,250 G, sweep width 200 G, temperature 20°C.

Preparation of mitochondria

Male SD rats (223–230 g) were purchased from Institute of Laboratory Animal Science, Chinese Academy of Medical Science (Beijing, China). Animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local animal care committee. Mitochondria were isolated from the rat liver following the procedure described previously with slight modifications [*19*]. In brief, the liver tissues were minced, washed and then homogenized at 4°C in 0.9% NaCl. The homogenate was centrifuged at 600 g for 7 min. The resulted supernatant was centrifuged again at 10,000 g for 10 min. Mitochondrial pellets was then washed twice with a buffer containing 3 mmol/L $MgCl_2$, 50 mmol/L Tris-HCL (pH 7.5) and 0.2 mmol/L sucrose and resuspended in the same buffer.

Statistical Analysis

All experiments were repeated six times. One-way ANOVA was used to estimate the overall significance followed by post hoc Tukey's tests corrected for multiple comparisons [*22*]. Data are presented as mean ± SEM. A probability level of 5% ($p<0.05$) is considered to be significant.

Results

Scavenging effects of dexrazoxane on superoxide free radical

Superoxide free radicals generated from irradiation of riboflavin were spin trapped by DMPO and exhibited a signal with 12 lines peaks (a_N = 14.2 G, a_H^β = 11.2 G, a_H^r = 1.3 G) on ESR spectrum (inset in Fig. 2). Addition of dexrazoxane to the reaction mixture effectively decreased the ESR signal generated from superoxide free radicals (Fig. 2). The IC_{50} of the scavenging effect of dexrazoxane on superoxide free radical is about 0.40 mg/mL.

Scavenging effect of dexrazoxane on hydroxyl radicals generated from photolysis of H_2O_2

The hydroxyl free radicals were generated from photolysis of H_2O_2 (0.1%) and spin trapped by DMPO (0.1 mol/L). The characteristic ESR spectrum exhibiting 4 peaks with 1:2:2:1 intensity (g = 2.0045, $a_N = a_H$ = 14.9 G) was obtained (inset in Fig. 3). Addition of dexrazoxane to the reaction mixture effectively scavenged the hydroxyl radicals generated from photolysis of H_2O_2 (Fig. 3) and the IC_{50} is about 2.10 mg/mL.

Scavenging effect of dexrazoxane on hydroxyl radicals generated from Fenton reaction

To compare the scavenging effects of dexrazoxane in the absence and presence of iron, we next examined the effect of dexrazoxane on hydroxyl free radicals generated from Fenton reaction using ESR spectroscopy. As shown in Fig. 4, dexrazoxane can effectively scavenge hydroxyl radical generated from Fenton reaction and the IC_{50} is about 1.60 mg/mL. It seams from the result that dexrazoxane is more effective for scavenging hydroxyl radicals which are generated in the presence of iron, but it is hard to keep the hydroxyl radical concentration is the same in the two different generation system, even the same concentration of H_2O_2 was used.

Scavenging effect of dexrazoxane on lipid free radicals

Lipid peroxidation has been implicated in many human diseases. We next studied the scavenging effect of dexrazoxane on lipid free radical generated in lipid peroxidation of mitochondria caused by hydroxyl radical from photolysis of H_2O_2. The lipid free radicals were spin trapped by 4-POBN and exhibited a signal with 6 lines on ESR spectrum (inset in Fig. 5). Dexrazoxane was added to the mitochondria before the hydroxyl radical stimulation and the production of lipid free radicals was measured again. It was found that dexrazoxane effectively scavenged lipid free radicals and the IC_{50} is about 4.35 mg/mL (Fig. 5).

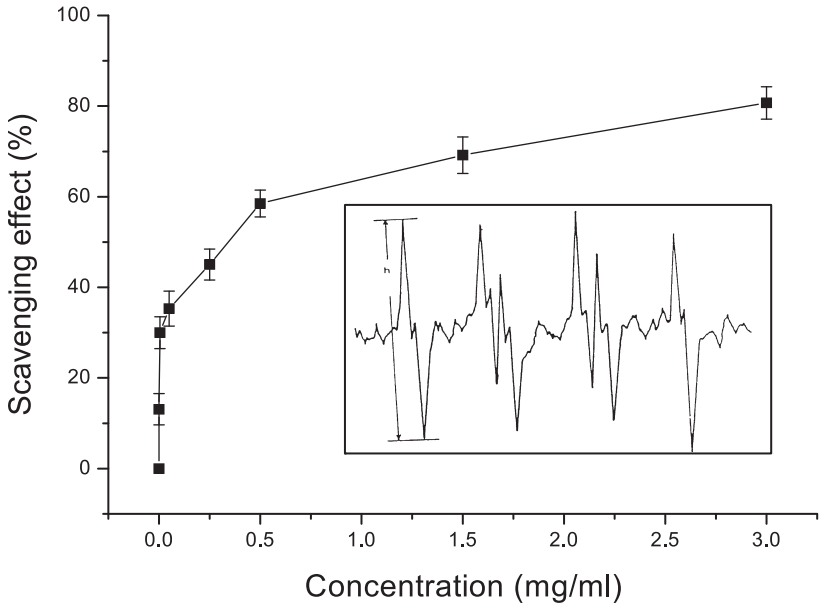

Fig. 2. Scavenging effects of dexrazoxane on superoxide free radicals. Superoxide free radicals were generated from irradiation of riboflavin and spin trapped by DMPO. (The ESR spectrum is shown in the inset). Different concentrations of dexrazoxane were added to the reaction mixture and the generation of superoxide free radicals was determined by ESR spectroscopy. Details of the procedure are described in the "Materials and Methods" ($n = 6$).

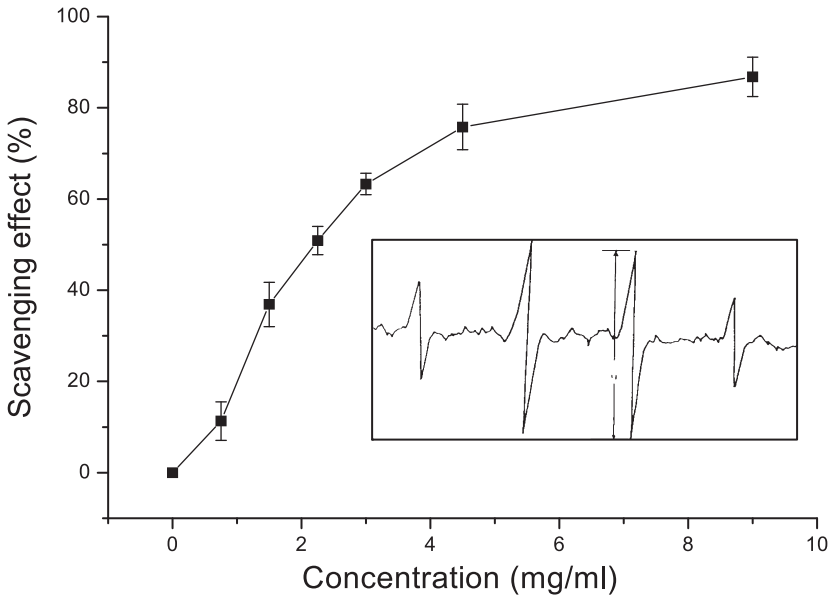

Fig. 3. Scavenging effects of dexrazoxane on hydroxyl free radicals generated from photolysis of H_2O_2. The inset shows the ESR spectrum of hydroxyl free radicals spin trapped by DMPO. Details of the procedure are described in the "Materials and Methods" ($n = 6$).

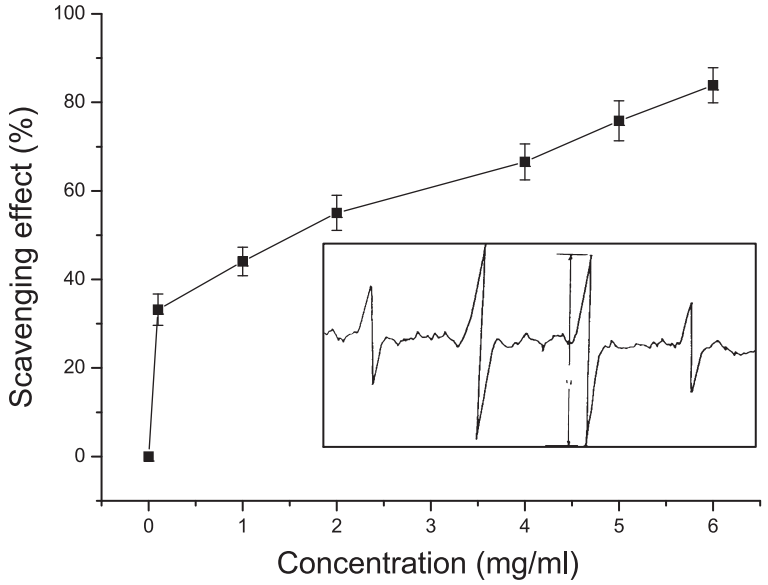

Fig. 4. Scavenging effects of dexrazoxane on hydroxyl free radicals generated from Fenton reaction. The inset shows the ESR spectrum of DMPO-OH. Details of the procedure are described in the "Materials and Methods" (n = 6).

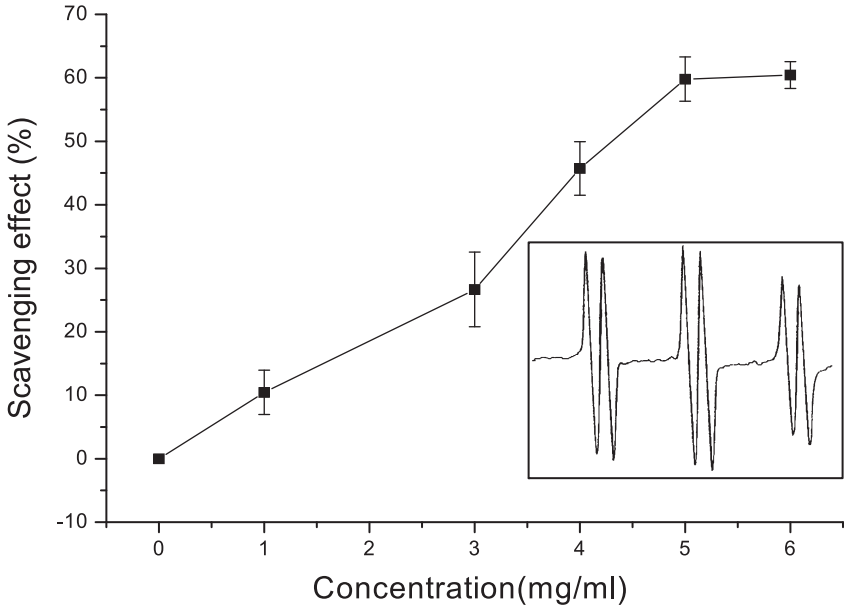

Fig. 5. Scavenging effects of dexrazoxane on lipid free radicals generated from lipid peroxidation of mitochondria. The inset shows the ESR spectrum of lipid free radicals generated from lipid peroxidation of mitochondria induced by photolysis of H_2O_2 and spin trapped by 4-POBN. Details of the procedure are described in the "Materials and Methods" (n = 6).

Scavenging effects of dexrazoxane on DPPH free radical

Stable free radical DPPH has been used to estimate the activity of antioxidants [*19*]. We examined the scavenging effect of dexrazoxane on DPPH free radicals using ESR spectroscopy. The ESR spectrum of DPPH free radical in ethanol solution is shown in the inset of Fig. 6. Adding dexrazoxane to the DPPH solution effectively decreased the intensity of the ESR signal generated from DPPH free radical and the IC_{50} is about 3.45 μg/mL (Fig. 6).

Scavenging effects of dexrazoxane on ABTS$^+$

ABTS assay is another method frequently used for

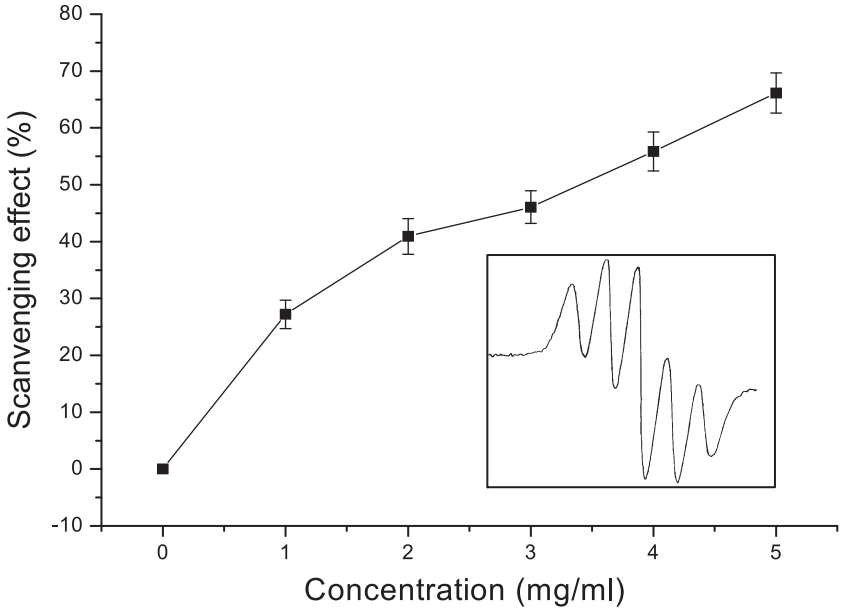

Fig. 6. Scavenging effects dexrazoxane on DPPH free radical. The inset shows the ESR spectrum of DPPH free radicals. Different concentrations of dexrazoxane were added to the DPPH free radical solution and analysed by ESR spectroscopy. Details of the procedure are described in the "Materials and Methods" ($n = 6$).

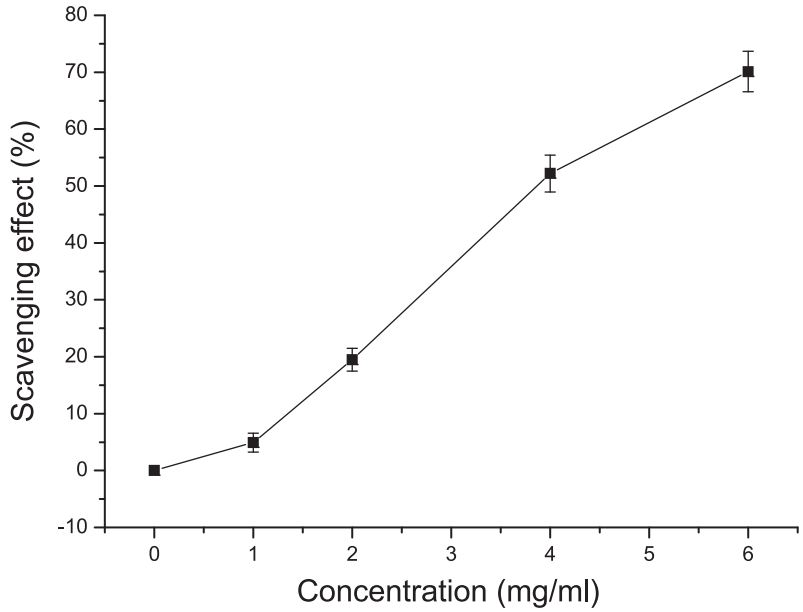

Fig. 7. Scavenging effects of dexrazoxane on $ABTS^+$ cation free radical. Different concentrations of dexrazoxane were added to the $ABTS^+$ working solution and measured at 734 nm by spectrophotometer. Details of the procedure are described in the "Materials and Methods" ($n = 6$).

measuring antioxidant activities. In the assay, the $ABTS^+$ cation free radical generated by oxidation of ABTS with potassium persulfate has blue color and absorbs light at 734 nm, which can be reduced by antioxidants and converted to its colorless neutral form. The discolorization is proportional to the antioxidant activity and can be monitored by spectrophotometer [*17*, *18*]. As shown in Fig. 7, dexrazoxane effectively scavenged $ABTS^+$ cation free radical and the IC_{50} is about 3.75 mg/mL.

Discussion

These results demonstrated that dexrazoxane was an antioxidant that could effectively scavenge superoxide, hydroxyl, lipid, DPPH and $ABTS^+$ free radicals *in vitro* reaction systems. It was found that dexrazoxane not only scavenged hydroxyl radical generated from Fenton reaction, but also effectively inhibited the generation of superoxide, hydroxyl radicals, lipid, DPPH and $ABTS^+$ in iron free reaction system, suggesting that iron chelation may be a factor contributing to the prevention of dexrazoxane on iron-based free radical generation, however, the antioxidant properties of dexrazoxane were not solely dependent on iron chelation. Furthermore, dexrazoxane directly scavenged oxygen free radicals in the *in vitro* solution system, suggesting that the scavenging effects of dexrazoxane did not require its enzymatic hydrolysis to its ring-opened forms. This is the first report that the antioxidant activity of dexrazoxane is not relied on its enzymatic conversion to its ring-opened hydrolysis products and dexrazoxane is effective in reducing oxygen free radicals generated from iron-independent processes. Therefore, dexrazoxane may be useful clinically for other diseases that are known to be benefited by antioxidant treatments [*23–32*] in addition to doxorubicin-induced cardiotoxicity. The application of dexrazoxane in those diseases should be evaluated in the future.

Vitamin C and E are water and lipid soluble vitamins and they can scavenge water and lipid soluble free radicals respectively. They can synergically inhibit lipid peroxidation of cell membrane, however, nether vitamin C or vitamin E alone can not inhibit lipid peroxidation [*33*]. Dexrazoxane can scavenge both water and lipid soluble free radicals and it may be used as combination of vitamin E and E in the future.

Free radicals are normal metabolites in biological systems and play important role in a variety of physiological functions including immunity, oxidative stress characterized by an imbalance between the production of reactive oxygen species and antioxidant defenses has been implicated in the pathogenesis of many human diseases such as cardiovascular diseases, cancer, neurodegenerative diseases. Free radicals such as Nitric Oxide (NO), hydroxyl, superoxide and lipid free radicals have closed relation with human health [*23–26*]. Free radicals have important biological functions such as oxygen free radicals in mitochondrial electron transfer chain, cell growth and differentiation, immunological response, NO free radical in blood vascular relaxation, blood pressure modulation, study and memory. But free radicals also have close relation with most of human diseases, such as heart and brain vascular diseases, cancers, aging and neurodegenerative diseases [*23–32*]. The generation and scavenging of free radicals or oxidation and reduction in human body should be balance for human health. If free radical generation is more than the scavenging, there will be extra free radicals able to damage cell components and lead to diseases. If antioxidants were supplied to help body to keep the balance, the diseases can be prevented. Dexrazoxane can scavenge free radicals alone suggests that it can be used as an antioxidant in all these kinds of diseases and open an new window to use dexrazoxane in clinical without need to do many pre-clinical experiments because it has been clinically used as a drug for more than 20 years.

In this paper, we studied the antioxidant activity of dexrazoxane by examining its scavenging effect on superoxide, hydroxyl, lipid, DPPH and $ABTS^+$ free radicals in *in vitro* reaction systems. The results showed that the scavenging effects of dexrazoxane on free radicals do not require its enzymatic hydrolysis. Furthermore, dexrazoxane was capable to inhibit the oxygen free radical generation in iron free reaction system, although iron chelation may be a factor contributing to the its decrease on iron-based free radical generation. These results suggest that the antioxidant properties of dexrazoxane are not solely dependent on iron chelation. Thus the application of dexrazoxane should not be limited to doxorubicin-induced cardiotoxicity. Instead, as an effective antioxidant that has been clinically proven safe, dexrazoxane may be used in a broader spectrum of diseases that are known to be benefited by antioxidant treatments.

Conclusion

In this paper, we showed that the scavenging effects of dexrazoxane on free radicals do not require its enzymatic hydrolysis and dexrazoxane was capable to inhibit the oxygen free radical generation in iron free reaction system. Thus the application of dexrazoxane should not be limited to doxorubicin-induced cardiotoxicity and, dexrazoxane may be used in a broader spectrum of diseases that are known to be benefited by antioxidant treatments.

Acknowledgments

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