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Comparison of superoxide detection abilities of newly developed spin traps in the living cells

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Abstract

This study compared the superoxide detection abilities of four spin traps, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO), 5-(diphenylphosphinoyl)-5-methyl-1pyrroline *N*-oxide (DPPMPO) and 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO) in living cells. Electron spin resonance (ESR) signals of the superoxide adducts were observed when spin traps were added to a suspension of human oral polymorphonuclear leukocytes (OPMNs) stimulated by phorbol 12-myristate 13-acetate. The ESR signal of the CYPMPO-superoxide adduct (CYPMPO-OOH) increased for 24 min after the initiation of the reaction, whereas the signals from DMPO-OOH and DPPMPO-OOH peaked at 6 and 10 min, respectively. The maximum concentrations of DMPO-OOH, DPPMPO-OOH and CYPMPO-OOH in OPMNs were 1.9, 6.0 and 10.7 μM, respectively. Furthermore, CYPMPO could more efficiently trap superoxide in blood PMNs compared with DEPMPO. From these results, it was concluded that CYPMPO performs better than DMPO, DPPMPO and DEPMPO for superoxide measurements in living cell systems because it has lower cytotoxicity and its superoxide adduct has a longer lifetime.

Keywords: *CYPMPO, diphenyl-PMPO, electron spin resonance, superoxide, spin trapping, polymorphonuclear leukocytes*

Abbreviations: *BPMN, blood polymorphonuclear leukocyte; CYPMPO, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline N-oxide; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DMSO, dimethyl sulphoxide; DPPMPO, 5-(diphenylphosphinoyl)-5-methyl-1-pyrrole N-oxide; DTPA, diethylenetriamine-N,N,N′,N″,N″-pentaacetic acid; ESR, electron spin resonance; HPX, hypoxanthine; OPMN, oral polymorphonuclear leukocyte; ROS, reactive oxygen species; SOD, superoxide dismutase; XOD, xanthine oxidase*

Introduction

Reactive oxygen species (ROS) such as superoxide and hydroxyl radical readily react with biomolecules in living cells and their excessive production is harmful to the living body. Although superoxide is not highly reactive, it is a precursor of the highly reactive hydroxyl radical, which is formed via the Fenton or Haber-Weiss reaction following dismutation of the superoxide into hydrogen peroxide [1]. Scavenging of hydroxyl radicals by exogenously administered antioxidants is very difficult, since hydroxyl radical immediately reacts with the compounds of the living body such as lipids, proteins and DNA [2]. On the other hand, scavenging of superoxide before formation of the hydroxyl radical would be possible since the reactivity of superoxide is much lower than that of the hydroxyl radical and it would be effective in preventing oxidative damage by ROS. Therefore, the superoxide scavenging activity of

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natural and synthetic antioxidants has been evaluated in numerous studies [3–9].

There are several methods of detecting superoxide, of which spin trapping coupled with electron spin resonance (ESR) is very useful for selective detection of oxygen radicals including superoxide. In this method, short-lived oxygen radicals can react with a spin trapping agent to form relatively stable spin adducts, which contain nitroxide radicals in most cases, and the adducts are then detected by an ESR spectrometer [10,11]. Among the various spin trapping agents, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO, Figure 1) is frequently used for detecting hydroxyl radicals and superoxides [12]. However, DMPO has disadvantages when it comes to measuring superoxide, because of its slow reaction rate with superoxide (reaction rate constant: 15.7 M^{-1} s^{-1}) and the poor stability of the DMPO-superoxide spin adduct (DMPO-OOH, half life: 1 min) [13,14]. To overcome these drawbacks, several analogues of DMPO were synthesized [15–22]. Recently, two novel spin traps that possess high reactivity with superoxide and spin adduct stability were synthesized [23,24] and used in cell-free systems [25–27]. These spin traps are 5-(diphenylphosphinoyl)-5-methyl-1-pyrroline *N*-oxide (DPPMPO) and 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO). The second-order reaction rate constants of DPPMPO and CYPMPO with superoxide are 39.5 and 48 M^{-1} s^{-1} and half-lives of their superoxide adducts are 8 and 15 min, respectively [23,24]. Based on these properties, it is expected that superoxide generation can be monitored more efficiently by using DPPMPO and CYPMPO as compared with DMPO. However, the results were obtained from cell-free superoxide generation systems. Living cells contain antioxidants, antioxidative enzymes, reducing substances and reducing enzymes and these substances would affect superoxide detection using spin traps. In particular, it is well known that reaction with reductants reduces the ESR signal of nitroxide radicals [28–30]. Furthermore, it is reported that several spin traps have cytotoxicity [31,32]. Therefore, it is necessary to evaluate the abilities of DPPMPO and CYPMPO in detecting superoxide in living cells for bioapplications. In this study, we investigated the stability of spin adducts in the presence of reductants and the cytotoxicity of DPPMPO and CYPMPO. We also compared the trapping abilities of four spin traps (DMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO), DPPMPO and CYPMPO) in model systems of superoxide generating cells, namely human oral and blood polymorphonuclear leukocytes (OPMNs and BPMNs).

Materials and methods

Reagents

DPPMPO (Diphenyl-PMPO), diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and a cell counting kit-8 (CCK-8) were obtained from Dojindo Laboratories (Kumamoto, Japan). CYPMPO was synthesized according to the protocol described in the previous report [24]. DMPO and xanthine oxidase (XOD, obtained from cow milk) were purchased from Labotec (Tokyo, Japan). Dimethyl sulfoxide (DMSO), ascorbic acid, glutathione (reduced form, GSH) and phorbol 12-myristate 13-acetate (PMA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Superoxide dismutase (SOD, obtained from bovine erythrocytes) and hypoxanthine (HPX) were purchased from Sigma-Aldrich (St. Louis, MO). DEPMPO was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). All other reagents used were of analytical grade.

Preparation of OPMNs

The OPMNs were collected from healthy volunteers according to the procedures of other reports [33–35] with a small modification. Briefly, 1 h after brushing the teeth, the volunteers thoroughly rinsed their oral cavity for 30 s with 20 mL of physiological saline six times. The collected suspension was centrifuged at 300 *g* for 10 min after passing it through a nylon net filter (NY30, Millipore, Bedford, MA). The pellets were suspended in 1 mL Hank's balanced salt solution (HBSS). OPMNs were counted and prepared with HBSS at 1×10^6 cells/mL.

BPMNs were isolated from heparinized blood of healthy volunteers using Mono-poly resolving medium (DS Pharma Biomedical Co., Ltd., Osaka, Japan). Heparinized blood (3.5 mL) was layered on

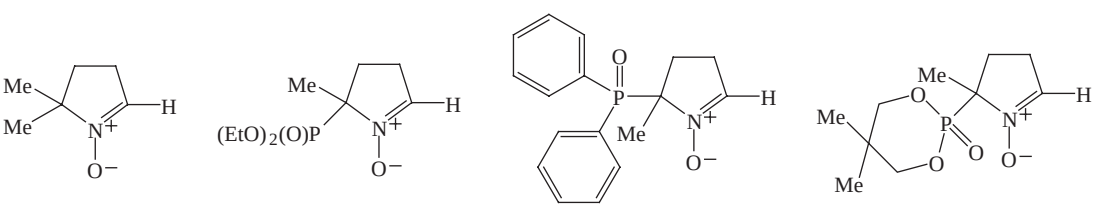

Figure 1. Structures of DMPO, DEPMPO, DPPMPO and CYPMPO

the resolving medium (3.0 mL) and centrifuged at 400 *g* for 20 min. After centrifugation, the layer of PMNs was collected, and washed in HBSS. BPMNs were counted and prepared with HBSS at 1×10^6 cells/mL. The Ethics Committee of Tohoku University approved this study's protocol.

Measurements of superoxide and hydroxyl radical in cell-free system

For the superoxide measurements, 50 μL of 2 mM HPX, 20 μL of a spin trapping agent, 30 μL of DMSO (or 20 mM DTPA), 50 μL of phosphate buffer and 50 μL of 0.4 U/mL XOD were mixed in a test tube. The mixture was transferred to an ESR quartz flat cell (Labotec) and ESR spectra were recorded after 1 min of reaction at room temperature. For the hydroxyl radical measurements, a test tube containing a 1 mL solution of spin trapping agent was irradiated with 1.0 MHz ultrasound for 1 min at 30°C. The reaction mixture was then transferred to an ESR quartz flat cell and the ESR spectra were immediately recorded.

Measurements of reactive oxygen species generated by PMNs

We prepared 2.23 M DMPO, 200 mM DEPMPO and 200 mM CYPMPO solutions in pure water. The 1.0 M DPPMPO solution was prepared in DMSO and diluted to the appropriate concentration with pure water in each experiment. We mixed 16 μL of spin trapping agent, 16 μL of 2 mM DTPA, 8 μL of 10 μg/mL PMA and 120 μL of 1×10^6 cells/mL PMNs in a test tube. The mixture was transferred to an ESR quartz flat cell and the ESR spectra were measured at room temperature.

The ESR spectra were recorded with a JES FA-100 spectrometer (JEOL, Tokyo, Japan). The conditions of the measurements were as follows: magnetic field, 335.5 ± 5 mT or 335.75 ± 7.5 mT; field modulation frequency, 100 kHz; field modulation width, 0.1 mT; time constant, 0.1 s; sweep time, 2 min; and microwave power, 8 mW. The concentration of spin adducts was estimated from the ESR signal intensity by using a calibration curve obtained from 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl.

Evaluation of cytotoxicity of spin trapping agents

Cell survivals were assessed 2 h after the addition of spin trapping agents by using CCK-8, according to the manufacturer's instructions; 8 μL of HBSS, 12 μL of a spin trapping agent and 90 μL of 1×10^6 cells/mL OPMNs were placed in each well of a 96-well plate and 10 μL of CCK-8 solution was added into each mixture. After 2 h of reaction at room temperature, the absorbance at 450 nm was measured using a DTX 880 multimode detector (Beckman Coulter, Tokyo, Japan) with a reference absorbance at 620 nm.

Results

Decrease in ESR signals of spin adducts in the presence of reductant

Figure 2A shows representative ESR spectra of DPPMPO-superoxide (DPPMPO-OOH) and hydroxyl radical (DPPMPO-OH) adducts obtained from the HPX/XOD superoxide generation system and ultrasound irradiation of water, respectively.

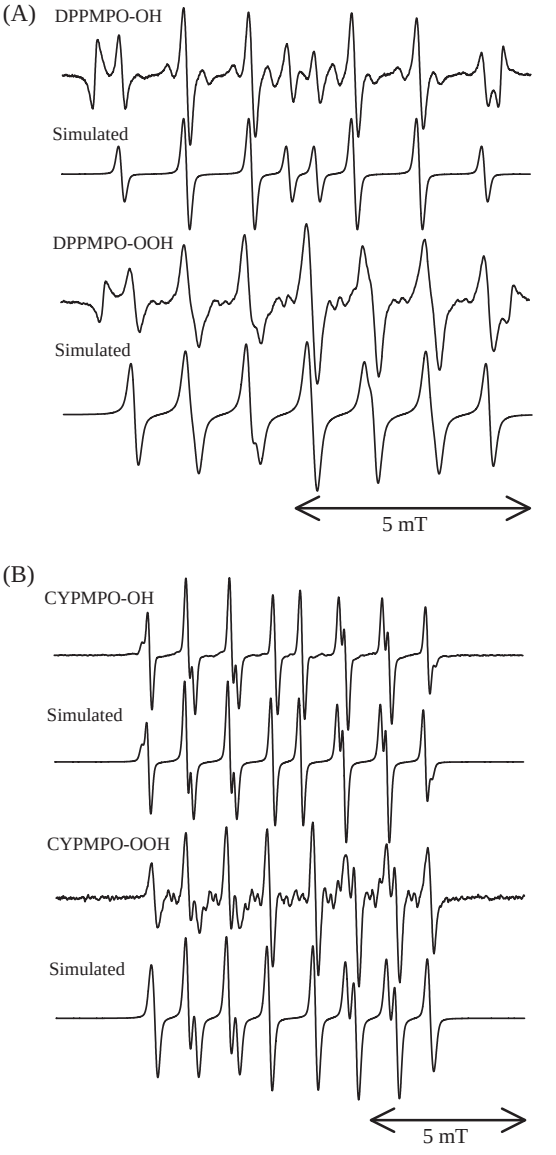

Figure 2. (A) ESR spectra of DPPMPO-OH obtained from the DPPMPO solution irradiated with ultrasound and DPPMPO-OOH from the HPX/XOD superoxide generation system and corresponding simulated spectra. The signals on both sides are of manganese oxide, which is an external standard. Computer simulations were performed by using hfcc listed in Table I. (B) ESR spectra of CYPMPO-OH and CYPMPO-OOH measured in the same reaction system as DPPMPO.

Figure 2B shows representative ESR spectra of CYPMPO-superoxide (CYPMPO-OOH) and hydroxyl radical (CYPMPO-OH) adducts obtained from the same systems as DPPMPO. CYPMPO-OOH and CYPMPO-OH are clearly identified by their centre signals since these signals do not overlap. The computer simulated spectra of each adduct, which were made by using hyperfine coupling constants (hfcc) summarized in Table I, are also shown in Figure 2.

We evaluated the stability of spin adducts of DMPO, DEPMPO, DPPMPO and CYPMPO in the presence of reductants. Figure 3 shows the changes in the peak height of the ESR signals of hydroxyl radical adducts after addition of ascorbic acid or GSH. The hydroxyl radical adducts were generated by irradiation of the spin trap solution with 1.0 MHz ultrasound and 5–10 μM hydroxyl radical adducts were obtained in each spin trap. Ascorbic acid readily reacted with the hydroxyl radical adducts of the four spin traps and decreased their ESR signals, whereas GSH didn't affect the stability of the hydroxyl radical adducts (Figure 3). DMPO-OH, DEPMPO-OH and CYPMPO-OH were more stable than DPPMPO-OH in the absence of a reductant. However, the half-life of DMPO-OH, DEPMPO-OH and CYPMPO-OH decreased from 57.5, 43.8 and 44.8 min to 7.3, 3.5 and 3.2 min, respectively, by adding 10 μM ascorbic acid (Table II). Superoxide adducts also decreased by adding a reductant (Figure 4). Although the half-lives of CYPMPO-OOH (30.4 min) and DEPMPO-OOH (24.4 min) were much longer than those of DMPO-OOH (1.3 min) and DPPMPO-OOH (6.7 min) in the absence of a reductant, they were decreased by the reaction with ascorbic acid and GSH. The half-life of CYPMPO-OOH became 2.4 and 5.0 min in the presence of 10 μM ascorbic acid and 100 μM GSH, respectively. Indeed, when GSH was added to the superoxide adducts of these four spin traps, the ESR signal of the superoxide adducts decreased and an ESR signal of hydroxyl radical adducts appeared (data not shown). We couldn't evaluate the half-life of DPPMPO-OOH in the presence of GSH, since the ESR signal of DPPMPO-OH and DPPMPO-OOH overlapped.

Table I. Hyperfine coupling constants of superoxide and hydroxyl radical adducts of DPPMPO and CYPMPO.

		a_N	a_H	a_P	
DPPMPO-OOH	A*	1.31	1.13	3.82	65%
	B	1.26	1.20	3.87	35%
DPPMPO-OH		1.39	1.39	3.58	
CYPMPO-OOH	A	1.31	1.04	5.10	65%
	B	1.29	1.15	5.24	35%
CYPMPO-OH	A	1.38	1.22	4.88	80%
	B	1.38	1.38	5.04	20%

*A and B are diastereomer.

ESR spectra from OPMN system with DPPMPO and CYPMPO

We used DPPMPO and CYPMPO for the detection of ROS generated in PMA-stimulated OPMNs. Figure 5 plots the ESR spectra obtained from the PMA-stimulated OPMNs suspension. These spectra have the same hfcc as DPPMPO-OOH and CYPMPO-OOH that were observed in the HPX/XOD superoxide generation system listed in Table I. The signal intensities of DPPMPO-OOH and CYPMPO-OOH in Figure 5 (0 SOD) were almost the same. The ESR signals decreased by adding SOD dependent on the concentration and they completely disappeared when 10 U/mL SOD was added. These results indicate that the superoxide adduct mainly contributed to the ESR spectra from the PMA-stimulated OPMNs system and that other radicals such as hydroxyl and carbon-centred radicals exerted little effect on the spectra.

Cytotoxicity of spin traps

The signal from the OPMNs system when 12.5 mM DPPMPO was used was much larger than the one observed when 6.25 mM DPPMPO used. However, the ESR signal decreased with DPPMPO concentrations higher than 12.5 mM and the signal was very small when 100 mM DPPMPO was used (Figure 6). These results indicate the possibility that DPPMPO has cytotoxic effects on OPMNs at these concentrations. Therefore, we evaluated the cytotoxicity of the spin traps. As shown in Figure 7, the OPMNs survival rate decreased with increasing concentration of DPPMPO. The cytotoxic effect was observed even when 12.5 mM DPPMPO was used and 100 mM DPPMPO decreased the survival rate of OPMNs by 55%. On the other hand, cytotoxicity was not observed at concentrations of 10, 20 and 33.3 mM CYPMPO and 223 mM DMPO, which are the concentrations used for superoxide detection in this study. Cytotoxicity of DEPMPO couldn't be estimated, since the absorbance at 450 nm of the mixture with DEPMPO (10, 20, 33.3 mM) became ~1.6-times higher than that of the control after 2 h of incubation (data not shown). The cytotoxic effect was also observed when 10% DMSO, which is present in the 100 mM DPPMPO solution, was used.

Concentrations of superoxide adducts of DMPO, DEPMPO, DPPMPO and CYPMPO in the PMA-stimulated PMNs system

First, we evaluated superoxide generation from PMA-stimulated OPMNs with three kinds of spin traps, namely, DMPO (223 mM), DPPMPO (12.5 mM) and CYPMPO (20 mM). DMPO is usually used at a higher concentration for superoxide detection since it has low reactivity with superoxide.

Therefore, we used DMPO at 223 mM for efficient trapping of superoxide. From the results shown in Figure 6, we chose to use 12.5 mM of DPPMPO, the concentration at which the highest ESR signal was obtained, even though it had some cytotoxic effect on OPMNs. CYPMPO was used at 20 mM. It has no cytotoxicity for OPMNs (Figure 7) and it efficiently trapped superoxide in the HPX/XOD and OPMNs systems (Figures 2 and 5).

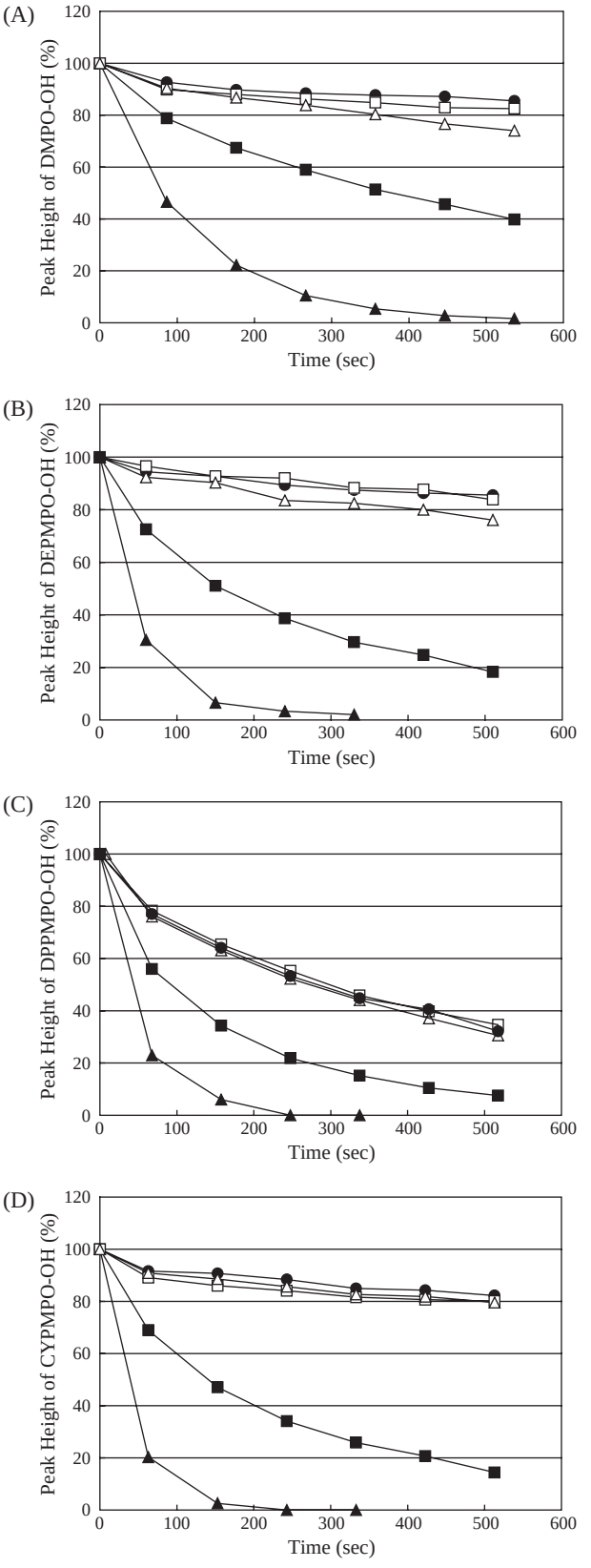

Figure 3 (*Continued*)

Table II. Half-life (min) of hydroxyl radical- and superoxide-spin adducts of four spin traps in the absence and presence of reductants.

		DMPO	DEMPO	DPPMPO	CYPMPO
$^{\cdot}OH$	Non	57.5	43.8	6.2	44.8
	GSH	54.2	42.9	6.3	41.3
	Asc	7.3	3.5	2.4	3.2
$O_2^{\cdot-}$	Non	1.3	24.4	6.7	30.4
	GSH	1.2	5.1		5.0
	Asc	0.9	2.1	1.9	2.4

The concentrations of the superoxide adduct of each spin trap are plotted in Figure 8A. When DMPO was used, its superoxide adduct (DMPO-OOH) increased for 6 min after the beginning of the reaction and then gradually decreased. The maximum concentration of DMPO-OOH was 1.9 μM. When DPPMPO was used instead of DMPO, the concentration of DPPMPO-OOH was greater than that of DMPO-OOH. The maximum concentration of DPPMPO-OOH was 6.0 μM at 10 min. Furthermore, when CYPMPO was used, the concentration of CYPMPO-OOH was almost the same as that of DPPMPO during the first few minutes and it increased for 24 min. The concentration of CYPMPO-OOH was greater than 10 μM after a reaction of 18 min.

Next, the superoxide detection abilities of CYPMPO and DEPMPO were compared by using PMA-stimulated BPMNs. CYPMPO and DEPMPO were of the same concentration (20 mM) in the experiments. The concentration of CYPMPO-OOH detected in BPMNs was lower than in OPMNs during first 10 min, but it increased over the course of 35 min and was 29.4 μM at 38 min (Figure 8B). DEPMPO-OOH also increased over the course of 35 min. However, its concentration was significantly lower than that of CYPMPO-OOH.

Discussion

Although DMPO is frequently used for detecting oxygen radicals in biological systems, it has disadvantages in measuring superoxide. Many spin

Figure 3. Peak heights of (A) DMPO-OH, (B) DEPMPO-OH, (C) DPPMPO-OH, (D) CYPMPO-OH in the absence (●) and presence of ascorbic acid and GSH. The spin trap solution was irradiated with 1.0 MHz ultrasound for 1 min and then ascorbic acid (10 μM (■), 50 μM (▲)) or GSH (100 μM (□), 500 μM (△)) was added. The peak heights of ESR signals were measured after adding a reductant. Each value represents the mean of duplicate measurements.

traps that can trap superoxide more quickly and form more stable adducts than DMPO were synthesized as possible alternatives to DMPO [15–22]. Of these, DEPMPO is a good spin trap and it has been used for ROS measurements in various ROS generation systems [36–39]. CYPMPO has properties (reaction rate with superoxide, half-life of superoxide adduct) similar to those of DEPMPO [24]; therefore, it is thought that the CYPMPO would give similar results to those of DEPMPO. In our results, the stabilities of the superoxide adduct and the hydroxyl radical adduct of CYPMPO were almost the same as that of DEPMPO in the presence and absence of ascorbic acid and GSH, except that the half-life of CYPMPO-OOH was a little longer than that of DEPMPO-OOH in the absence of a reductant. In the measurements using PMA-stimulated BPMNs, CYPMPO could detect superoxide more efficiently than DEPMPO

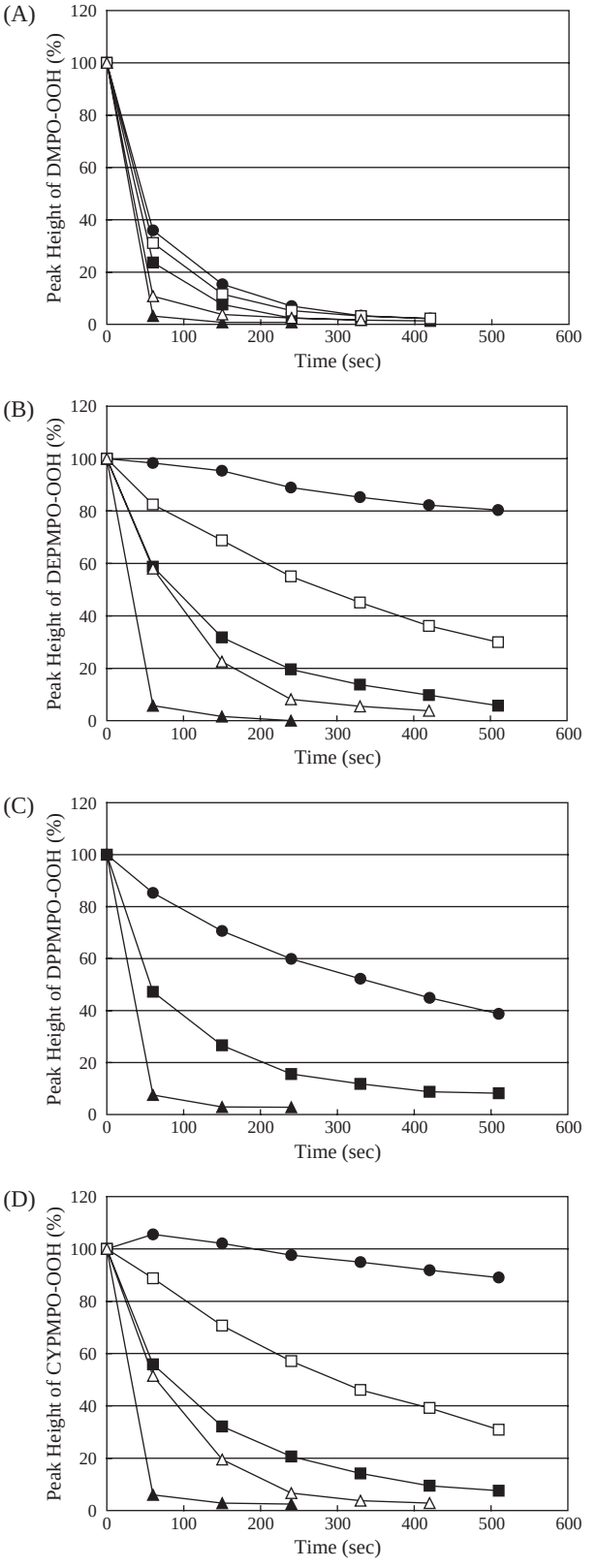

Figure 4 (*Continued*)

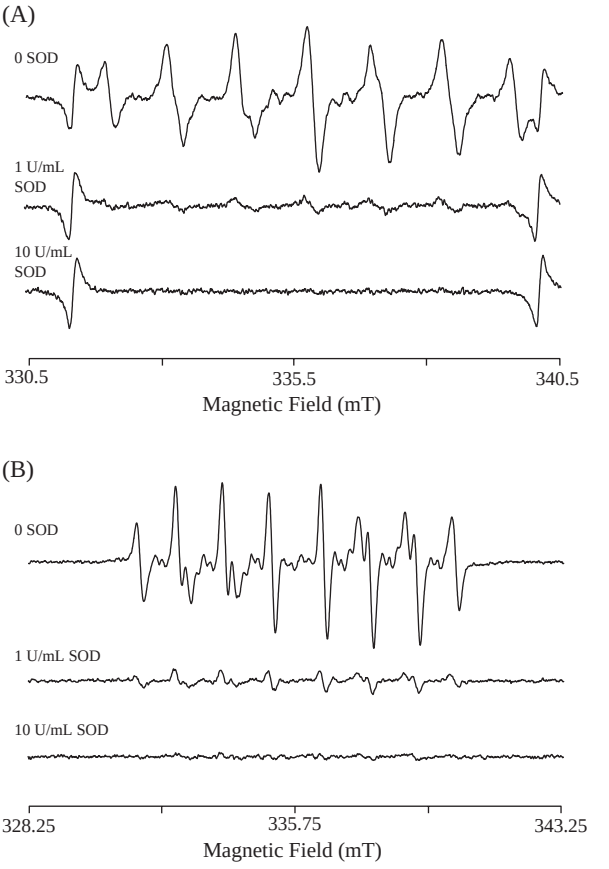

Figure 5. (A) ESR spectra from a mixture of OPMNs (7.5×10^5 cells/mL), PMA (0.5 μg/mL), DTPA (0.2 mM) and DPPMPO (12.5 mM) in the absence and presence of SOD (1, 10 U/mL). The spectra were recorded after 5 min reaction at room temperature. The signals on both sides are of manganese oxide, which is an external standard. (B) Same as (A) except CYPMPO (20 mM) was added instead of DPPMPO.

Figure 4. Peak heights of (A) DMPO-OOH, (B) DEPMPO-OOH, (C) DPPMPO-OOH, (D) CYPMPO-OOH in the absence (●) and presence of ascorbic acid and GSH. Superoxide adducts were formed by the 1 min reaction in HPX/XOD system. Ascorbic acid (10 μM (■), 50 μM (▲)) or GSH (100 μM (□), 500 μM (△)) was added after stopping superoxide generation by allopurinol (2 mM) and the peak heights of the ESR signals were measured. Each value represents the mean of duplicate measurements.

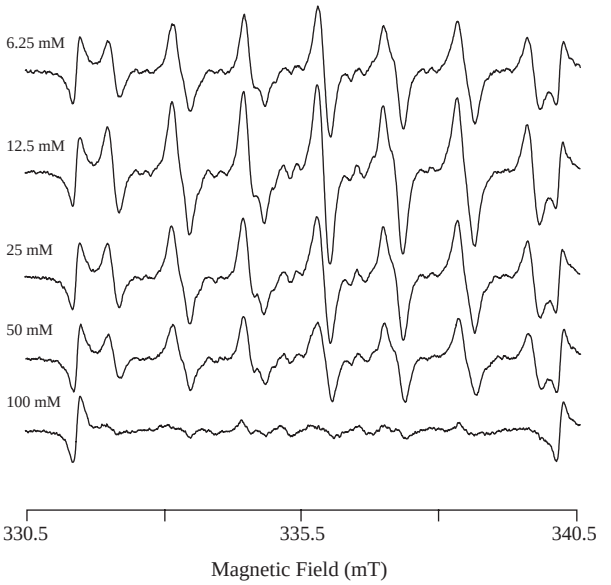

Figure 6. ESR spectra from a mixture of OPMNs (7.5×10^5 cells/mL), PMA (0.5 μg/mL), DTPA (0.2 mM) and DPPMPO (6.25, 12.5, 25, 50 and 100 mM). The signals on both sides are of manganese oxide, which is an external standard.

(Figure 8B). This may be attributed to the faster (1.5-times) reaction rate of CYPMPO with superoxide compared with DEPMPO [24]. Furthermore, the differences in half-life between CYPMPO-OOH (30.4 min) and DEPMPO-OOH (24.4 min) in the absence of reductants may contribute to the difference in spin concentrations of CYPMPO-OOH and DEPMPO-OOH detected in the BPMNs system. We also think that CYPMPO is advantageous over DEPMPO with respect to ease of handling and purification, as suggested in the previous report [24].

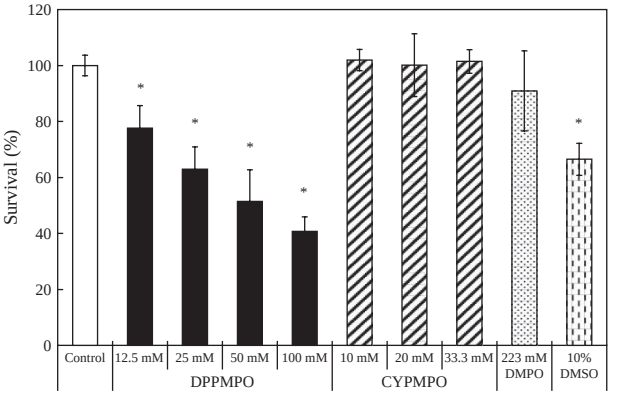

Figure 7. Cell survival after adding spin traps. DPPMPO (12.5, 25, 50, 100 mM), CYPMPO (10, 20 and 33.3 mM), DMPO (223 mM) or DMSO (10%) were mixed with OPMNs (7.5×10^5 cells/mL) and the cell counting kit-8 solution was added to the mixtures. The control contained pure water instead of spin traps. The absorbance at 450 nm and 620 nm were measured after 2 h of reaction at room temperature. Cell survivals were estimated with the absorbance differences between 450 nm and 620 nm and are represented by the means ± SD of three experiments. $^\star p < 0.05$ as compared with control.

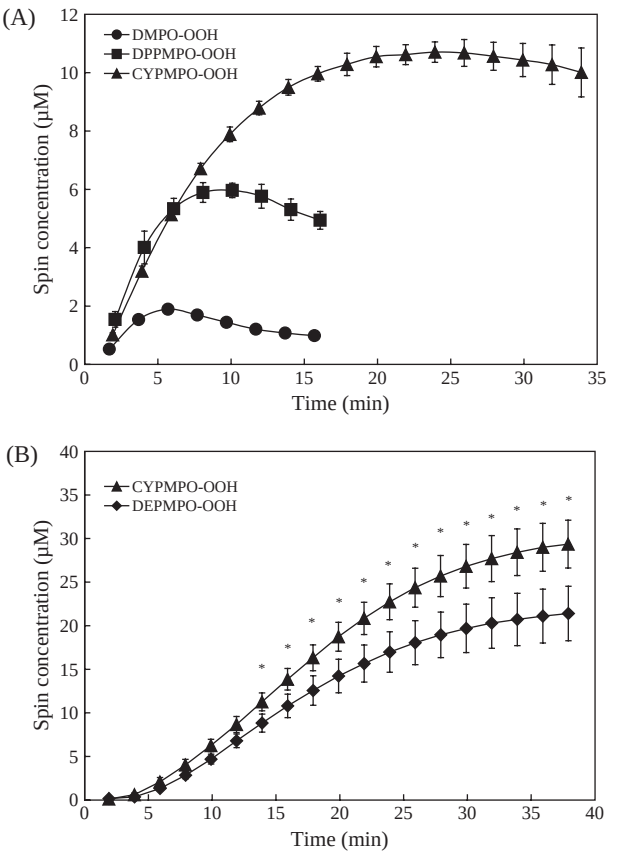

Figure 8. (A) Differences between DMPO, DPPMPO and CYPMPO in detecting superoxide in OPMNs. The spin trap (223 mM DMPO (●), 12.5 mM DPPMPO (■) or 20 mM CYPMPO (▲)) was mixed with DTPA (0.2 mM), PMA (0.5 μg/mL) and OPMNs (7.5×10^5 cells/mL). (B) Differences between DEPMPO and CYPMPO in detecting superoxide in BPMNs. DEPMPO (20 mM, ◆) or CYPMPO (20 mM, ▲) was mixed with DTPA (0.2 mM), PMA (0.5 μg/mL) and BPMNs (7.5×10^5 cells/mL). ESR spectra were recorded at 2 min intervals during the reaction at room temperature and the concentration of DMPO-OOH, DPPMPO-OOH and CYPMPO-OOH were estimated from the ESR signal intensities. Values are the means ± SD of three measurements. $^\star p < 0.05$ between CYPMPO-OOH and DEPMPO-OOH.

The ESR signal of CYPMPO-OOH increased until 24 min in the PMA-stimulated OPMNs system. This indicates that superoxide was generated for at least 24 min. In contrast, the ESR signals of DMPO-OOH and DPPMPO-OOH start to decrease at 6 and 10 min, respectively. Furthermore, the DMPO-OOH signal was much smaller than the DPPMPO-OOH and CYPMPO-OOH signals even during the first few minutes of the reaction. These results suggest that the estimated superoxide concentration is less than the actual concentration when DMPO is used. Although DMPO has a lower reaction rate constant with superoxide than those of CYPMPO and DPPMPO, it was used at a considerably higher (greater than 10-times) concentration than CYPMPO and DPPMPO in this study. Therefore, superoxide would be efficiently trapped by DMPO in the same way as CYPMPO and DPPMPO. The lower concentration of DMPO-OOH is probably

attributed to its low stability. DMPO-OOH may decay during the first few minutes, since its half-life is 1.3 min, even in the absence of a reductant (Table II). The concentration of DPPMPO-OOH was almost the same as that of CYPMPO-OOH up to 6 min, but it decreased after 10 min of reaction, whereas the concentration of CYPMPO-OOH continued to increase. We consider that this difference in the superoxide adduct concentration might also be due to the different stabilities of DPPMPO-OOH and CYPMPO-OOH.

As described above, when GSH was added to the superoxide adducts, the ESR signal from them decreased and an ESR signal from hydroxyl radical adducts appeared. This phenomenon was observed in all four spin traps. It is known that the glutathionyl radical adduct of DMPO gives an ESR signal similar to that of the hydroxyl radical adduct [12]. However, the ESR signals observed here were identified as hydroxyl radical adducts and not glutathionyl adducts from their hyperfine coupling constants. It was reported that DMPO-OH is generated from DMPO-OOH by the reaction with GSH [40]. Our results suggest that the same reaction as DMPO can occur with DEPMPO, DPPMPO and CYPMPO.

When the concentration of the spin trapping agent increased, the trapping efficiency for radicals increased. Consequently, the ESR signal should also increase. However, the ESR signal of DPPMPO-OOH decreased when the concentration of DPPMPO increased (Figure 7). It is known that certain nitrone-type spin traps have cytotoxic effects at various concentrations [31,32]. We believe that the cytotoxicity of DPPMPO at high concentrations is responsible for the decrease in DPPMPO-OOH. This expectation was clearly realized in the measurements of cell survivals (Figure 8). Even a low concentration (12.5 mM) of DPPMPO affected cell survival, whereas 33.3 mM CYPMPO and 223 mM DMPO did not affect it at all. Furthermore, the DPPMPO solution was prepared in DMSO since it is not very soluble in water. High DMSO concentrations present in higher DPPMPO concentrations may also affect cell survival. Therefore, a high concentration of DPPMPO is not suitable for experiments with living cells, since both DPPMPO and the solvent DMSO adversely influence cell survival. The mechanism of the cytotoxicity of DPPMPO has yet to be elucidated. However, some reports have indicated that lipophilic spin traps such as 5-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide are more toxic than other spin traps such as DMPO and DEPMPO [31,32]. DPPMPO is lipophilic compared with DMPO and CYPMPO [23,41]. Therefore, the effects on the cell membrane may be responsible for the cytotoxicity of these lipophilic spin traps.

In this study, we evaluated the stabilities of superoxide and hydroxyl radical adducts of four spin traps in the presence of ascorbic acid and GSH. It was revealed that CYPMPO-OOH and DEPMPO-OOH are more stable than DMPO-OOH and DPPMPO-OOH in the absence of a reductant, but their stabilities decreased rapidly in the presence of ascorbic acid and GSH. We applied these spin traps to measurements of superoxide in a living cell system. DEPMPO, DPPMPO and CYPMPO could detect superoxide more efficiently than DMPO. In particular, CYPMPO gave a larger ESR signal and a more stable superoxide adduct compared with other three spin traps in the experiments using PMNs. Furthermore, no cytotoxicity was observed with CYPMPO at the concentration used in this study. From these results, we conclude that (1) DEPMPO, DPPMPO and CYPMPO perform better than DMPO because their superoxide adducts have a longer lifetime than DMPO's and (2) CYPMPO is better than DPPMPO because it has lower cytotoxicity; i.e. in the presence of CYPMPO, live PMNs continue to produce superoxide. Furthermore, the results shown in Figure 8B indicate that CYPMPO can detect superoxide in living cells as well as or better than DEPMPO. Therefore, we believe that superoxide generation in living cells can be more precisely monitored by using CYPMPO rather than DMPO, DEPMPO or DPPMPO. However, it is necessary to consider the existence of reductants and the reduction of spin adducts in living cells when we interpret the results.

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References

[1] Halliwell B, Gutteridge JMC. The chemistry of free radicals and related 'reactive species'. In: B Halliwell and JMC Gutteridge, editors. Free radicals in biology and medicine. 3rd ed. New York: Oxford University Press; 1999. p 36–104.

[2] Halliwell B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. Free Radic Res 1992;25:57–74.

[3] Sichel G, Corsaro C, Scalia M, Di Bilio AJ, Bonomo RP. *In vitro* scavenger activity of some flavonoids and melanins against $O_2^{-(\cdot)}$. Free Radic Biol Med 1991;11:1–8.

[4] Cotelle N, Bernier JL, Henichart JP, Catteau JP, Gaydou E, Wallet JC. Scavenger and antioxidant properties of ten synthetic flavones. Free Radic Biol Med 1992;13:211–219.

[5] Guo Q, Zhao BL, Shen SR, Hou JW, Hu JG, Xin WJ. ESR study on the structure-antioxidant activity relationship of tea catechins and their epimers. Biochim Biophys Acta 1999;1427:13–23.

[6] Wood JE, Senthilmohan ST, Peskin AV. Antioxidant activity of procyanidin-containing plant extracts at different pHs. Food Chem 2002;77:155–161.

[7] Sato E, Kohno M, Hamano H, Niwano Y. Increased antioxidative potency of garlic by spontaneous short-term fermentation. Plant Foods Hum Nutr 2006;61:157–160.

[8] Saito K, Kohno M, Yoshizaki F, Niwano Y. Extensive screening for edible herbal extracts with potent scavenging

activity against superoxide anions. Plant Foods Hum Nutr 2008;63:65–70.
[9] Saito K, Kohno M, Yoshizaki F, Niwano Y. Antioxidant properties of herbal extracts selected from screening for potent scavenging activity against superoxide anions. J Sci Food Agric 2008;88:2707–2712.
[10] Iwamura M, Inamoto N. Reactions of nitrones with free radicals. II. Formation of nitroxides. Bull Chem Soc Jpn 1970;43:860–863.
[11] Janzen EG. Spin trapping. Acc Chem Res 1971;4:31–40.
[12] Buettner GR. Spin trapping: ESR parameters of spin adducts. Free Radic Biol Med 1987;3:259–303.
[13] Buettner GR, Oberley LW. Considerations in the spin trapping of superoxide and hydroxyl radical in aqueous systems using 5,5-dimethyl-1-pyrroline-1-oxide. Biochem Biophys Res Commun 1978;83:69–74.
[14] Finkelstein E, Rosen GM, Rauckman EJ. Spin trapping. Kinetics of the reaction of superoxide and hydroxyl radicals with nitrones. J Am Chem Soc 1980;102:4994–4999.
[15] Frejaville C, Karoui H, Tuccio B, Le Moigne F, Culcasi M, Pietri S, Lauricella R, Tordo P. 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline n-oxide: a new efficient phosphorylated nitrone for the *in vitro* and *in vivo* spin trapping of oxygen-centered radicals. J Med Chem 1995;38:258–265.
[16] Olive G, Mercier A, Le Moigne F, Rockenbauer A, Tordo P. 2-Ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide: evaluation of the spin trapping properties. Free Radic Biol Med 2000;28:403–408.
[17] Zhang H, Joseph J, Vasquez-Vivar J, Karoui H, Nsanzumuhire C, Martasek P, Tordo P, Kalyanaraman B. Detection of superoxide anion using an isotopically labeled nitrone spin trap: potential biological applications. FEBS Lett 2000;473:58–62.
[18] Zhao H, Joseph J, Zhang H, Karoui H, Kalyanaraman B. Synthesis and biochemical applications of a solid cyclic nitrone spin trap: a relatively superior trap for detecting superoxide anions and glutathiyl radicals. Free Radic Biol Med 2001;31:599–606.
[19] Chalier F, Tordo P. 5-Diisopropoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide, DIPPMPO, a crystalline analog of the nitrone DEPMPO: synthesis and spin trapping properties. J Chem Soc Perkin Trans 2002;2:2110–2117.
[20] Stolze K, Udilova N, Rosenau T, Hofinger A, Nohl H. Synthesis and characterization of EMPO-derived 5,5-disubstituted 1-pyrroline N-oxide as spin traps forming exceptionally stable superoxide spin adducts. Biol Chem 2003;384:493–500.
[21] Tsai P, Ichikawa K, Mailer C, Pou S, Halpern HJ, Robinson BH, Nielsen R, Rosen GM. Esters of 5-carboxyl-5-methyl-1-pyrroline N-oxide: a family of spin traps for superoxide. J Org Chem 2003;68:7811–7817.
[22] Villamena FA, Rockenbauer A, Gallucci J, Velayutham M, Hadad CM, Zweier JL. Spin trapping by 5-carbamoyl-5-methyl-1-pyrroline *N*-oxide (AMPO): theoretical and experimental studies. J Org Chem 2004;69:7994–8004.
[23] Nishizawa M, Shioji K, Kurauchi Y, Okuma K, Kohno M. Spin-trapping properties of 5-(diphenylphosphinoyl)-5-methyl-4,5-dihydro-3H-pyrrole N-oxide (DPPMDPO). Bull Chem Soc Jpn 2007;80:495–497.
[24] Kamibayashi M, Oowada S, Kameda H, Okada T, Inanami O, Ohta S, Ozawa T, Makino K, Kotake Y. Synthesis and characterization of a practically better DEPMPO-type spin trap, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline N-oxide (CYPMPO). Free Radic Res 2006;40:1166–1172.
[25] Shimamura T, Fujimura Y, Ukeda H. Electron spin resonance analysis of superoxide anion radical scavenging activity with spin trapping agent, Diphenyl-PMPO. Anal Sci 2007;23:1233–1235.
[26] Kobayashi HP, Watanabe T, Oowada S, Hirayama A, Nagase S, Kamibayashi M, Otsubo T. Effect of CV-159-Ca2+/calmodulin blockade on redox status hepatic ischemia-reperfusion injury in mice evaluated by a newly developed in vivo EPR imaging technique. J Surg Res 2008;147:41–49.
[27] Ogura A, Oowada S, Kon Y, Hirayama A, Yasui H, Meike S, Kobayashi S, Kuwabara M, Inanami O. Redox regulation in radiation-induced cytochrome c release from mitochondria of human lung carcinoma A549 cells. Can Lett 2009;277:64–71.
[28] Quintanilha AT, Packer L. Surface localization of sites of reduction of nitroxide spin-labeled molecules in mitochondria. Proc Natl Acad Sci USA 1977;74:570–574.
[29] Eriksson UG, Brasch RC, Tozer TN. Nonenzymatic bioreduction in rat liver and kidney of nitroxyl spin labels, potential contrast agents in magnetic resonance imaging. Drug Metab Dispos 1987;15:155–160.
[30] Iannone A, Bini A, Swartz HM, Tomasi A, Vannini V. Metabolism in rat liver microsomes of the nitroxide spin probe TEMPOL. Biochem Pharmacol 1989;38:2581–2586.
[31] Khan N, Wilmot CM, Rosen GM, Demidenko E, Sun J, Joseph J, O'Hara J, Kalyanaraman B, Swartz HM. Spin traps: *in vitro* toxicity and stability of radical adducts. Free Radic Biol Med 2003;34:1473–1481.
[32] Rohr-Udilova N, Stolze K, Marian B, Nohl H. Cytotoxicity of novel derivatives of the spin trap EMPO. Bioorg Med Chem Lett 2006;16:541–546.
[33] Ashkenazi M, Dennison DK. A new method for isolation of salivary neutrophils and determination of their functional activity. J Dent Res 1989;68:1256–1261.
[34] Al-Essa L, Niwa M, Kohno K, Tsurumi K. A proposal for purification of salivary polymorphonuclear leukocytes by combination of nylon mesh filtration and density-gradient method: a validation by superoxide- and cyclic AMP-generating responses. Life Sci 1994;55:PL333–PL338.
[35] Sato EF, Utsumi K, Inoue M. Human oral neutrophils: isolation and characterization. Methods Enzymol 1996;268:503–509.
[36] Roubaud V, Sankarapandi S, Kuppusamy P, Tordo P, Zweier JL. Quantitative measurement of superoxide generation using the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide. Anal Biochem 1997;247:404–411.
[37] Roubaud V, Sankarapandi S, Kuppusamy P, Tordo P, Zweier JL. Quantitative measurement of superoxide generation and oxygen consumption from leukocytes using electron paramagnetic resonance spectroscopy. Anal Biochem 1998;257:210–217.
[38] Timmins GS, Liu KJ, Bechara EJH, Kotake Y, Swartz HM. Trapping of free radicals with direct *in vivo* EPR detection: a comparison of 5,5-dimethyl-1-pyrroline-N-oxide and 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide as spin traps for $HO^{\cdot}$ and $SO_4^{\cdot-}$. Free Radic Biol Med 1999;27:329–333.
[39] Stocker P, Lesgards JF, Vidal N, Chalier F, Prost M. ESR study of a biological assay on whole blood: antioxidant efficiency of various vitamins. Biochim Biophys Acta 2003;1621:1–8.
[40] Jones CM, Lawrence A, Wardman P, Burkitt MJ. Electron paramagnetic resonance spin trapping investigation into the kinetics of glutathione oxidation by the superoxide radical: re-evaluation of the rate constant. Free Radic Biol Med 2002;10:982–990.
[41] Shioji K, Iwashita H, Shimomura T, Yamaguchi T, Okuma K. ESR measurement using 2-diphenylphosphinoyl-2-methyl-3,4-dihydro-2H-pyrrole N-oxide (DPhPMPO) in human erythrocyte ghosts. Bull Chem Soc Jpn 2007;80:758–762.

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