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Evaluation of superoxide anion radical scavenging activity of shikonin by electron spin resonance

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Abstract

The scavenging activity of shikonin (SK) for superoxide anion radical ($O_2^{\cdot -}$) was evaluated by electron spin resonance (ESR) to clarify the mechanism of the enhancing effect of SK on wound healing and its anti-inflammatory effect. SK quenched $O_2^{\cdot -}$ in a dose-dependent manner. Values for the $O_2^{\cdot -}$ scavenging activity of SK were converted to values representing the equivalent activity of superoxide dismutase (SOD) and were termed SOD-like activity values. SK exhibited potent $O_2^{\cdot -}$ quenching activity and its SOD-like activity was calculated to be about 920 U/mg. These results suggested that the $O_2^{\cdot -}$ scavenging activity of SK played an important role in enhancing wound healing and in the anti-inflammatory effect of SK. No difference in $O_2^{\cdot -}$ scavenging activity was observed between SK and its optical isomer alkannin. SK was reduced to SK semiquinone radical by $O_2^{\cdot -}$. The SK semiquinone radical may be responsible for the anti-tumor and anti-bacterial effects of SK.

Keywords: Shikonin; Alkannin; Superoxide anion radical; Superoxide dismutase; Semiquinone radical; ESR

1. Introduction

'Shiun-ko' is a traditional herbal medicine (Kampo medicine) and has been used to treat burns and hemorrhoids by external application. Shikonin (SK) and its derivatives are the active components in 'Shiun-ko' and have been reported to have various pharmacological effects (Hayashi, 1977a,b,c). The pharmacological effects of SK and its derivatives were reported to be the acceleration of the proliferation of granulation tissue

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(Ozaki et al., 1994, 1996), an anti-bacterial effect (Tanaka and Odani, 1972), an anti-inflammatory effect (Tanaka et al., 1986) and an anti-tumor effect (Sankawa et al., 1977). In previous studies, SK ointment had an accelerating effect on wound healing and also exhibited an anti-inflammatory effect in an experimental wound healing model in rats (Sekine et al., 1998a). In addition the SK ointment exhibited an anti-bacterial effect in vivo (Sekine et al., 1998b). However, the mechanisms underlying the pharmacological effects are not known.

Free radicals such as the superoxide anion radical ($O_2^{\cdot -}$) and the hydroxyl radical ($^{\cdot}OH$) are considered to play important roles in the pathogenesis of many diseases. There have been many reports concerned with the role of free radicals in wound healing (Melikian et al., 1987; White and Heckler, 1990). Takami et al. (1993) reported that superoxide dismutase (SOD) ointment had a healing effect on open wounds and burn ulcers in rats. In this study, we evaluated the $O_2^{\cdot -}$ scavenging activities of SK and its optical isomer alkannin (AK) in vitro using electron spin resonance (ESR) in order to clarify the mechanisms underlying their pharmacological effects. Values for the $O_2^{\cdot -}$ scavenging activities of SK and AK were converted to values representing the equivalent activity of SOD and were termed SOD-like activity values (Mitsuta et al., 1990).

2. Experimental

2.1. Materials

SK and AK were obtained from Maruzen Pharmaceutical Co. (Hiroshima, Japan) and used after being purified to over 99% pure (chemical structure is shown in Fig. 1). SOD (3750 units (U)/mg protein) was obtained from Funakoshi (Tokyo, Japan). 5,5-Dimethyl-1-pyrroline-1-oxide (DMPO), hypoxanthine (HPX) and xanthine oxidase (XOD) were obtained from Labotec (Tokyo, Japan). All other chemicals used were of reagent grade.

2.2. Determination of the composition of the optical isomers SK and AK

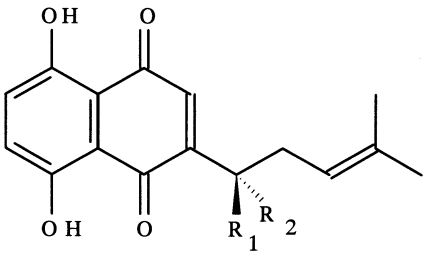
The composition of shikonin (*R*-configuration) (*R*-SK) and alkannin (*S*-configuration) (*S*-AK) in SK and AK was determined by HPLC according to Ikeda's method (Ikeda et al., 1991).

2.3. ESR spectrometry conditions

ESR spectra were recorded on a JEOL JES RE-1X spectrometer (JEOL, Tokyo). The ESR spectrometry conditions used to estimate the $O_2^{\cdot -}$ were as follows: magnetic field: 337.1 ± 5 mT; power: 4 mW, 9.42 GHz; sweep time: 2 min; modulation: 100 kHz, 0.079 mT; amplitude: 1×200; time constant 0.3 s. The ESR spectrometry condition used to estimate the SK semiquinone radical was as follows: magnetic field: 337.1 ± 5 mT; power: 1 mW, 9.42 GHz; sweep time: 4 min; modulation: 100 kHz, 0.01 mT; amplitude: 1×200; time constant 0.03 s.

2.4. Evaluation of $O_2^{\cdot -}$ scavenging activity

$O_2^{\cdot -}$ were generated using the HPX-XOD reaction system. The procedure for the determination of O_2^{-} yield, O_2^{-} being detected as a spin adduct (DMPO-O_2^{-}), is shown in Fig. 2. SOD was dissolved in phosphate buffered saline (PBS) (pH 7.4) and the concentrations were in the range of 0.8–25.0 U/ml (final concentrations were 0.18–5.8 U/ml). SK and AK were dissolved in dimethyl



(*R*-) Shikonin: R1=OH, R2=H
(*S*-) Alkannin: R1=H, R2=OH
MW: 288.3

Fig. 1. Chemical structure of shikonin and alkannin.

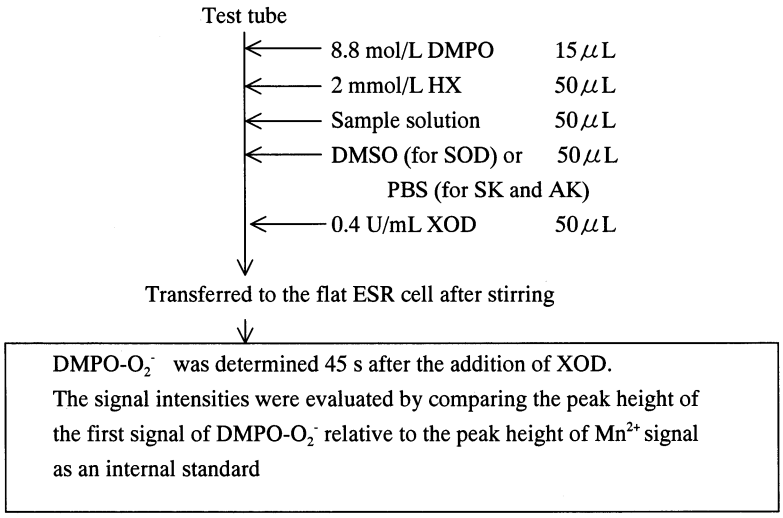


Fig. 2. Procedure for the determination of DMPO-O_2^- yield.

sulfoxide (DMSO) and the concentrations were in the range of 3.9×10^{-3}–39.0 mmol/l (final concentrations were 9.0×10^{-4}–9.0 mmol/l). All other chemicals were dissolved in PBS. The amount of DMPO-O_2^- formed was determined exactly 45 s after the addition of XOD. The signal intensities were evaluated by comparing the peak height of the first DMPO-O_2^- signal relative to the peak height of the Mn^{2+} signal as an internal standard. The $O_2^{\cdot -}$ scavenging activities of SK and AK were evaluated to estimate the concentrations required to reduce the relative peak height of the DMPO-O_2^- by 50% (ID_{50}). The second-order rate constants of SK and AK for the reaction with O_2^- were calculated according to the method of Mitsuta et al. (1990). In addition, the SOD-like activities of SK and AK were determined according to the kinetic competition model (Finkelstein et al., 1979, 1980).

3. Results and discussion

3.1. Composition of optical isomers in SK and AK

The optical composition of *R*-SK and *S*-AK was determined to be 82 and 18% (82:18) in SK, and 15 and 85% (15:85) in AK, respectively. Namely, SK and AK were not optically pure but contained respective optically rich isomers. Therefore, it was considered that SK and AK could be used to evaluate the $O_2^{\cdot -}$ scavenging activity of each optical isomer.

3.2. $O_2^{\cdot -}$ scavenging activity of SOD

When DMPO was added to the HPX-XOD reaction system, $O_2^{\cdot -}$ were detected as DMPO-O_2^- (Finkelstein et al., 1979). The relationship between the concentrations of SOD and $F/(1-F)$ (F, inhibitory ratio; $0 < F < 1$) on the formation of DMPO-O_2^- is shown in Fig. 3. The linear regression line for estimating the $O_2^{\cdot -}$ scavenging activity of SOD was expressed as Eq. (1), and the ID_{50} value ($F = 0.5$) of SOD was calculated to be 1.92 U/ml according to Eq. (1):

$$F/(1-F) = 0.52X$$

X, concentration of SOD (U/ml) (1)

The SOD-like activity was expressed according to Eq. (2) (Mitsuta et al., 1990):

$$\text{SOD-like activity (U/mg)} = 1.92\ (\text{U/ml})/ID_{50}\ (\text{mg/ml}) \quad (2)$$

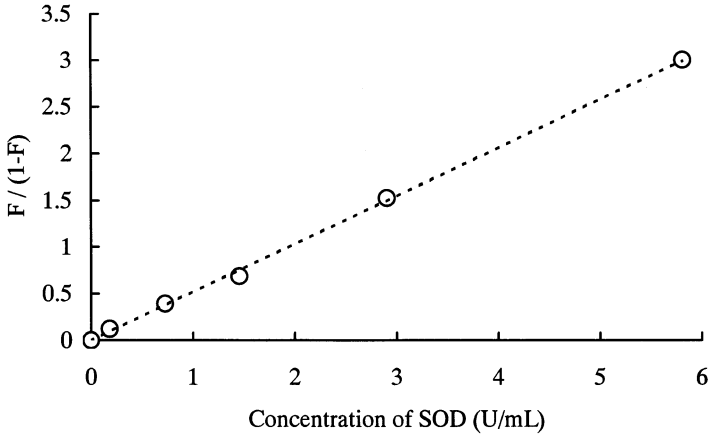


Fig. 3. Relationship between the concentration of standard SOD and the inhibitory ratio (F) on the formation of DMPO-O_2^-.

3.3. $O_2^{\cdot -}$ scavenging activity of SK and AK

The relationship between the concentration of SK (or AK) and the relative signal intensity of DMPO-O_2^- is shown in Fig. 4. The signal intensities of DMPO-O_2^- decreased with increasing concentrations of SK and AK. The ID_{50} values of SK and AK were calculated to be 7.2×10^{-6} and 5.5×10^{-6} mol/l, respectively, from the linear equation at around 50% yields of DMPO-O_2^- in Fig. 4. The second-order rate constants of SK and AK were calculated to be 1.4×10^6 and 1.9×10^6 $(mol/l)^{-1} \cdot s^{-1}$, respectively (Table 1). From these results, the $O_2^{\cdot -}$ scavenging activities of SK and AK were considered to be almost the same, but the rate constant of SK was somewhat lower than that of AK. Mitsuta et al. (1990) reported that the value of the second-order rate constant of L-ascorbic acid was 3.5×10^5 $(mol/l)^{-1} \cdot s^{-1}$. Therefore, the $O_2^{\cdot -}$ scavenging activities of SK and AK were estimated to be higher than that of L-ascorbic acid.

The SOD-like activities of SK and AK were calculated to be 920 and 1200 U/mg, respectively, according to Eq. (2) using the ID_{50} value of each. In previous study, 0.1% SK ointment had an enhancing effect on the healing of burn and open wounds in rats (Sekine et al., 1998a). The SOD-like activity of 0.1% SK ointment was calculated to be 920 U/g. Abe et al. (1987) reported that SOD ointment had an anti-inflammatory effect on skin inflammation in rats. They also reported that the minimal effective concentration of SOD ointment was 0.01% (310 U/g). Takami et al. (1993) reported that 0.06% SOD ointment (1920 U/g) had a healing effect on open wounds and burn ulcers in rats. These reports suggest that SOD-like activity plays an important role in the wound healing enhancing effect and the anti-inflammatory effect of SK ointment. Tanaka and Odani (1972) reported that 'Shiun-ko' contained about 0.2% SK derivatives. Therefore, the effectiveness of 'Shiun-ko' for the treatment of burn may also be due to SOD-like activity.

The ESR spectra of DMPO-O_2^- observed following the addition of SK are shown in Fig. 5. In addition to the normal signals of DMPO-O_2^-, signals representing new radical (*, Fig. 5) were detected. The signal intensities increased cumulatively. The ESR spectrum obtained 20 min after addition of 187 μmol of SK is also shown in Fig. 6. Dodd and Mukherjee (1984) reported that 5,8-dihidroxy-1,4-naphthoquinone (naphthazarin) formed semiquinone radical. Because SK has a naphthazarin structure, the generated radical was considered to be SK semiquinone radical. If this is the case, it means that SK is reduced to SK semiquinone radical by $O_2^{\cdot -}$. Öllinger and Brunmark (1991) reported that the cytotoxicity of naphthazarin was due to the formation of semiquinone radical. This reaction was also reported for adriamycin and other anthracycline

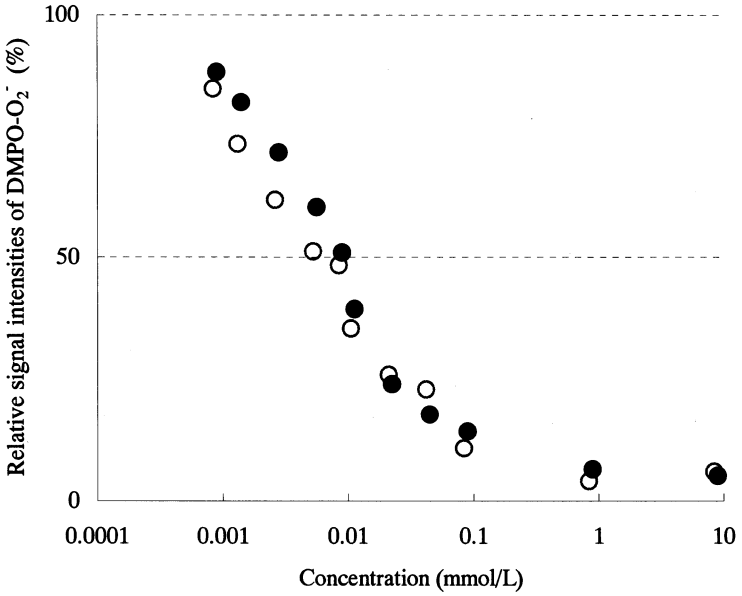


Fig. 4. Inhibitory effects of SK and AK on the formation of DMPO-O_2^- (●, SK; ○, AK).

antibiotics. The semiquinone radical may exhibit cytotoxicity via the generation of endogenous $O_2^{\cdot-}$ (Kalyanaraman et al., 1980). These results suggest that the anti-bacterial and anti-tumor effects of SK are due to semiquinone radical formation. However, the $O_2^{\cdot-}$ generating activity of SK semiquinone radical was not evaluated in this study. In future studies, therefore, it will be necessary to evaluate the endogenous $O_2^{\cdot-}$ generating activity of SK semiquinone radical.

Recently, Tsujita et al. (1997) reported that SK semiquinone radical was generated by the electrochemical reduction in methanol solution. They showed the ESR spectrum of SK semiquinone radical. And they speculated that the one electron reduction process occurring between $O_2^{\cdot-}$ and SK was coupled with the $O_2^{\cdot-}$ scavenging behavior of SK. In the present study, it was confirmed that SK was converted to the SK semiquinone radical in the HPX-XOD reaction system. We considered that the reaction was due to the one electron reduction of SK by $O_2^{\cdot-}$ generated in the HPX-XOD reaction system. Therefore, the reaction between $O_2^{\cdot-}$ and DMPO was inhibited by SK (or AK), indicating a competitive reaction between DMPO and SK (or AK) for $O_2^{\cdot-}$. These results suggested that the scavenging activities of SK and AK for $O_2^{\cdot-}$ was not produced by the inhibition of the $O_2^{\cdot-}$ generation system, but produced by the competitive reaction between DMPO and SK (or AK) for $O_2^{\cdot-}$.

Table 1
ID_{50} values, rate constants (k) and SOD-like activity values of SK and AK

	ID_{50} (mol/l)	k ((mol/l)$^{-1}$s^{-1})	SOD-like activity (U/mg)
SK	7.2×10^{-6}	1.4×10^{6}	920
AK	5.5×10^{-6}	1.9×10^{6}	1200

On the other hand, SOD was a catalyst for decomposition from $O_2^{\cdot-}$ to hydrogen peroxide and molecular oxygen (McCord and Fridovich, 1969). Therefore, the mechanisms underlying the scavenging activities of SK and SOD for $O_2^{\cdot-}$ might be different. In this experiment, however, the $O_2^{\cdot-}$ scavenging activity of SK was converted to the activity of SOD in order to estimate the relative $O_2^{\cdot-}$ scavenging activity of SK to SOD.

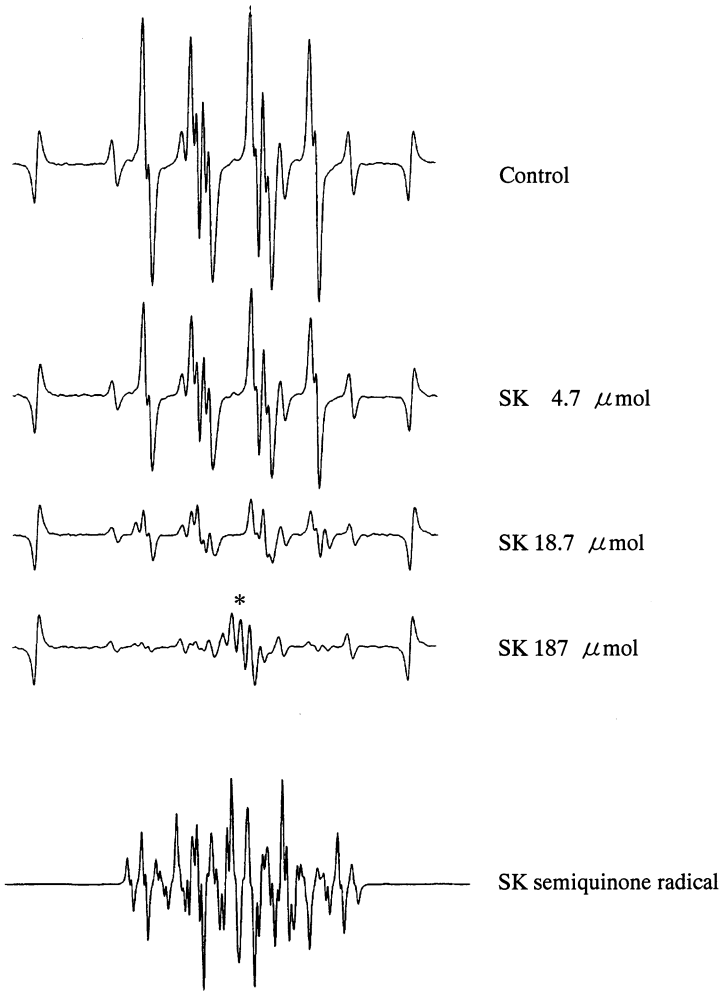


Fig. 5. The ESR spectra of DMPO-O_2^- observed upon the addition of SK and the ESR spectrum of the SK semiquinone radical.

4. Conclusions

SK and its optical isomer AK exhibited the same level of $O_2^{\cdot-}$ scavenging activity, which was more potent than the activity of L-ascorbic acid. The $O_2^{\cdot-}$ scavenging activity of SK was considered to play an important role in the wound healing enhancing and in the anti-inflammatory effect of SK. SK was reduced to SK semiquinone radical by $O_2^{\cdot-}$. SK semiquinone radical may be related to the anti-tumor and anti-bacterial effects of SK. Thus, the $O_2^{\cdot-}$ scavenging activity and semiquinone radical formation play important roles in the various pharmacological effects of SK.

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