

Serum Hydroxyl Radical Scavenging Capacity as Quantified with Iron-Free Hydroxyl Radical Source

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Summary We have developed a simple ESR spin trapping based method for hydroxyl (OH) radical scavenging-capacity determination, using iron-free OH radical source. Instead of the widely used Fenton reaction, a short (typically 5 seconds) *in situ* UV-photolysis of a dilute hydrogen peroxide aqueous solution was employed to generate reproducible amounts of OH radicals. ESR spin trapping was applied to quantify OH radicals; the decrease in the OH radical level due to the specimen's scavenging activity was converted into the OH radical scavenging capacity (rate). The validity of the method was confirmed in pure antioxidants, and the agreement with the previous data was satisfactory. In the second half of this work, the new method was applied to the sera of chronic renal failure (CRF) patients. We show for the first time that after hemodialysis, OH radical scavenging capacity of the CRF serum was restored to the level of healthy control. This method is simple and rapid, and the low concentration hydrogen peroxide is the only chemical added to the system, that could eliminate the complexity of iron-involved Fenton reactions or the use of the pulse-radiolysis system.

Key Words: hydroxyl radical, antioxidant capacity, ESR spin trapping, hydrogen peroxide, photochemistry

Introduction

Because hydroxyl (OH) radicals are known to be ultimate injurious species in biological systems [1, 2], the ability to remove OH radicals is considered to be an important factor for homeostasis. Thus, serum OH radical scavenging activity could be an index for patho-physiological conditions. In OH radical scavenging assay, the selection of OH radical source has been limited and most studies employed the Fenton

reaction, *i.e.*; ferrous ion plus hydrogen peroxide. A majority of OH radical quantification methods are indirect and utilizes OH radical's ability to damage chromophores or fluorophores, resulting in the loss of color or fluorescence [3, 4]. In contrast, ESR spin trapping method directly measures OH radical level [5–7]. The decrease in OH radical level by the addition of the specimen with antioxidant activity can be readily converted into OH radical scavenging capacity. Nevertheless, the spin trapping data for serum OH radical scavenging activity have been scarce probably because of the lack of appropriate OH radical source.

Patho-physiological OH radical production is believed to be through the Fenton reaction-type process; therefore we

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speculate that the addition of exogenous iron for the purpose of OH radical generation would make the system more complicated. Tanigawa reported that in ESR spin trapping, when the Fenton reaction was employed as OH radical source, various factors such as ferric ion/hydrogen peroxide concentration, buffer, and chelating agent could affect the outcome [8]. Complex reaction mechanism in the Fenton reaction was suspected as a reason for these variations [9]; furthermore, the interaction between spin trapping agent and iron ion seems make the reaction system more complicated [10]. In summary, drawbacks of the use of the Fenton reaction as the OH radical source are: 1) the reaction is unstoppable and initiated as soon as ferrous ion and hydrogen peroxide are mixed; 2) OH radical yield depends on the sequence of the addition of reactants and the reaction time [8]; and 3) OH radical level could be decreased either by antioxidant's scavenging activity or inhibition of the Fenton reaction. Other drawbacks specific for spin trapping include: 1) ferric ion catalyzes the formation of OH radical adducts via non-free radical reactions [11]; and 2) the interference of the iron with the reaction between OH radical and antioxidant [10]. The use of iron-free OH radical generating system could eliminate most of these drawbacks.

We have developed a simple method of OH radical scavenging-capacity measurement by using photolytic OH radical generating system. It has been known for many years that UV photolysis of dilute hydrogen peroxide aqueous solution efficiently produces OH radicals. Harbour *et al.* reported that hydroperoxyl (HO_2) and OH radicals could be detected through the spin trapping method with the spin trap PBN (phenyl *N*-*tert*-butyl nitron) or DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) [12]. When hydrogen peroxide concentration was lower than approximately 300 mM (1%), UV photolysis produced OH radical adduct alone. In addition, the concentration of OH radical adduct was proportional to the illumination time as long as it was kept relatively short to avoid photolytic decomposition of the spin adduct [13]. In the present study, OH radical was produced with *in situ* photolysis in the absence or presence of antioxidant or serum. The amount of OH radical was quantified using an improved spin trap, CYPMPO (5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide) [14]. Recent spin trapping studies employed either DMPO [15] or DEPMPO (5-diehoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide) [16] as a spin trap, but unlike DMPO and DEPMPO, CYPMPO is non-hygroscopic solid at room temperature (mp 126°C) and the spin trap solution has a long shelf-life. Moreover, the stability of the spin adducts of OH and superoxide is comparable to those of DEPMPO. Thus, the decrease of the ESR signal of OH radical adduct by the addition of antioxidant was converted into OH radical scavenging capacity after simple kinetic calculation.

We confirmed the validity of this method in several pure

bio-chemicals with antioxidant activities. Through the use of iron-free OH radical source, new results were obtained in the sera from chronic renal failure (CRF) patient before and after hemodialysis. Serum could contain various factors that form metal complex with iron; therefore, we expected that the iron-free method could provide more reliable serum OH radical scavenging capacity data.

Experimental Procedures

Materials and equipment

Hydrogen peroxide, dimethyl sulfoxide, mannitol, sodium benzoate, and sodium salicylate were purchased from Kanto Chemical Co. (Tokyo, Japan). Acetyl salicylic acid, glutathione, *N*-acetylcysteine (NAC), diethylenetriamine pentaacetic acid (DETAPAC), ferrous sulfate, and sodium phosphate were obtained from Nacalai Tesque (Kyoto, Japan); PBN and CYPMPO were from Radical Research Inc. (Tokyo, Japan); and DMPO from Labotec Co. (Tokyo, Japan).

All human subject procedures were conducted with individual consent form and strictly following the protocol that had been approved by Asao Clinic Human Subject Use Committee. Blood was obtained from chronic kidney disease (CKD) patients ($n = 10$) under going stable maintenance hemodialysis (HD) and healthy volunteers ($n = 10$). Ten HD patients (5 females and 5 males, mean age 67.1 ± 9.4 years, time on dialysis 71.2 ± 45.8 months, receiving three weekly HD using polysulphone dialysis membrane) were enrolled. The underlying disease was chronic glomerulonephritis in 5, diabetic nephropathy in 2, polycystic kidney disease in 2 and nephrosclerosis in 1. Ten normal controls (5 females and 5 males, mean age 40.6 ± 14.9 years) were enrolled. Serum was separated and stored in the -20°C freezer until use. HD patients' blood was collected before and after hemodialysis.

A JEOL JES-TE300 X-band ESR spectrometer (Tokyo, Japan) equipped with 100 kHz field modulation and a WIN-RAD operation software (Radical Research Inc.) was employed for ESR measurements. Typical spectrometer settings were: field modulation width 0.1 mT; microwave power 8 mW; field scan width and rate ± 7.5 mT/2 min; time constant 0.1 s. UV light source was a medium pressure mercury/xenon arc (RUVF-203SR UV illuminator, Radical Research Inc.).

Photolytic production of OH radical

UV light from an RUVF-203SR UV illuminator that is installed with 200 W medium pressure mercury/xenon arc lamp was illuminated *in situ* to aqueous hydrogen peroxide solution. This UV illuminator is equipped with a quartz-fiber optical guide and its far-end was mechanically fitted to the hole in front of the ESR cavity, therefore, the distance

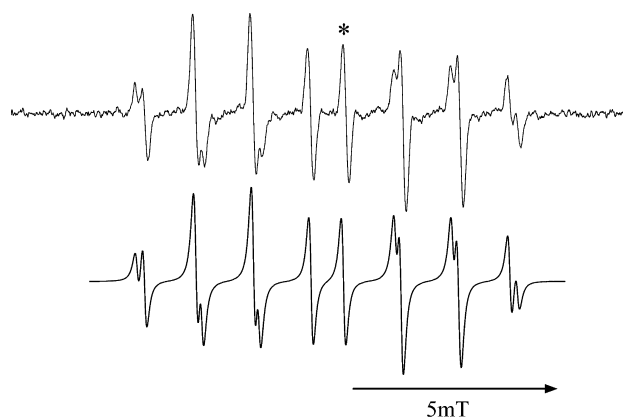


Fig. 1. ESR spectrum of OH radical adduct of CYPMPO (top). OH radical was produced with 5 s UV illumination to 10 mM H₂O₂ phosphate buffer (50 mM) solution. ESR spectrum was recorded immediately after the illumination was ceased. ESR spectrometer settings were: field modulation width 0.1 mT; microwave power 8 mW; field scan width/rate 15 mT/2 min; time constant 0.1 s. The intensity of the line marked with * was adopted as a measure for the adduct concentration. The bottom chart is a computer simulated ESR spectrum using hyperfine coupling constants; Isomer 1: A_N = 1.37 mT, A_H = 1.37 mT, A_P = 4.88 mT; Isomer 2: A_N = 1.25 mT, A_H = 1.23 mT, A_P = 4.70 mT.

between the light source and the sample can be kept fixed. The illuminator is also equipped with a mechanical shutter, and the illumination period can be controlled within ± 0.01 sec. A typical ESR spectrum obtained in this photolysis is shown in Fig. 1 along with computer-simulated spectrum. This spectrum was assigned to OH radical adduct of CYPMPO. The optimization of the photolytic conditions is described in the results section.

Measurement of antioxidant's OH radical scavenging capacity

OH radical scavenging capacity (rate) measurement was conducted as follows: 1) *in situ* illuminate UV light to the solution containing 10 mM hydrogen peroxide and 10 mM CYPMPO in 100 mM sodium phosphate buffer (pH 7.4), and record ESR signal intensity of OH radical adduct (I_0); 2) record the ESR signal intensity (I) after UV illumination from the same solution in the presence of a known amount of the selected antioxidant (AOX); and 3) according to the equation shown in the next section, plot $I_0/I-1$ against $[AOX]_0/[CYPMPO]_0$ and calculate k_2/k_1 (k_1 : CYPMPO's OH radical trapping rate constant, k_2 : AOX's OH radical scavenging rate constant).

Measurement of serum OH radical scavenging capacity

Serum was dissolved at the rate of 10, 20, and 30% in 100 mM sodium phosphate buffer (pH 7.4), containing 10 mM hydrogen peroxide, 10 mM CYPMPO, and 1 mM DETAPAC. In ambient conditions, UV light was illuminated *in situ* and the ESR signal intensity I was recorded. The signal intensity I_0 in the absence of serum was also recorded, and $I_0/I-1$ was calculated. Serum OH radical scavenging capacity was calculated by dividing $I_0/I-1$ with $[\text{serum \%}]/100([CYPMPO]$ in mM), e.g., in the case of 30% serum with 10 mM CYPMPO, $I_0/I-1$ will be divided by $30/100 \times 10 = 0.03$, and the scavenging capacity was obtained in the unit of mM-CYPMPO (mM-CYPMPO equivalent). This number can be converted into mM-DMPO equivalent unit by dividing with 1.34, the ratio of scavenging capacity of CYPMPO and DMPO (Table 1). For the sake of comparison, the Fenton reaction was used to generate OH radicals. Hydrogen peroxide (1 mM), ferrous sulfate (0.1 mM), DETAPAC (1 mM), and CYPMPO (10 mM) were sequentially dissolved in sodium phosphate buffer (20 mM, pH 7.4); 60 s after mixing ESR signal from OH radical

Table 1. Relative OH radical scavenging capacity as measured with photo-generated OH radicals

	vs CYPMPO	vs DMPO		absolute k_{AOX} (M-1 s-1) ^b
		this work	published ^a	
acetylsalicylic acid	1.99 ± 0.05	1.48	1.8–3.6	5–10E9 [22]
DMPO	1.34 ± 0.07	(1)	(1)	2.8–3.6E9 [23–25]
dimethylsulfoxide	2.26 ± 0.33	1.69	1.9 [8]	7E9 [26]
ethanol	0.55 ± 0.02	0.41	0.19–0.31 [8]	0.7–1.1E9 [27]
glutathione	2.48 ± 0.22	1.85	2.9	8E9 [28]
mannitol	0.62 ± 0.01	0.46	0.27–0.50 [8]	1–1.9E9 [29, 30]
N-acetylcysteine	3.27 ± 0.11	2.44	4.9	1.36E10 [31]
PBN	2.32 ± 0.12	1.73	0.93–3.6	2.6–10E9 [24, 25]
sodium benzoate	2.00 ± 0.06	1.49	1.6 [8]	5.7E9 [32]
sodium salicylate	2.62 ± 0.02	1.96	4.3	1.2E10 [33]

^a Numbers without the reference were calculated from absolute $k_{AOX} = 2.8E9$ of DMPO. ^b For example, 5E5 means 5×10^5 .

adduct was recorded. The same measurement was performed in the presence of serum, and OH radical scavenging capacity was calculated.

Data analysis

Kinetic formulation of the competitive reaction between the spin trap and the antioxidant against OH radical has been published elsewhere [17, 18]. Briefly, in the presence of the spin trap (ST) and AOx, OH radical scavenging reactions that should occur are:



When the ESR peak intensity of OH radical adduct in the presence of ST alone and both ST and AOx is I_0 and I , respectively, the amount of ESR-silent product must be $I_0 - I$. Thus,

$$I_0/I - 1 = k_2[\text{OH}][\text{AOx}] / k_1[\text{OH}][\text{ST}] \quad (3)$$

Because the concentrations of ST and AOx are much higher than OH radical concentration and barely consumed in this reaction, we can replace $[\text{ST}]$ and $[\text{AOx}]$ with $[\text{ST}]_0$ and $[\text{AOx}]_0$, respectively, where $[\]_0$ denotes the initial concentration of the component. Thus,

$$I_0/I - 1 = k_2/k_1 \cdot [\text{AOx}]_0 / [\text{ST}]_0 \quad (4)$$

In conclusion, the plot of $I_0/I - 1$ against $[\text{AOx}]_0/[\text{ST}]_0$ will give a zero-crossing line with the slope of k_2/k_1 . When the same spin trap and the free radical-generating condition are used, k_1 becomes common for all antioxidants. Thus, k_2 can be compared each other using k_1 as a reference.

In the case of serum specimens, the sera were volumetrically diluted with the rate of by 10, 20, and 30% with phosphate buffer and the scavenging capacity k_2/k_1 was calculated by dividing $I_0/I - 1$ with $[\text{serum-vol}\% / 100] / ([\text{ST}]_0 \text{ in mM})$, and those three values were averaged and expressed with standard deviation (SD). In order to evaluate the significance level of difference, control group vs CRF group and pre-HD vs post HD were subjected to Student's *t* test and paired Student's *t* test, respectively.

Results

Formation of OH radical adduct and its ESR spectrum

When the phosphate buffer (100 mM, pH 7.4) solution containing 10 mM hydrogen peroxide and 10 mM CYPMPO (spin trap) was UV-illuminated for 5 s, we obtained an ESR spectrum shown in Fig. 1. ESR signal was absent when the solution containing 10 mM CYPMPO alone was UV-illuminated for 5 s (data not shown). Based on the previous

study this spectrum was assigned to the OH radical adduct of CYPMPO [14]. A computer-simulated spectrum is shown in the bottom panel of Fig. 1. The relative concentration of this species can be obtained using the peak-to-peak line height of the selected line (*e.g.*, marked by * in Fig. 1).

Optimization of the illumination conditions

The crucial point of the present method is to establish the illumination condition to produce reproducible ESR signal intensity for OH radical adduct. The reproducibility of the level of OH radical adducts was determined by loading a fresh 10 mM hydrogen peroxide/10 mM CYPMPO solution into the same sample cuvette, illuminated for exactly 5 s (± 0.01 s), and the ESR signal was recorded. This was repeated for five times and the experimental error of the ESR intensity was $\pm 4\%$. The decay of the ESR signal after the photolysis was ceased was negligible because previous experiments indicated that the half life of the OH radical adduct of CYPMPO in the same condition was approximately 70 min [14]. The wavelength range in the UV light (from 200 W medium pressure mercury/xenon arc) was 250–450 nm; however, the illumination using band-pass filters (RU-330 (250–390 nm), RU-340 (280–370 nm), RU-350 (320–370 nm), and RU-360 (320–380 nm); Radical Research Inc) indicated that OH radical adduct was the sole product at any wavelength. Although molar absorption coefficient of hydrogen peroxide monotonically decreases from $100 \text{ M}^{-1}\text{m}^{-1}$ at 250 nm to $0.001 \text{ M}^{-1}\text{m}^{-1}$ at 400 nm [19], the yield of OH radical adduct was slightly dependent on the wavelength, indicating that the light intensity was sufficient to override the absorbance difference.

OH radical adduct of CYPMPO (or any nitron spin traps) but not CYPMPO itself is moderately UV-sensitive and prolonged illumination diminished its ESR intensity [13]. The dependence of the yield of OH radical adduct against the illumination time was measured in 10 mM H_2O_2 /10 mM CYPMPO- sodium phosphate buffer (100 mM) solution (data not shown). The ESR intensity of OH radical adduct linearly increased as a function of the illumination period up to 30 s, and completely leveled off after 180 s illumination. Thus, we adopted 5 s as the illumination time where the photochemical decomposition of OH radical adducts was negligible. When higher H_2O_2 concentrations were used one could further shorten the illumination time, however, in such a case, errors in the mechanical-shutter speed may become a concerning factor.

The arbitrary selection of relative AOx/ST concentrations was possible because the kinetic equation (4) contains only the ratio $[\text{AOx}]/[\text{ST}]$, but not absolute concentrations. Using NAC as AOx, we tested the three cases of $[\text{NAC}]_0/[\text{CYPMPO}]_0 = 0.5$, *i.e.*, $[\text{NAC}]_0/[\text{CYPMPO}]_0 = 2.5 \text{ mM}/5 \text{ mM}$, $5 \text{ mM}/10 \text{ mM}$, and $10 \text{ mM}/20 \text{ mM}$. We obtained $k_2/k_1 = 3.5$, 3.4 , and 3.8 , respectively, indicating that the

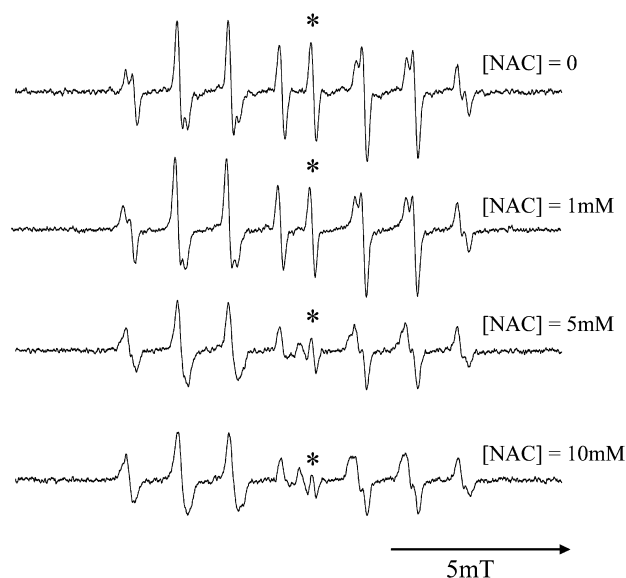


Fig. 2. Changes in the level of OH radical adduct of CYPMPO as a function of the concentration of the antioxidant *N*-acetylcysteine (NAC). Five-second *in situ* UV photolysis was carried out to the sodium phosphate buffer (50 mM) solution of H₂O₂ (10 mM) and CYPMPO (10 mM). The peak-to-peak intensity of the line marked with * was adopted as I_0 or I to calculate relative OH radical scavenging rates.

selection of the spin trap concentration (10 mM) was justifiable.

In summary, once ESR signal of OH radical adduct with the signal-to-noise ratio higher than 100 is obtained was obtained using a UV-illuminator, the experimental parameters that determines the accuracy of the ESR intensity measurement are the illumination time and the concentrations of spin trap and hydrogen peroxide.

Antioxidant's OH radical scavenging capacity measurement

ESR spectrum recorded after UV illumination to 10 mM hydrogen peroxide in 100 mM sodium phosphate buffer was readily assigned to OH radical adduct of CYPMPO (Fig. 1). In the presence of antioxidant, the signal level decreased as a function of the antioxidant concentration. Fig. 2 shows an example when NAC was added as an OH radical scavenging antioxidant. There was a possibility that the OH radical production could be decreased by antioxidant's UV-absorption. In such a case, the data point for higher antioxidant concentration in the plot like in Fig. 2 should go above the line passing through the origin, because I_0 and I would be decreased. But there were no such discrepancies in the plot.

Signal intensities obtained from Fig. 2 were plotted according to Eq. 4 (Fig. 3) and the slope of the line was calculated. Thus, scavenging rate constants (scavenging

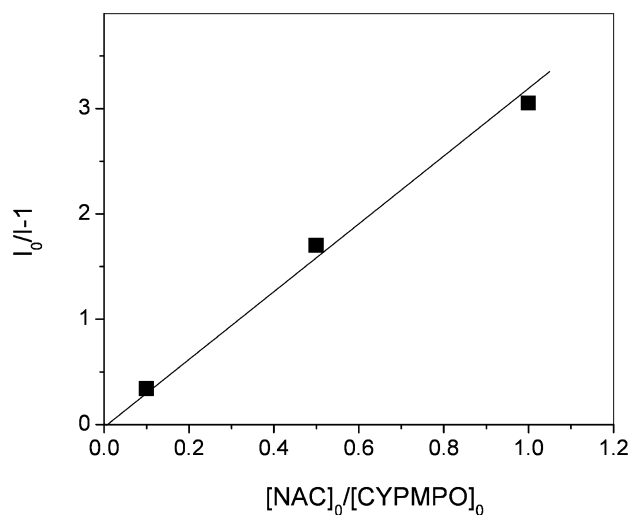


Fig. 3. A plot of $(I_0 - I)/I$, where I_0 and I denote ESR intensity in the absence and presence of NAC, respectively, against $[AOx]_0/[ST]_0$, where $[AOx]_0$ and $[ST]_0$ denote the initial concentrations of NAC and CYPMPO, respectively. The slope of the zero-crossing line represents the ratio of scavenging rate constants k_2/k_1 .

capacities) relative to CYPMPO for several antioxidants are listed in Table 1. OH radical scavenging capacities of the selected antioxidants were previously measured mainly using pulse radiolysis or ESR spin trapping with the Fenton reaction as OH radical source and DMPO as a spin trap. The present OH radical scavenging capacity was measured with respect to CYPMPO scavenging rate, but in order to facilitate the comparison with the previous data the rates are also expressed in reference to DMPO scavenging capacity (Table 1).

Serum OH radical scavenging capacity measurement

The present method of OH radical scavenging capacity determination was applied to the sera that were collected from 10 HD patients and 10 healthy volunteers. Serum was diluted with phosphate buffer at the rate of 10, 20, and 30%, and the ESR intensity was plotted according to Eq. (4). In CRF group, blood was collected before (pre-HD) and after hemodialysis (post-HD). Fig. 4 shows data points for 30 serum specimens in the unit of mM-CYPMPO equivalent. The data point before and after HD from the same subject is connected with a solid line, indicating that scavenging capacity of 9 out of 10 subjects increased after HD. Fig. 5 is a graph for median values and SDs for control, pre-HD, and post-HD, *i.e.*, 79 ± 21 , 45 ± 10 , and 77 ± 19 mM-CYPMPOeq, respectively. The significance level of difference was estimated using unpaired or paired Student's *t* test. *p* Values for control vs pre-HD (unpaired) and pre-HD vs post-HD (paired) are 0.0003 and 0.003, respectively (Fig. 5), indicating that the differences are statistically significant.

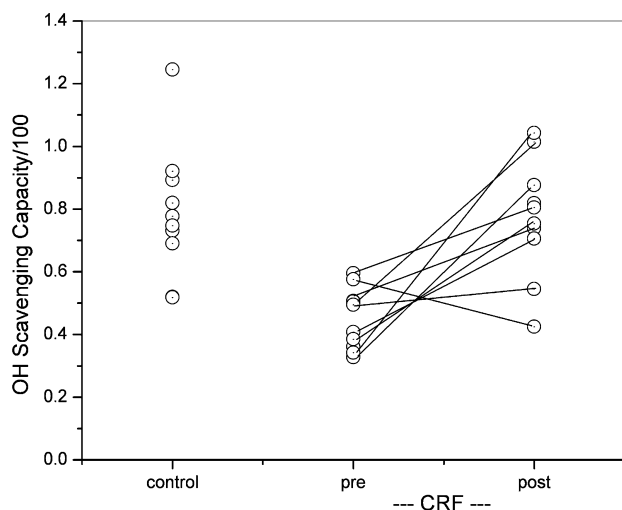


Fig. 4. Serum OH radical scavenging capacity of control group ($n=10$) and CRF subject ($n=10$, Pre and post hemodialysis) in the unit of mM-CYPMPOeq. Serum was volumetrically diluted in buffer solution by 10, 20, and 30%. Each point represents the mean value for the three concentrations. In control, each point represents individual subject. In CRF group, pre-HD and post-HD data points that were obtained from the same individual were connected with a solid line.

Regarding the effect of hemo-concentration after HD on OH-scavenging capacity, the pre/post-dialysis ratios of serum total protein concentrations (TP) and body weight (BW) were calculated to be 1.12 ± 0.04 and 1.04 ± 0.01 , respectively. Whereas, the pre/post-dialysis ratio of OH-scavenging capacity was 1.84 ± 1.10 , that was significantly higher than that of TP and BW ($p < 0.001$). Table 2 lists the present and past Fenton reaction results in both mM-CYPMPOeq unit and mM-DMPOeq unit.

Free radical production from antioxidants.

Some reagents such as glutathione and NAC produced free radicals after scavenging OH radical [20]. Those free radicals were spin trapped by CYPMPO, yielding typical

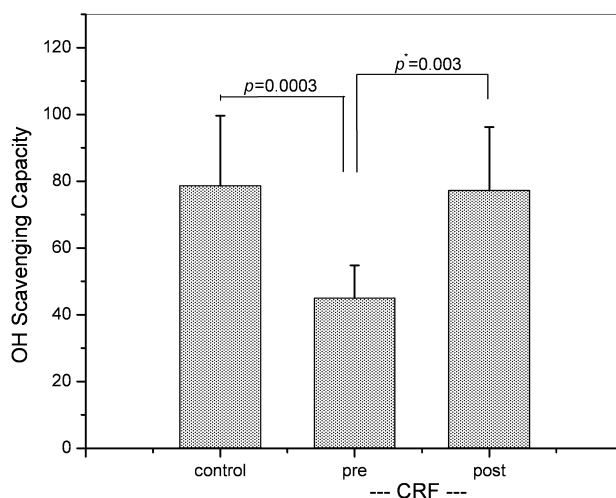


Fig. 5. A graph for mean values of OH radical scavenging capacity for control and pre-HD/post-HD CRF groups with error bars (SD). Post statistical test for the difference with unpaired Student's t test indicated that the difference between control and pre-HD groups was significant ($p = 0.0003$). The difference between pre-HD and post-HD was analyzed with paired Student's t test and also significant ($p^* = 0.003$).

characteristics of S-centered radical adduct. Major component in the bottom spectrum of Fig. 2 is assignable to sulfur-centered NAC radical adduct [20]. Fortunately, when the antioxidant concentration was low, the levels of secondary radical adducts were negligible as compared to OH radical adduct. Dimethyl sulfoxide produced methyl radical adduct after the reaction with OH radical [21], but methyl adduct signal has little overlap with that of OH radical adduct, especially in the central region (data not shown). When PBN and DMPO were tested as antioxidants, naturally OH radical adducts of PBN and DMPO were visible, but the overlap did not interfere with the peak-height measurements (data not shown). Serum specimens did not produce spin adducts other than OH adducts even at 30% concentration.

Table 2. Serum OH radical scavenging capacity

Method	Control	Pre-HD	Post-HD	Unit	Source
UV	79 ± 21 ($n = 10$)	45 ± 10 ($n = 10$)	77 ± 19 ($n = 10$)	mM-CYPMPOeq ^a	this work
	59	36	55	mM-DMPOeq ^b	this work
Fenton	131 ± 52 ($n = 10$)	226 ± 34 ($n = 10$)	214 ± 25 ($n = 10$)	mM-CYPMPOeq ^a	this work
	98	120	160	mM-DMPOeq	ref [8]
	110 to 163			mM-DMPOeq ^b	this work
Fenton	54.0 (plasma)	59.9 (plasma)	61.5 (plasma)	mM-DMPOeq	ref [34]

^a Errors are in SD. ^b These values were calculated by dividing the above numbers with 1.34 (DMPO's OH radical scavenging capacity in Table 1).

Discussion

Using iron-free, photochemical OH radical source we were able to measure *in vitro* OH radical scavenging capacity for several antioxidants as well as sera at room temperature. The accuracy of this method relies on the UV illuminator that produces reproducible amount of OH radicals in repeated experiments. While, in the Fenton reaction, the concentration of OH radical adduct depends on a few laboratory parameters, including the sequence of mixing the reactants and the time interval between the mixing and the ESR measurement. In pure antioxidants, we obtained reasonable agreement to the previous data which were obtained using Fenton reaction/spin trapping or pulse radiolysis (Table 1).

The proposed method is relatively simple and rapid as compared to the Fenton reaction-based method; in addition, the selection of experimental conditions was flexible. We obtained similar results by changing the experimental conditions such as the ratio of hydrogen peroxide/spin trap concentrations and UV intensity/illumination-time within a reasonable range. As opposed to the Fenton system, start and stop of OH radical production was controllable simply by turning on and off the UV-light. Most data from previous Fenton/spin trapping studies were obtained using a single antioxidant concentration, but our results are based on the data taken in three different antioxidant concentrations, thus experimental error could be calculated (Table 1). Absolute second order OH radical scavenging rate constants listed in Table 1 were obtained mainly with pulse radiolysis; this technique is not a method of easy access for a majority of biomedical investigators. Most plots for $I_0/I-1$ vs $[AOx]/[ST]$ like Fig. 3 showed good linear dependence, suggesting that the possibility of reactions other than Eqs. (1) and (2) were unlikely. Dimethylsulfoxide, ethanol, NAC, and glutathione reacted with OH radical, and the resultant products were spin trapped by CYPMPO to show ESR signal. In Fig. 2 (bottom panel), in the presence of high concentration of NAC (10 mM) one can see the appearance of spin adducts that was assignable to sulfur-centered radical adduct from NAC. These observations are the direct evidence that the antioxidant and the spin trap were competing to react with OH radical according to Eqs. (1) and (2). Possible drawback of the present method is photochemical decomposition of the specimen. However, the present protocol used very short illumination period in order to avoid such decomposition. Another drawback could be that this method is not applicable to non-transparent or turbid sample solutions.

Use of the Fenton reaction for OH radical source in OH radical scavenging capacity assay may increase the possibility that exogenous iron modifies the biological system of interest. The present method completely eliminated such concern, thus we obtained clear-cut results for human serum

OH radical scavenging capacity. In the sera of CKD patients, we clearly showed that OH radical scavenging capacity of pre-HD specimens were significantly lower than control, *i.e.*, 45 unit as compared to 79 unit (Fig. 5, Table 2); whereas, in post-HD sera, it increased to 77 unit, suggesting that hemodialysis was able to restore OH radical scavenging capacity to the control level. The correlation scheme for pre-HD/post-HD pairs in Fig. 4 shows that each individual CRF subject gained the restoration. Paired Student's *t* test achieved *p* value of 0.003, indicating that the restoration was not the result of random averaging. Our Fenton reaction data (Table 1) did show the decrease from control to pre-HD, but there was no change from pre-HD to post-HD. Previously, the similar results were obtained in CKD plasma using the Fenton reaction (Table 2) [34]. It is possible that CKD may cause the increase of certain serum component(s) that inhibits OH radical scavenging activity, and HD can remove such components. The pre/post-dialysis ratios of serum TP and BW were significantly lower than that of OH radical scavenging capacity; *i.e.*, the recovery of this capacity could not be explained by hemo-concentration after dialysis alone; however, it is possible that some dialyzable molecules contribute to this process. Further studies are required to identify such components. Also, there is a possibility that OH radical scavenging assay using the Fenton reaction detects the activity against modified OH radical species.

In conclusion, use of iron-free, photochemical OH radical source in the spin trapping determination of OH radical scavenging capacity may be useful specifically for intact biological fluid.

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Abbreviations

ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DEPMPO, 5-diehoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide; CYPMPO, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide; PBN, phenyl *N*-tert-butyl nitron; CRF, chronic renal failure; NAC, *N*-acetylcysteine; DETAPAC, diethylenetriamine pentaacetic acid; CKD, chronic kidney disease; HD, hemodialysis; AOx, antioxidant; TP, total protein concentrations; BW, body weight.

References

- [1] McCord, J.M. and Wong, K.: Oxygen free radicals and tissue damage. *Excerpta Medica*, **65**, 343–351, 1979.
- [2] Halliwell, B. and Gutteridge, J.M.C.: *Free radicals in biology and medicine*. Japan Scientific Societies Press, Tokyo, 1988.
- [3] Prior, R. and Cao, G.: *In vivo* total antioxidant capacity: comparison of different analytical methods. *Free Radic. Biol. Med.*, **27**, 1173–1181, 1999.
- [4] Taysi, S., Polat, F., Gul, M., Sari, R.A., and Bakan, E.: Lipid peroxidation, some extracellular antioxidants, and antioxidant enzymes in serum of patients with rheumatoid arthritis. *Rheumatol. Int.*, **21**, 200–204, 2004.
- [5] Janzen, E.G.: Spin trapping. *Methods Enzymol.*, **105**, 188–198, 1984.
- [6] Finkelstein, E., Rosen, G.M., and Rauckman, E.J.: Spin trapping. kinetics of the reaction of superoxide and hydroxyl radicals with nitrones. *J. Am. Chem. Soc.*, **102**, 4994–4999, 1980.
- [7] Finkelstein, E., Rosen, G.M., and Rauckman, E.J.: Spin trapping of superoxide and hydroxyl radical: practical aspects. *Arch. Biochem. Biophys.*, **200**, 1–16, 1980.
- [8] Tanigawa, T.: Determination of hydroxyl radical scavenging activity by electron spin resonance. *J. Kyoto Pref. Univ. Med.*, **99**, 133–143, 1990.
- [9] Goldstein, S., Meyerstein, D., and Czapski, G.: The Fenton reagents. *Free Radic. Biol. Med.*, **15**, 435–445, 1993.
- [10] Jiang, J., Bank, J.F., and Scholes, C.P.: Subsecond time-resolved spin trapping followed by stopped-flow EPR of Fenton reaction products. *J. Am. Chem. Soc.*, **115**, 4742–4746, 1993.
- [11] Finkelstein, E., Rosen, G.M., and Rauckman, E.J.: Production of hydroxyl radical by decomposition of superoxide spin trapped adducts. *Mol. Pharmacol.*, **21**, 262–265, 1982.
- [12] Harbour, J.R., Chow, V., and Bolton, J.R.: An electron spin resonance study of the spin adducts of OH and HO₂ radicals with nitrones in the ultraviolet photolysis of aqueous hydrogen peroxide solution. *Can. J. Chem.*, **52**, 3549–3553, 1974.
- [13] Kotake, Y. and Janzen, E.G.: Decay and fate of the hydroxyl radical adduct of α -phenyl *N*-tert-butyl nitron in aqueous media. *J. Am. Chem. Soc.*, **113**, 9503–9506, 1991.
- [14] Kamibayashi, M., Oowada, S., Kameda, H., Okada, T., Inanami, O., Ohta, S., Ozawa, T., Makino, K., and Kotake, Y.: Synthesis and characterization of a practically better DEPMPO-type spin trap, 5-(2,2-dimethyl-1,3-propanoate cyclophosphoryl)-5-methyl-1-pyrroline N-oxide (CYPMPO). *Free Radic. Res.*, **40**, 1166–1172, 2006.
- [15] Janzen, E.G. and Liu, J.I.-P.: Radical addition reactions of 5,5-dimethyl-1-pyrroline-1-oxide. ESR spin trapping with a cyclic nitron. *J. Magn. Reson.*, **9**, 510–512, 1973.
- [16] Frejaville, C., Karoui, H., Tuccio, B., Le Moigne, F., Culcasi, M., Pietri, S., Lauricella, R., and Tordo, P.: 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide: a new efficient phosphorylated nitron for the *in vitro* and *in vivo* spin trapping of oxygen-centered radicals. *J. Med. Chem.*, **38**, 258–265, 1995.
- [17] Buettner, G.R. and Mason, R.P.: Spin-tapping methods for detecting superoxide and hydroxyl free radicals *in vitro* and *in vivo*. *Methods Enzymol.*, **186**, 127–133, 1990.
- [18] Huang, S.H., Leonard, S., Shi, X., Goins, M.R., and Vallyathan, V.: Antioxidant activity of lazard (U-75412E) and its protective effects against crystalline silica-induced cytotoxicity. *Free Rad. Biol. Med.*, **24**, 529–536, 1998.
- [19] Phipps, M.K. and Giguère, P.A.: Hydrogen peroxide and its Analogues. III. Absorption spectrum of hydrogen and deuterium peroxides in the near ultraviolet. *Can. J. Chem.*, **29**, 490–493, 1951.
- [20] Zhao, H., Joseph, J., Zhang, H., Karoui, H., and Kalyanaraman, B.: Synthesis and biochemical applications of a solid cyclic nitron spin trap: a relatively superior trap for detecting superoxide anions and glutathyl radicals. *Free Radic. Biol. Med.*, **31**, 599–606, 2001.
- [21] Burkitt, M.J. and Mason, R.P.: Direct evidence for *in vivo* hydroxyl-radical generation in experimental iron overload: an ESR spin-trapping investigation. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 8440–8444, 1991.
- [22] Hiller, K.O., Hodd, O.L., and Willson, R.L.: Anti-inflammatory drugs: protection of a bacterial virus as an *in vitro* biological measure of free radical activity. *Chem. Biol. Interact.*, **47**, 293–305, 1983.
- [23] Madden, K.P. and Taniguchi, H.: *In situ* radiolysis time-resolved ESR studies of spin trapping by DMPO: Re-evaluation of hydroxyl radical and hydrated electron trapping rates and spin adduct yields. *J. Phys. Chem.*, **100**, 7511–7516, 1996.
- [24] Sueishi, Y., Yoshioka, C., Olea-Azar, C., Reinke, L.A., and Kotake, Y.: Substituent effect on the rate of the hydroxyl and phenyl radical spin trapping with nitrones. *Bull. Chem. Soc. Jpn.*, **75**, 2043–2047, 2002.
- [25] Sridhar, R., Beaumont, P.C., and Powers, E.L.J.: Fast kinetics of the reaction of hydroxyl radicals with nitron spin traps. *Radioanal. Nucl. Chem.*, **101**, 227–237, 1986.
- [26] Cederbaum, A.I., Dricker, E., Rubin, E., and Cohen, G.: The effect of dimethylsulfoxide and other hydroxyl radical scavengers on the oxidation of ethanol by rat liver microsomes. *Biochem. Biophys. Res. Commun.*, **78**, 1254–1262, 1984.
- [27] Anbar, M. and Neta, P.: A compilation of specific bimolecular rate constants for the reactions of hydrated electrons, hydrogen atoms, and hydroxyl radicals with inorganic and organic compounds in aqueous solution. *Int. J. Appl. Radiat. Isot.*, **18**, 493–523, 1967.
- [28] Lin, W.S. and Armstrong, D.A.: Glutathione mediation of papain inactivation by hydrogen peroxide and hydroxyl radicals. *Radiat. Res.*, **69**, 434–441, 1977.
- [29] Goldstein, S. and Czapski, G.: Mannitol as an OH scavenger in aqueous solutions and in biological systems. *Int. J. Radiat. Biol. Relat. Biol.*, **46**, 725–729, 1984.
- [30] Rehman, A., Whiteman, M., and Halliwell, B.: Scavenging hydroxyl radicals but not of peroxynitrite by inhibitors and substrates of nitric oxide synthases. *Br. J. Pharmacol.*, **122**, 1702–1706, 1997.
- [31] Aruoma, O.I., Halliwell, B., Hoey, B.M., and Butler, J.:

- The antioxidant action of N-acetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic. Biol. Med.*, **6**, 593–597, 1989.
- [32] Franzini, E., Sellak, H., Hakim, J., and Pasquier, C.: Comparative sugar degradation by (OH)· produced by the iron-driven Fenton reaction and gamma radiolysis. *Arch. Biochem. Biophys.*, **309**, 261–265, 1994.
- [33] Amphlett, C.B., Adams, G.E., and Michael, B.D.: Pulse radiolysis studies of deaerated aqueous salicylate solutions. *Advances in Chemistry Series*, **81**, 231–250, 1968.
- [34] Naito, O.: Plasma hydroxyl radical scavenging activity in chronic renal failure patients. *St. Marianna Med. J.*, **23**, 519–530, 1995.