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〔ORIGINAL〕

ESR spin trapping study on ascorbic acid as a scavenger of hydroxyl and hydrogen radicals

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Abstract

The present study was carried out to confirm the usefulness of the ESR spin trapping technique for detecting and measuring short-lived radicals and to clarify the ability of ascorbic acid as a radical scavenger against radicals generated from irradiated human serum. One hundred μl of human serum was irradiated with 1 Gy of X-ray radiation coexisting with 35 μl of DETAPAC, 50 μl of various concentrations (2×10^{-4}M～60×10^{-4}M) of ascorbic acid, and 15 μl of DMPO. After irradiation at each concentration, the ESR signals were recorded using an ESR spectrometer. These signals were identified, and then normalized as the relative signal intensities against the standard signal intensity of manganese oxide marker. The relative signal intensities were plotted, and the results showed that the signals were from DMPO-OH and DMPO-H, showing the generation of ·OH and ·H, and that the relative signal intensities obtained at each concentration decreased as the concentration of ascorbic acid increased.

Key words : ESR spin trapping technique, ascorbic acid, hydroxyl radical, hydrogen radical, radical scavenger

Introduction

The major constituent of living tissue is water, and X-ray irradiation of tissue results in the production of hydroxyl radicals , hydrogen radicals, and aqueous electrons[1]. Of these, the hydroxyl radicals is the most hazardous species, because this radical initiates the chain reaction of lipid peroxidation which causes damage to living tissue[2]. Therefore, removing hydroxyl radicals in the early stages may be an effective defense for living tissue against damage caused by irradiation. The administration of antioxidants seems effective to remove hydroxyl radicals. Ascorbic acid is one such antioxidants that may react as a radical scavenger.

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Pure ascorbic acid is a white crystalline solid which is very soluble in water[3,4]. The most striking chemical activity of ascorbic acid is its ability to act as a reducing agent. When ascorbic acid is added to reaction systems, hydroxyl radicals generated by irradiation is scavenged by reduction or interference of electron transfer[2,5]. Thus, by scavenging hydroxyl radicals, ascorbic acid plays an important role in protecting living tissue. The characteristics of ascorbic acid as a radical scavenger has been studied and documented[2,5], however, the relationship between the concentration of ascorbic acid added and its scavenging ability at different concentrations has not been well studied and there are few reports in the literature. The absence of procedures to detect the short-lived radical species has made it difficult to study the relationship that may exist between them.

Recently, ESR spin trapping has been used as a useful modality to detect short-lived radicals[6]. This technique can also be applied in discriminating trapped radicals without complex procedures. To detect or study hydroxyl radicals by ESR spectrometry, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) has frequently been employed as a spin trapping reagent[7-9]. The advantage of this technique is that the short-lived radicals stabilize into comparatively long-lived radical adducts such as DMPO-OH. The technique has many other advantages. For instance, the detection and measurement of the radicals are not obstructed by coexistence of insoluble particles and colored impurities. Further, the ESR spin trapping technique can also be applied to examine the potential of radical scavengers[3-5,9]. The signal intensities obtained by ESR spectometry must be different with various scavengers in different concentrations. A comparison of these differences would allow a determination of quantitative relationships.

The study here was carried out using the ESR spin trapping technique to determine the quantitative relationship between various concentrations of ascorbic acid added to the reaction system at different concentrations and the scavenging ability against hydroxyl radicals generated by X-ray radiation.

Materials and Methods

Sodium dihydrogenphosphate dehydrate and disodium hydrogenphosphate•12 water for the sodium phosphate buffer (PBS, pH=7.5) were from Wako Pure Chemical Industry Ltd. Diethylenetriamine-N, N, N′, N″N″-pentaacetic acid (DETAPAC) to chelate the metal ions was by Dojin Chemical Laboratory Ltd. The spin trapping reagent, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) with no detectable impurities was from Labotec Corporation Ltd.

Freezer stored human serum was used in this study after melting at room temperature. Hydroxyl radicals were generated by X-ray irradiation using Sofron BST-1500 CX equipment (Sofron X-ray Industry Corporation Ltd.).

To detect the radicals generated from the irradiated human serum, an identification of the radicals was carried out. One hundred μl of human serum, 35 μl of DETAPAC, 50 μl of PBS without ascorbic acid, and 15 μl of DMPO were placed into a test tube and were mixed by

Table 1 Irradiation conditions

Tube voltage	60 kVp
Tube current	3 mA
FSD	30 cm
HVL	0.31 mm Al
Dose rate	1 Gy/min
Dose	1.0 Gy

Table 2 ESR Spectrometry conditions

Frequency	9.4075 GHz
Power	8 mW
Center field	334.5 mT
Sweep width	±10.0 mT
modulation width	0.5×0.1 mT
Receiver gain	0.4×100
Time constant	0.1 sec
Sweep time	2.0 min
Temperature	RT

stirring. The mixture was then irradiated by 1 Gy of X-ray radiation under the conditions shown in Talbe 1. Tlhe detection and measurement of radicals were carried out using an ESR spectrometer (JEOL JES-FR 80) under the conditions in Talbe 2.

After identification of the radicals, the same procedures were carried out using various concentrations (2×10^{-4}M~60×10^{-4}M) of ascorbic acid diluted by PBS. All chemical procedures and measurements were made at room temperature.

The ESR spectra obtained from the irradiated human serum were recorded, and the signal heights were normalized to the relative signal intensities against the standard signal height of the manganese oxide marker. The numerical values of the relative signal intensities were analyzed statistically.

Results

Fig. 1 shows an ESR spectrum obtained from the irradiated human serum under controlled conditions without addition of ascorbic acid. This spectrum shows two kinds of signal: one, hyperfine coupling constants were analyzed as AN=1.4875 mT and AHβ=1.4833 mT, as in Table 3-(a), showing that this signal was from DMPO-OH, the other, those were analyzed as AN=1.6385 mT and AHβ=2.2500 mT, as in Table 3-(b), showing it to be from DMPO-H.

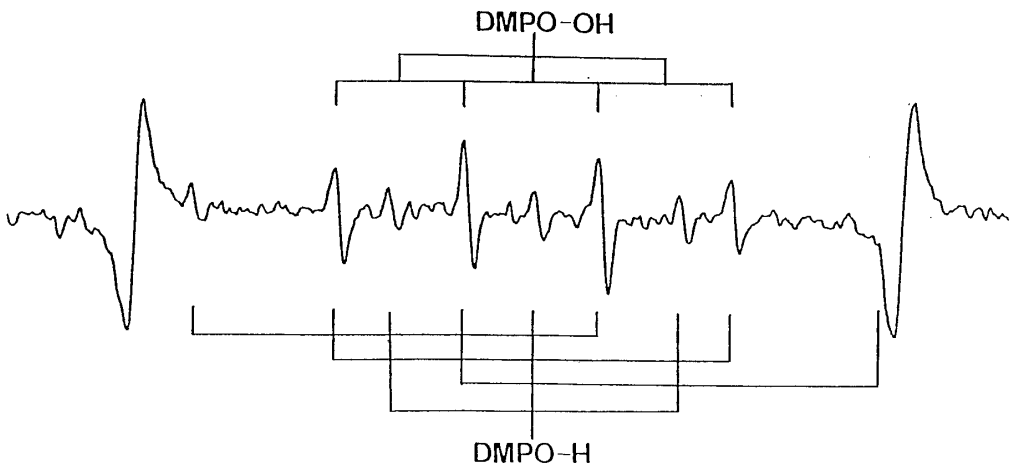

Fig. 1 ESR spectrum from the irradiated human serum. Two kinds of signals were observed: one of DMPO-OH and the other of DMPO-H.

Table 3 Hyperfine coupling constants of the radicals obtained from the irradiated human serum.

Adduct	AN	AHβ	g	Identification
a	1.4875	1.4833	2.0056	DMPO-PH
b	1.6375	2.2500	2.0055	DMPO-H

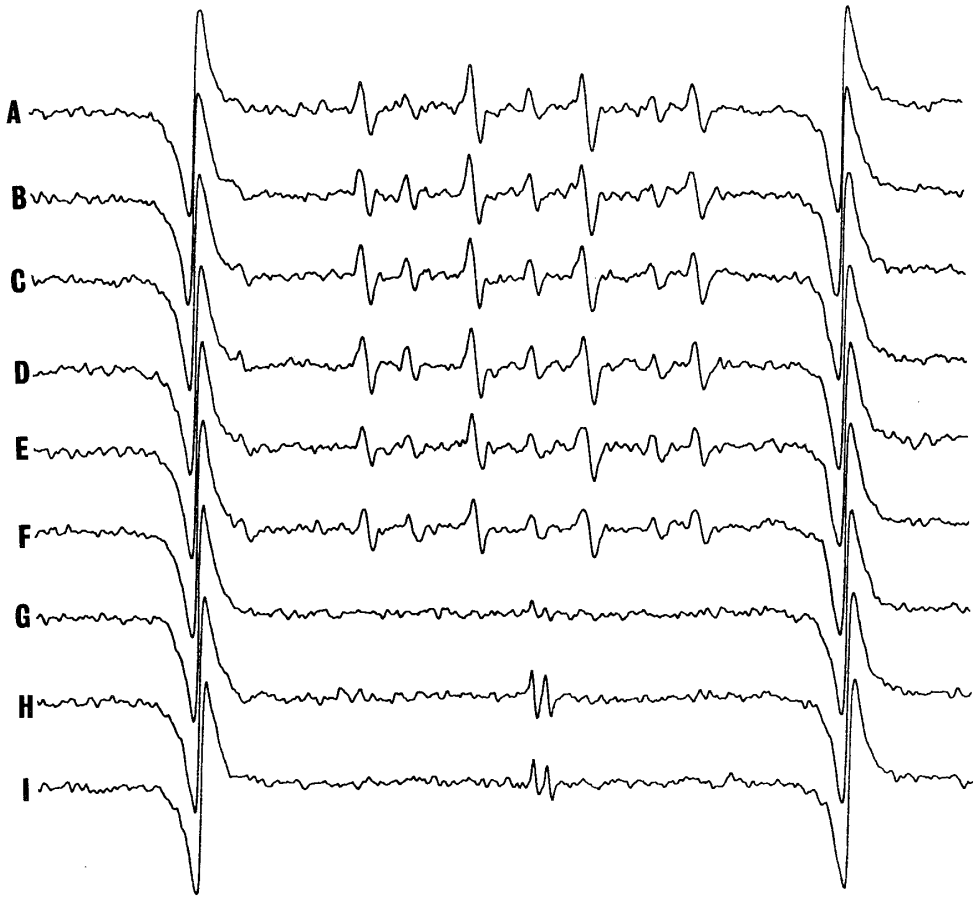

Fig. 2 ESR spectra obtained from the irradiated human serum at various ascorbic acid concentrations.
(A) : No ascorbic acid, (B) : 2×10^{-4}M, (C) : 4×10^{-4}M, (D) : 6×10^{-4}M, (E) : 8×10^{-4} M, (F) : 10×10^{-4}M, (G) : 20×10^{-4}M, (H) : 40×10^{-4}M, and (I) : 60×10^{-4}M.

Table 4 Relative signal intensities of •OH and •H obtained at various concentrations of ascorbic acid.

Ascorbic acid ($\times 10^{-4}$M)	•OH	•H
0	0.1641±0.0099	0.0665±0.0245
2	0.1509±0.0092	0.0684±0.0117
4	0.1540±0.0207	0.0703±0.0168
6	0.1594±0.0177	0.0724±0.0154
8	0.1216±0.0096	0.0551±0.0095
10	0.1131±0.0118	0.0514±0.0105
20	0.0125±0.0125	0.0050±0.0050
40	0.0257±0.0160	0.0123±0.0081
60	0.0173±0.0167	0.0108±0.0072

AV±SD(n=5)

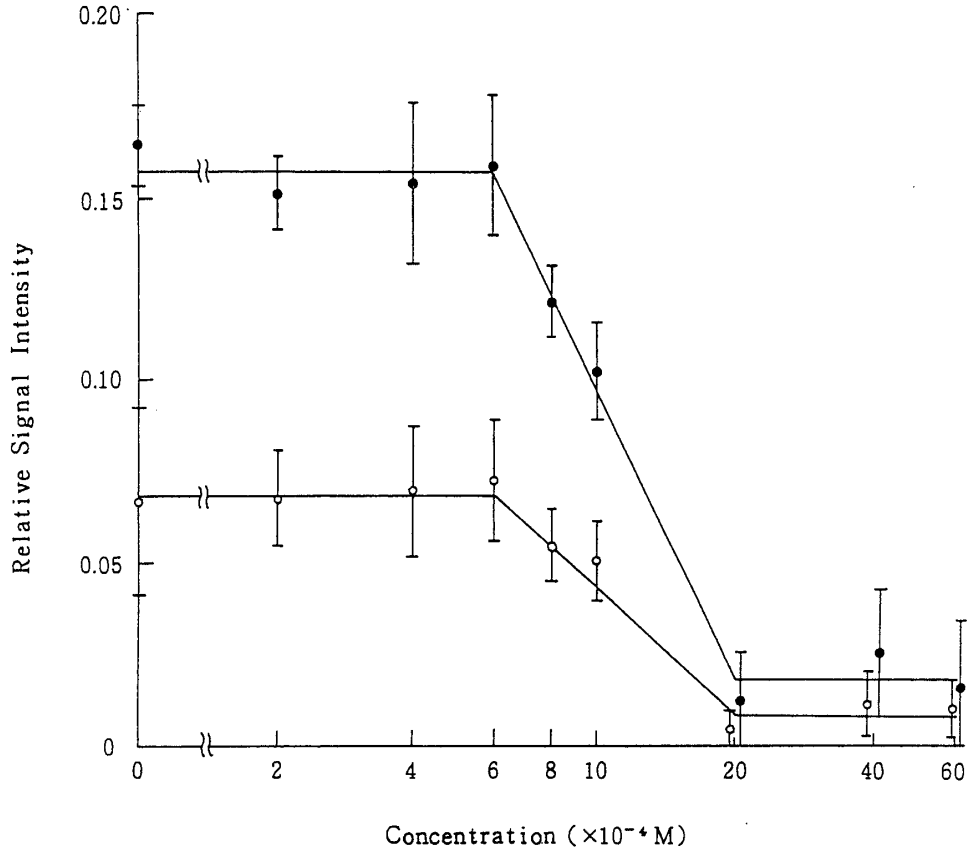

Fig. 3 Relative signal intensities of •OH and •H generated from the irradiated human serum at various ascorbic acid concentrations (AV±SD, n=5).

These results confirm that the radicals generated from the irradiated human serum were hydroxyl and hydrogen radicals.

As shown in Fig. 2-(A~D), in the presence of low concentrations of ascorbic acid, between 2 to 6 × 10^{-4}M, the signal intensities of both DMPO-OH and DMPO-H were at almost as same values as under the controlled coditions. When the concentration of ascorbic acid was over 6 × 10^{-4}M, the signal intensities decreased markedly with increases in the concentration as shown in Fig. 2-(E~I). The relative signal intensities calculated with the manganese oxide marker are shown in Table 4, which plots the curves in Fig. 3.

Discussions

In routine dental examinations, X-ray radiography has been preferentially used for a long time. When irradiating by X-ray radiation, there is first ionization of the water in living tissue, followed by hydroxyl and hydrogen radical generation[1,2].

These radicals are commonly detected and measured by optical spectrometry[10-13]. However, because of the low selectivity of these radicals and obstruction by insoluble particles or colored inpurities, measurements have been difficult. When ESR spectrometry has been used to detect the radiclas[1,2], direct detection of short-lived radicals by ESR is possible only with

relatively high radical concentrations in the ESR cavities. Such dense concentrations of radicals can only be produced by intense irradiation with high energy radiation or by rapid mixing of flow systems. Some ESR equipment has also been substatially modified to increase sensitivity and resolution, but the complexy of these procedures still make it difficult to use direct ESR spectrometry to detect short-lived radicals.

Recently, indirect techniques for detecting low concentrations of free or short-lived radicals have been developed[1,2], and ESR spin trapping is one such technique which detects the radicals indirectly. The principle of this technique was first described by Janzen[6]. Using spin trapping reagents such as DMPO or PBN, short-lived radical species such as HO• and H• are stabilized to DMPO-OH, DMPO-H, PBN-OH and PBN-H, and the hyperfine coupling constants of these permit the identification of the initial radicals. This technique allows the use of commonly available ESR equipment[3,4,7-9,14-16], and the ESR spectra obtained in the present study could easily identify what radicals were generated in the irradiated serum. The relationship between the concentration of ascorbic acid added to the reaction system and its scavenging ability at different concentrations could also be determined. As shown in Table 4 and Fig. 3, the relative signal intensities were almost the same as those obtained under the controled conditions when the concentration of ascorbic acid was below 6×10^{-4}M, at higher concentrations the relative signal intensities of the radicals decreased.

In conclusion, the results reconfirmed that the ESR spin trapping technique was able to identify short-lived radicals and determine the role of various concentrations of ascorbic acid in relation to hydroxyl and hydrogen radicals. The scavenging ability of ascorbic acid for the two radicals was concentrations dependent.

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