PERGAMON

Radiation Physics and Chemistry 66 (2003) 17–25

Radiation Physics and Chemistry

www.elsevier.com/locate/radphyschem

Test for antioxidant ability by scavenging long-lived mutagenic radicals in mammalian cells and by blood test with intentional radicals: an application of gallic acid

Jun Kumagai[a,*], Tomoko Kawaura[a], Tetsuo Miyazaki[a], Michel Prost[b], Emmanuelle Prost[b], Masami Watanabe[c], Joëlle Quetin-Leclercq[d]

[a] *Department of Applied Chemistry, Graduate School of Engineering, Nagoya University, Furo-Cho Chikusa-ku, Nagoya 464-8603, Japan*
[b] *Centre Européen de Recherche et d'Analyse (CEDRA), 3 rue des Mardors, 21560 Couternon, France*
[c] *School of Pharmaceutical Science, Nagasaki University, 1-14 Bunkyo, Nagasaki 852-8521, Japan*
[d] *Cham Unit, School of Pharmacy, Faculty of Medecine, Université Catholique de Louvain, avenue E. Mounier, 72, B-1200 Bruxelles, Belgium*

Received 27 August 2001; received in revised form 29 January 2002; accepted 29 May 2002

Abstract

Antioxidant ability of gallic acid (GA) are determined both by electron spin resonance measurement of long-lived radicals produced in γ-ray irradiated Syrian golden hamster embryo cells with GA and by hemolysis measurement with GA when blood cells are submitted to radicals. Scavenging properties of GA are determined by the reaction rate constant with long-lived mutagenic radicals in the cells while the blood test allows to analyze the global effects of this compound: radical scavenger + metal ion chelator + regeneration of intra- and extra-cellular antioxidant.

Keywords: Gallic acid; Long-lived radical (LLR); Antioxidant; ESR; SHE

1. Introduction

Determining the antioxidants properties of a compound is one of the most important topics in biology, because oxidative stresses in biological systems have been proven to be responsible for several damages and diseases (Thomas and Kalyanaraman, 1997). There are two types of methods to determine the efficacy of antioxidants up to now. The first one measures the modification of some biological marker or index in cell systems, such as blood cells when antioxidants are introduced in these systems. Although these measurements directly determine the effects of antioxidants, it is impossible to have information on all the reactions which might occur in these complex systems.

The other set of methods measures the amounts of reactive oxygen species (ROS) trapped by spin-trapping reagents. This is one of the most popular techniques for chemists to determine antioxidant properties (Niki et al., 1985). Targets of the analysis are ROS, and dilute aqueous solutions are favored for the measurements. An antioxidant and a spin-trapping reagent are mixed in the solution, and the antioxidant efficacy is determined by the amount of ROS trapped by the spin-trapping reagent.

In 1993, we employed electron spin resonance (ESR) spectroscopy and highly sensitive measurement techniques (Yoshimura et al., 1993) to identify long-lived radicals (LLRs), called also slow release radicals, in Syrian hamster embryo (SHE) cells γ-irradiated at room temperature. LLR decayed with a half lifetime of about

*Corresponding author. Tel.: +81-52-789-2591; Fax: +81-52-789-25914612.
E-mail address: kumagai@apchem.nagoya-u.ac.jp (J. Kumagai).

0969-806X/02/$ - see front matter
PII: S0969-806X(02)00288-8

20 h. ESR spectroscopy showed that vitamin C added to SHE cells after irradiation, removed LLRs from irradiated cells. Since vitamin C was added 2 h after irradiation, it did not scavenge highly ROS like OH radicals which have a half lifetime of nano seconds (Ohno, 1991; Yoshimura et al., 1993). We showed that vitamin C added 20 min after irradiation and left in for 2 h did not change the survival irradiated mouse or human embryo cells or the levels of chromosome aberrations induced in them, but it reduced the frequency of *HPRT*-mutants to background levels (Koyama et al., 1998). We also showed that LLRs were effectively scavenged by vitamin C during this time. We also have shown that epigallocatechin-3-*O*-gallate (EGCG), an ingredient of green tea, added after the irradiation, scavenges LLRs and reduces mutation (Kumagai et al., 2002b). Treatment with EGCG also did not affect radiation-induced cell death and chromosomal aberration formation (Ise et al., 2002). This points out the important role of LLRs in the induction of mutation in mammalian cells. Very recently, we have succeeded in the assignment of LLRs as sulfinyl radicals produces in some proteins (Kumagai et al., 2002a).

In this study, we propose to use the reaction between antioxidants and LLRs in mammalian cells in order to determine the antioxidant efficacy of a compound. The advantage of this method is as follows. First, their long lifetime (ca. 20 h) makes them easier to observe quantitatively and the rate constant for their reaction with antioxidants can also be measured. Second, since this method uses cells, the reaction between radicals and antioxidants mimics in vivo systems. Thus, the rate constant for the reaction could be applied directly to biological systems. A previous paper has shown that irradiation of an aqueous egg-albumin solution also produces LLRs and can be used vitamin C model for irradiated mammalian cells (Miyazaki et al., 1995; Matsumoto et al., 1997). This is the reason why we also studied the effects of antioxidants on egg-albumin irradiated solutions.

As antioxidant, we choose gallic acid (GA). GA is a natural phenolic antioxidant present in numerous plants (Senter et al., 1983). It is a frequent constituent of hydrolysable tannins in many edible plants and is also present in *Vitis vinifera* (Castillo et al., 2000; Haraguchi et al., 2000). GA has been shown to possess scavenging activities against several radicals, for example: superoxide anion, hydroxyl radicals, singlet oxygen or peroxyl radicals, and to protect cells from damage induced by UV-B or ionizing irradiation (Richards and Adams, 1987; Masaki et al., 1994, 1995a, b; Sawa et al., 1999).

The first aim of this study is to determine if GA possesses scavenging properties against LLRs and could protect irradiated cells from the deleterious effects of these radicals and estimate the rate constant. The second aim is to determine if GA can protect blood cells from an oxidant stress which causes hemolysis. The third aim is to compare both methods if a correlation exists between the reaction kinetics of GA with LLRs and the protective effect on red blood cells.

2. Materials and methods

2.1. Blood test

Aliquots of 100 ml of diluted human blood in saline solution are treated in 96-well plates with 90 ml of an aqueous solution of 2,2′-azobis(2-amidinopropane) dihydrochloride ($0.4333\,kg\,dm^{-3}$) as a source of radicals and 80 ml of 0, 10, 25, 50 or $100\,mg\,dm^{-3}$ GA monohydrate in saline solution prepared from a stock solution of $5\,kg\,dm^{-3}$. Final GA concentrations were then, respectively, 0, 2.7, 6.7, 13.4 and $26.8\,mg\,dm^{-3}$. The lysis of red blood cells is recorded over a 5-h time course by turbidimetry using a 96-well microplate reader. Results are expressed as percentages of increase on half hemolysis time as compared to controls (without GA) as a function of GA concentration.

2.2. Measurement of LLRs

Syrian golden hamster embryo (SHE) cells and 10% egg-albumin solutions were used for the ESR measurement. SHE cells (1 g), which approximately include 3×10^8 cells, in a test glass tube was irradiated with γ-rays from ^{60}Co source to a total dose of 5 kGy at room temperature at the ^{60}Co radiation facility in Nagoya University, Japan. The tubes were then maintained in a thermostat at 297 ± 0.5 K for 2 h. An aqueous solution of GA (1 ml, $21.7\,g\,dm^{-3}$) was added to the irradiated SHE cells, and quickly mixed. The concentration of GA in the cell suspension was about $10.9\,g\,dm^{-3}$. Irradiated control and treated suspensions were immediately introduced to high purity Suprasil quartz tubes (4.7 mm × 3.1 mm of outer and inner diameters) and sealed in air, the tubes were then put into liquid nitrogen in order to measure X-band ESR spectra. The tubes were maintained in a thermostat at 297 ± 0.5 K for 15 min to 1 h after every ESR measurements to proceed the reaction between LLRs and GA.

Solutions of 10% egg-albumin were prepared as follows: Egg-albumin powder (1 g, NACALAI TESQUE, INC.) was dissolved in 9 ml distilled water, then the volume of the solution was finally adjusted as 10 ml with distilled water. The albumin solution was γ-irradiated with 5 kGy at room temperature in glass tubes. The tubes were maintained in a thermostat at 297 ± 0.5 K during 2 h after the irradiation. GA monohydrate (solid form) was added to the solutions to give 4.5 and $5.4\,g\,dm^{-3}$, respectively. The solutions were immediately introduced to high purity Suprasil quartz

tubes, sealed in air, and then the tubes were placed into liquid nitrogen. The ESR spectra of LLRs in the 10% albumin solution were measured as same as in the SHE cells.

The ESR spectra were measured by using a JEOL JES-RE1X ESR spectrometer at 77 K. We carefully chose microwave power (0.1 mW) and field modulation (0.79 mT) to avoid saturation of the signals. The yields of radicals were obtained by double integration of the signals using an ES-PRIT425 computer system (JEOL).

3. Results

Fig. 1 shows the effect of GA on the hemolysis of red blood cells, and Fig. 2, the ESR spectra of LLRs in SHE cells γ-irradiated with 5 kGy at room temperature when GA at 10.9 g dm^{-3} was added to the cells at 2 h after the irradiation. The relative amount of radicals decreased to 55% of that of the control sample at 47 min after addition of GA.

Fig. 3 shows the effect of GA on the relative yields of LLRs in the SHE cells γ-irradiated with 5 kGy and stored at 297 K. GA (10.9 g dm^{-3}) was added to the cells at 2 h after the irradiation ($t = 0$). We observe that the

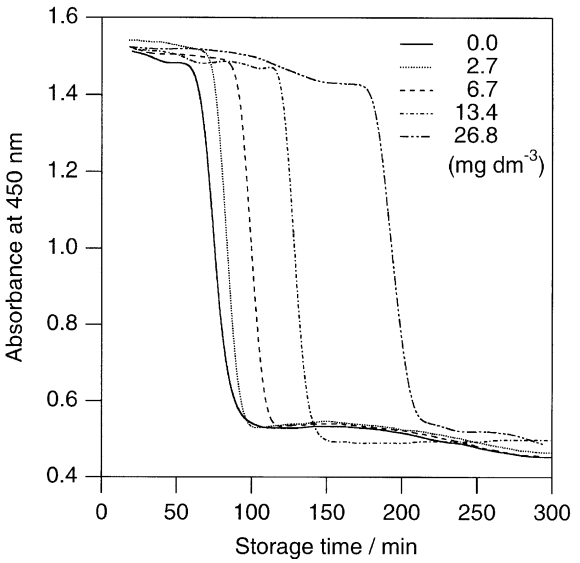


Fig. 1. Effect of gallic acid on the hemolysis of red blood cells. Aliquots of 100 µl of 1/50 diluted human blood in saline solution are treated in 96-well plates with 90 µl of an aqueous solution of 0.4333 kg dm^{-3} of 2,2′ azobis(2-amidinopropane) dihydrochloride and 80 µl of saline solution containing 0 (control), 10, 25, 50 and 100 mg dm^{-3} gallic acid monohydrate prepared from a stock solution at 5 kg dm^{-3} in water. The lysis of red blood cells is recorded over a 5-h time course by turbidimetry using a 96-well microplate reader.

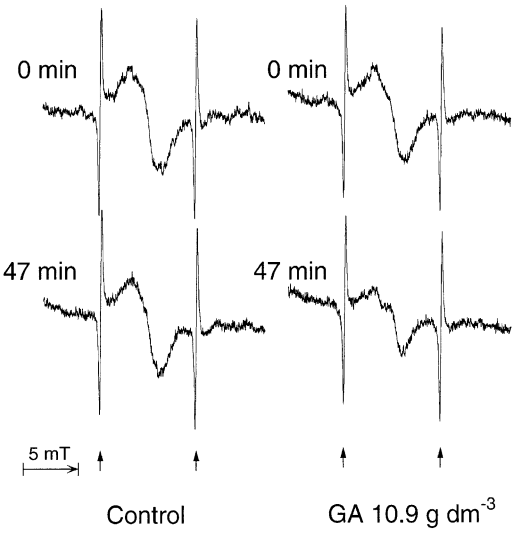


Fig. 2. ESR spectra of LLRs in SHE cells γ-irradiated with 5 kGy at 297 K and stored at 297 K. GA in 10.9 g dm^{-3} was added to the cells at 2 h after the irradiation. Spectra were recorded when GA was added ($t = 0$) and after 47 min at 297 K. The arrows indicate the signals due to Mn^{2+} used as an ESR marker.

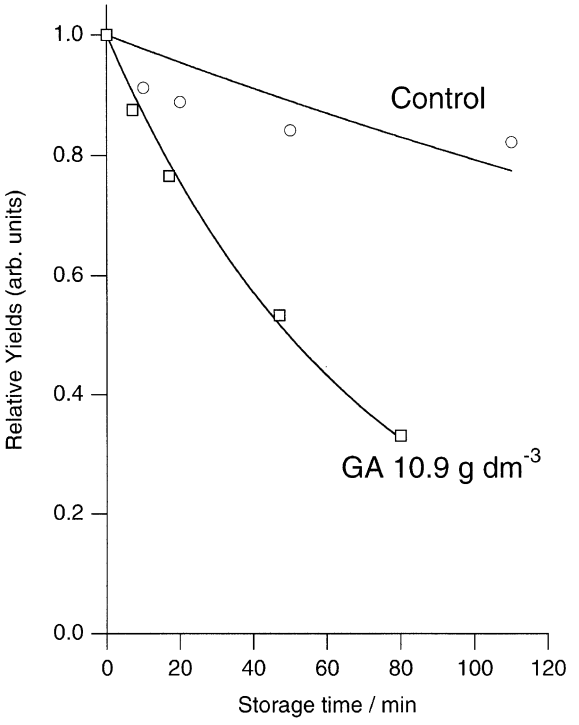


Fig. 3. Effect of GA on the relative yields of LLRs in SHE cells γ-irradiated with 5 kGy and stored at 297 K. GA in 10.9 g dm^{-3} was added to the cell at 2 h after the irradiation.

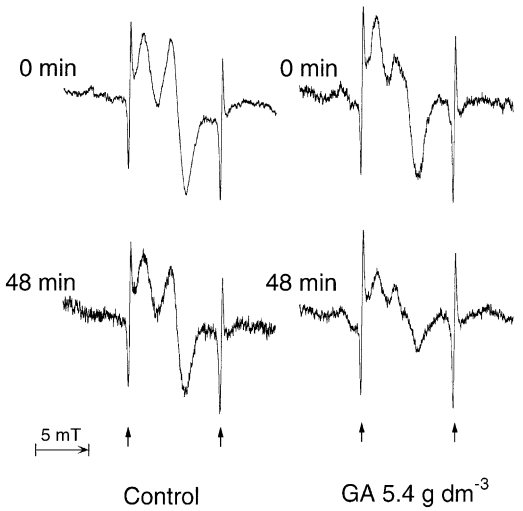


Fig. 4. ESR spectra of LLRs in albumin aqueous solution ($0.1\,kg\,dm^{-3}$) γ-irradiated with 5 kGy at 297 K and stored at 297 K. GA ($5.4\,g\,dm^{-3}$) was added to the solution at 2 h after the irradiation ($t = 0$). The arrows indicate the signals due to Mn^{2+} used as an ESR marker.

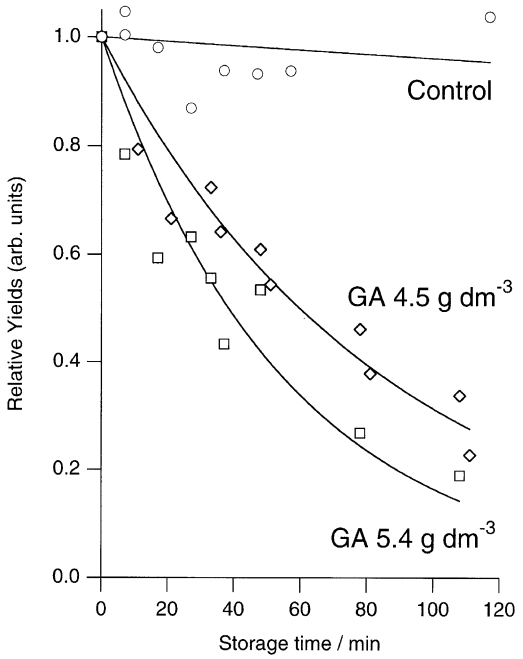


Fig. 5. Effect of GA on the relative yields of LLRs in the albumin aqueous solutions γ-irradiated with 5 kGy at 297 K and stored at 297 K. GA at 4.5 and $5.4\,g\,dm^{-3}$ was added to the solution at 2 h after the irradiation ($t = 0$).

relative yield of LLRs in the γ-irradiated SHE decays much faster than that of control after GA addition.

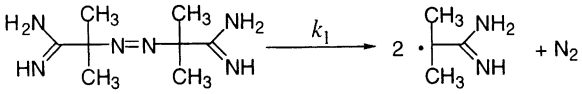
Fig. 4 shows the ESR spectra of long-lived albumin radicals in albumin aqueous solution ($0.1\,kg\,dm^{-3}$) γ-irradiated with 5 kGy at 297 K and stored at 297 K. GA was added at 2 h after the irradiation ($t = 0$). We observe that GA induces a 50% decrease in the relative amount of radicals at 48 min after the addition of GA as compared to the control sample, and that the addition of GA immediately modifies the shape of the ESR spectrum.

Fig. 5 shows the effect of two concentrations of GA on the relative yields of LLRs in the albumin aqueous solutions γ-irradiated with 5 kGy and stored at 297 K. GA was added to the solutions at 2 h after the irradiation ($t = 0$). The relative yield of LLRs in γ-irradiated albumin solution rapidly decreased as compared to the controls in a dose-dependant manner.

4. Discussion

4.1. Effect of gallic acid for preventing hemolysis in blood and scavenging long-lived radicals in SHE cells

In the blood test, the radical generator 2,2′ azobis(2-amidinopropane) dihydrochloride is added to the blood solution, and produces two 2-amidinopropyl radicals as follows:



(1)

$$R^{\bullet} + O_2 \xrightarrow{k_2} ROO^{\bullet} \quad (2)$$

$$2ROO^{\bullet} \xrightarrow{k_3} 2RO^{\bullet} + O_2. \quad (3)$$

These radicals rapidly react with oxygen to give peroxyl radicals ($k_1 = 2 \times 10^{-4}\,min^{-1}$, $k_2 \cong 1 \times 10^{9}\,dm^3\,mol^{-1}\,s^{-1}$) which can decompose to alkoxyl radicals (Miki et al., 1987; Prost, 1989, 1992).These radicals attack red blood cells, and produce hemolysis. When GA is added in this system we observe a reduction of the hemolysis rate. As shown in Fig. 1, the time necessary for half hemolysis increase in parallel with the concentration of GA in the medium. Thus, this test clearly shows that GA is very effective to protect red blood cells from hemolysis. It measures the effect of GA induced by different possible protective mechanisms: radical scavenging, chelation of transition metals, protection or regeneration of intra- and extra-cellular antioxidants.

We also analyzed the effect of GA on LLRs produced by γ-ray irradiation. Our group previously studied the effect of vitamin C for scavenging the LLRs produced in the γ-irradiated SHE cell (Matsumoto et al., 1997). Vitamin C scavenged the LLRs very effectively, and the rate of mutation and transformations were simultaneously decreased (Koyama et al., 1998). If GA

scavenges the LLRs in the irradiated SHE cells, it probably decreases mutations and/or transformations. As shown in Figs. 2 and 3, the relative yield of LLRs in γ-irradiated SHE decays much faster than in the controls when GA is added. GA is also effective for scavenging the LLRs generated in the γ-irradiated 10% egg-albumin aqueous solution as shown in Figs. 4 and 5. The reaction between the LLRs and GA can be described as

$$LLR^{\bullet} + GA \xrightarrow{k} P, \tag{4}$$

where $LLR^{\bullet}$ and P represent a long-lived radical and a reaction product, respectively. If we assume that the reaction proceeds with a pseudo-first-order kinetics, the decay rate of $LLR^{\bullet}$ can be expressed as

$$-\frac{d[LLR^{\bullet}]}{dt} = k[LLR^{\bullet}][GA] \equiv k'[LLR^{\bullet}], \tag{5}$$

where k and k' are the rate constants of the pseudo-first-order reaction. Table 1 shows the rate constants for reactions of LLRs with GA or vitamin C in SHE cells and albumin solutions at 297 K. The rate constants of the reaction between LLRs and GA are approximately half of those of LLRs and vitamin C in the same conditions. These values are in the order of $10^{-3}\,dm^3\,mol^{-1}\,s^{-1}$, which is much smaller than that of ROS in dilute aqueous solutions of 10^6–$10^{10}\,dm^3\,mol^{-1}\,s^{-1}$ (Thomas and Kalyanaraman, 1997). We have previously studied on the rate constant for reaction of vitamin C with protein LLRs in γ-irradiated albumin aqueous solution at 295 K (Miyazaki et al., 1995). Theoretical rate constant estimated from the viscosity of the 10% albumin aqueous solution at 295 K was $9 \times 10^9\,dm^3\,mol^{-1}\,s^{-1}$. Although this value is rather close to experimental rate constants of ROS in dilute aqueous solution: 10^8–$10^{10}\,dm^3\,mol^{-1}\,s^{-1}$, the rate constant of vitamin C with protein LLRs is only $1.4 \times 10^{-2}\,dm^3\,mol^{-1}\,s^{-1}$. This large difference between theoretical and experimental rate constants is probably due to the reason that the reaction of vitamin C does not take place effectively in polymer coils or that a concentration of vitamin C in the polymer coil is lower than that in bulk solution. Our recent study of LLRs in SHE and albumin aqueous solution by the analysis of electron spin echo envelope modulation spectroscopy has elucidated that the LLRs were located insight of polymer chain in hydrophobic region (Kumagai et al., 2000). Therefore, the rate constants of the reactions between additives and LLRs are much smaller than those between additives and ROS in dilute aqueous solution.

Table 1
Rate constants of reactions of LLRs and additives (GA[a], vitamin C) at 297 K in SHE cells and egg-albumin (10%) solutions

Medium of LLRs	Additives	k_S ($dm^3\,mol^{-1}\,s^{-1}$)
SHE	GA	3.5×10^{-3}
SHE	Vitamin C[b]	7.0×10^{-3}
Albumin	GA	8.5×10^{-3}
Albumin	Vitamin C[b]	14.0×10^{-3}

[a] GA: Gallic acid.
[b] Taken from Matsumoto et al. (1997).

As the present results clearly indicate GA is very effective in preventing hemolysis of blood cells and scavenging LLRs produced by γ-irradiated egg-albumin solutions or SHE cells, we tried to correlate these results. Although the decay of the LLRs is monotonous in SHE cells and/or egg-albumin solutions, the decay of absorbance at 450 nm in the blood test decreases drastically after some lag time. This may be explained by the fact that a cell has intrinsic protective mechanisms preventing hemolysis after a single damage from a single radical. Hemolysis is then only observed when these defense mechanisms are overcome. Possible mechanisms are discussed in next section.

4.2. Correlation between the biological measurement and the measurement of long-lived radicals

This study shows that both methods are very effective to determine the protective effects of compounds in mimics "in vivo" systems. However, it is not easy to compare both results directly because the systems and methods are rather different. We compare the two systems assuming two cases.

4.2.1. Consumption model (GA is completely consumed when hemolysis of the blood cells starts)

The consumption model means half hemolysis time is corresponding to the consumption of entire GA by the reaction with the radicals. In this case, lag times to half hemolysis time in the absence of GA can be explained by consumption of intrinsic radical scavengers such as vitamin E. If this model is correct, the blood test and the measurement of LLRs are independent, and show the effects of GA on protection of hemolysis and scavenging of LLRs in SHE cells and/or egg-albumin solution, respectively.

4.2.2. Multi-damage hit model (concentration of GA is almost constant during the hemolysis)

If excess amount of GA exists in the system during the blood test, the consumption model described above cannot explain why the lag time to hemolysis increased with increasing the concentration of GA. We explain the increase in lag time by increase in the concentration of GA by using reaction kinetics including the multi-damage hit theory (Zimmer, 1961; Dyson, 1993). This apply may find an interface between both measurements qualitatively and propose a plausible mechanism to

explain hemolysis. In this theory, we assume that the protective effect of GA in the blood test may be correlated, to some extent, to the reaction rate of GA with the LLRs.

Let us take a number N_0 of biological individuals which are identical with respect to the attack of radicals. It is expected that in a volume of v dm^3, after a dose D, vD hits producing free radicals should occur (hits per dm^3). Since free radicals are assumed to be produced homogeneously by radical generators, the dose is proportional to the number of hits by radicals. The probability $P(n)$ that exactly n hits occur in a volume v when the expected number of hits is vD is given by

$$P(n) = \frac{(vD)^n e^{-vD}}{n!}, \tag{6}$$

where the $P(n)$ is the probability when the Poisson's law of distribution can be applied to the hit events. If n hits are required to destroy an individual, the survival ratio N/N_0 can be observed by summation of Eq. (6) when $n = 1, 2, \ldots, n-1$, as

$$\frac{N}{N_0} = e^{-vD} \sum_{j=0}^{n-1} \frac{(vD)^j}{j!}, \tag{7}$$

where N is a number of surviving individuals. If we assume that each biological unit possesses m targets, each of which has the formal volume v and that each of these m targets must receive n hits to destroy the unit, then the ratio N/N_0 after delivery of dose D is given by

$$\frac{N}{N_0} = \left[1 - e^{-vD} \sum_{j=0}^{n-1} \frac{(vD)^j}{j!}\right]^m. \tag{8}$$

If a single hit is enough to inactivate each of these m targets, Eq. (8) can be simplified as

$$\frac{N}{N_0} = 1 - (1 - e^{-vD})^m. \tag{9}$$

Here, we apply Eq. (9) to the system of blood test as

$$\frac{N_B}{N_{B0}} = 1 - (1 - e^{-v_B \alpha t})^m, \tag{10}$$

where N_{B0} and N_B are the numbers of red blood cells which are not hemolyzed in the blood test at initial time, and at the time t, respectively, and v_B is the volume of a reaction site in the red blood cell. α the rate of the generation of oxygenated radicals in the system as shown in reactions (2) and (3). Thus, the dose D in Eq. (9) is substituted by the amount of radicals αt. As the decomposition rate k_1 in reaction (1) is low (only 12% of decomposition in 10 h) and the reaction of radicals with oxygen in reaction (2) is very rapid, the radical flux remains constant during the blood test. It means that the rate of the generation of oxygenated radicals (α) can be treated as constant (Miki et al., 1987; Prost, 1989, 1992).

Let us take into account GA as radical scavenger in this system. The radicals $R_P^\bullet$ in this system react with both red blood cells and GA molecules competitively as

$$R_p^\bullet + C \xrightarrow{k_C} \text{damage} \tag{11}$$

$$R_p^\bullet + GA \xrightarrow{k_S} R_p + GA^\bullet, \tag{12}$$

where C is a reaction site in red blood cells, k_C and k_S are the reaction rate constants of reactions (11) and (12), respectively. The reaction rates of (11) and (12) can be expressed as $k_C[C][R_P^\bullet]$ and $k_S[GA][R_P^\bullet]$, respectively. The k_S can be related to the rate constant of the reaction between LLRs and GA in SHE cells or in albumin solutions. Thus, the rate for the generation of radicals in the presence of GA can be expressed as

$$\alpha = \alpha_0 \frac{k_C[C][R_p^\bullet]}{k_C[C][R_p^\bullet] + k_S[GA][R_p^\bullet]}, \tag{13}$$

where α_0 is the rate of generation of radicals when no GA is present in the system. Thus, Eq. (10) can be expressed as

$$\frac{N_B}{N_{B0}} = 1 - \left\{1 - \exp\left(-v_B \alpha_0 t \frac{k_C[C][R_p^\bullet]}{k_C[C][R_p^\bullet] + k_S[GA][R_p^\bullet]}\right)\right\}^m. \tag{14}$$

Fig. 6 shows the plot of Eq. (14). Since the results of the blood test as shown in Fig. 1 are measured by absorbance of red blood cells, N_B/N_{B0} is linear to the absorbance. Values and units of $[R_p^\bullet][C], k_S, k_C$, and $v_B\alpha_0$ are arbitrarily chosen, a value of m is assumed as 1×10^5, and [GA] is varied from 0 to 100 in arbitrary units. As shown in the simulation in Fig. 6, value of N_B/N_{B0} begins to decrease after some lag time even if [GA] = 0, and the lag time increased upon addition of GA. The shape of simulated lines shown by solid lines in Fig. 6 is qualitatively similar to that of the experimental data shown in Fig. 1. This means that the protective effect of GA against hemolysis of red blood cells can be related to the reaction of GA with radicals. A simulation result of single-damage hit model ($m = 1$ and [GA] = 100) is shown in dotted line in Fig. 6. In this case value of N_B/N_{B0} begins to decrease just after $t = 0$ even if GA is added. Thus, introduction of the multi-damage hit model is necessary to account for the lag time. The half hemolysis times is related to the rate constant (k_S) of the reaction between antioxidants and radicals in mammalian cells or albumin solutions. In fact, numerous antioxidants show different hemolysis times (Prost, 1989, 1992; Blache and Prost, 1992). The introduction of this multi-damage hit theory has succeeded to correlate the effect of GA in the blood

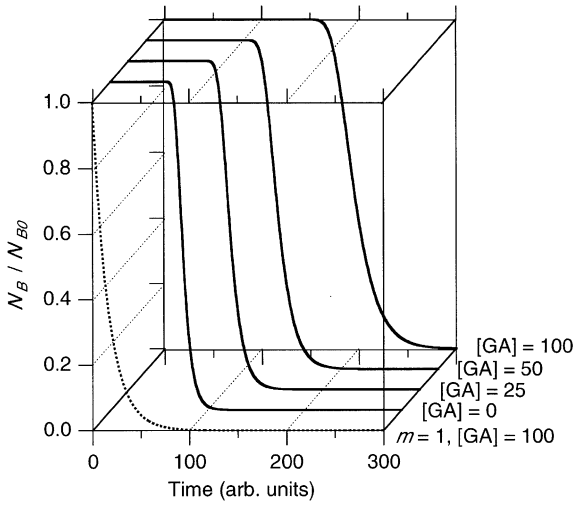


Fig. 6. A simulation of the blood test by using multiple-damage hit theory. Survival rates of red blood cells are plotted in solid lines versus to the storage time of the solution at different concentrations of GA. Details of the simulation are explained in discussion.

test and the rate constant (k_S) of the reaction of GA with LLRs.

4.3. Comparison of both techniques used for determining antioxidant properties

In this paper, we have shown that it was possible to use the reaction between antioxidants and LLRs in mammalian cells or albumin solutions as new method to determine the radical scavenging properties of these antioxidants. Two other types of methods are usually used to determine the antioxidant efficacy. The first set of methods measures some biological marker or index in cell systems, such as blood cells submitted to an oxidant stress when antioxidants are introduced to the medium. Another set of methods measures the amounts of ROS trapped by spin-trapping reagents in the systems. We compare these three methods for determining the antioxidative properties of compounds.

Table 2 summarizes the characteristics of the three types of measurements for determining the antioxidant properties. Biological measurements directly give information on the global effect of the compound in the biological system used. For example, the blood test directly measures the degree of hemolysis of red blood cells in the presence of radical generator and different concentrations of GA. Although the results of the measurement clearly showed that GA prevents hemolysis effectively, there are no information on the reaction rates between GA and radicals. Furthermore, the observed effect may be related to several properties of the tested compound: radical scavenger, heavy metal chelator, protective effect on the intrinsic defense mechanisms of the cell or regenerative of the substances implicated in this mechanisms. Thus, it is impossible to determine the reaction kinetics between radicals and GA. Nevertheless, this type of test allows to measure, in a dynamic way, the global effects of a reagent. The blood test is simple to carry out, rapid, inexpensive and can be automated.

Measurement of ROS by spin-trapping reagents is another typical way for determining the antioxidative capacities of antioxidants. The advantage of this method is that ROS, which are very important in biological effects, can be caught by spin-trapping reaction. However, there are some problems with this method. First, most of these experiments are done in in vitro systems such as dilute aqueous systems for the ease of the experiment. Because most of biological reactions in vivo proceed in concentrated systems with biopolymers, there is no guarantee that reactions in the dilute systems reflect the actual reaction in in vivo. Secondly, when spin-trapping reagents are used in in vivo systems, some reagents may show unwanted biological effect such as toxicity. (Yamashita et al., 1996; Li et al., 1997; Mesenge et al., 1997; Miyajima and Kotake, 1997; Pazos et al., 1999).

Table 2
Comparison of the three methods for determining the antioxidative ability of antioxidants

	Biological measurement	Measurement of reactive oxygen species by spin-trapping reagents	Measurement of LLRs
Measurement of biological effect	Available	Not available	Not available
Measurement of radicals	Not available	Available (in vitro)	Available (in vivo)
Measurement of kinetics of radicals	Not available	Available (in vitro)	Available (in vivo)
Side effect of spin-trapping reagents	None	Possible	None
Applicable systems	In vivo	In vitro	In vivo

The measurement of LLRs also cannot give information on biological effects. However, it has the following advantages: first, LLRs are easy to measure directly because of their long lifetime (ca. 20 h) even in mimics in vivo systems. The rate constant for the reaction of antioxidants can be obtained quantitatively from the decay rates of the LLRs with or without antioxidants. Secondly, the LLRs themselves are biologically very important because they induce mutation and transformation in the cells (Koyama et al., 1998). Studies on the scavenging properties of antioxidants on the LLRs are very important for reducing mutation and transformation in the cells. Consequently, the measurement of LLRs is a very interesting new method for determining antioxidative properties and has been shown to give complementary information to the biological tests and the spin-trapping methods.

Acknowledgements

This work is supported in part by grants from Ministry of Education, Science, Sports and Culture, Japan and in part by grants of "Ground Research Announcement for Space Utilization" promoted by Japan Space Forum. The authors thank Prof. B. Tilquin of Université Catholique de Louvain, Belgium, for helpful discussions and financial support.

References

Blache, D., Prost, M., 1992. Free radical attack: biological test for human resistance capability. In: Proceedings of the IX College Park Colloquium on Chemical Evolution, NASA, Washington, DC.

Castillo, J., Benavente-Garcia, O., Lorente, J., Alcaraz, M., Redondo, A., Ortuno, A., Del-Rio, J.A., 2000. Antioxidant activity and radioprotective effects against chromosomal damage induced in vivo by X-rays of flavan-3-ols (procyanidins) from grape seeds (*Vitis vinifera*): comparative study versus other phenolic and organic compounds. J. Agri. Food Chem. 48, 1738–1745.

Dyson, N.A., 1993, Radiation Physics with Applications in Medicine and Biology. Horwood, New York, Tokyo.

Haraguchi, H., Honda, S., Kamikawa, T., Kondou, Y., Kubo, Y., Kubo, I., 2000. Structural criteria of antioxidative activities of gallic acid esters. In: Proceedings of 219th ACS Meeting, San Francisco, CA.

Ise, T., Kodama, S., Suzuki, K., Tanaka, T., Kumagai, J., Miyazaki, T., Watanabe, M., 2002. A role of long-lived radicals in radiation mutagenesis and its suppression by epigallocatechin gallate. Int. Congr. Ser. 1236, in press.

Koyama, S., Watanabe, M., Kodama, S., Suzuki, K., Matsumoto, T., Miyazaki, T., 1998. Radiation-induced long-lived radicals which cause mutation and transformation. Mutat. Res. 421, 45–54.

Kumagai, J., Kumada, T., Watanabe, M., Miyazaki, T., 2000. Electron spin echo study of long-lived radicals which cause mutation in γ-ray irradiated mammalian cells. Spectrochim. Acta Part A 56, 2509–2516.

Kumagai, J., Miyazaki, T., Kodama, S., Watanabe, M., 2002a. Long-lived radicals responsible for delayed effects of genomic instability in irradiated mammalian cells. In: Proceedings of An International Workshop on "Radiation-Induced Genetical Instability", Nagasaki, Japan.

Kumagai, J., Nakama, M., Miyazaki, T., Ise, T., Kodama, S., Watanabe, M., 2002b. Scavenging of long-lived radicals by (−)-epigallocatechin-3-*O*-gallate and simultaneous suppression of mutation in irradiated mammalian cells. Radiat. Phys. Chem. 64 (4), 293–297.

Li, G.L., Forooque, M., Holtz, A., Olsson, Y., 1997. Effects of α-phenyl-*N*-tertbutyl nitrone (PBN) on compression injury of rat spinal cord. Free Radical Res. 27 (2), 187–196.

Masaki, H., Atsumi, T., Sakurai, H., 1994. Hamamelitannin as a new potent active oxygen scavenger. Phytochemistry 37, 337–343.

Masaki, H., Atsumi, T., Sakurai, H., 1995a. Peroxyl radical scavenging activities of hamamelitannin in chemical and biological systems. Free Radical Res. 22, 419–430.

Masaki, H., Atsumi, T., Sakurai, H., 1995b. Protective activity of hamamelitannin on cell damage of murine skin fibroblasts induced by UVB irradiation. J. Dermat. Sci. 10, 25–34.

Matsumoto, T., Miyazaki, T., Kosugi, Y., Kumada, T., Koyama, S., Kodama, S., Watanabe, M., 1997. Reaction of long-lived radicals and vitamin C in γ-irradiated mammalian cells and their model system at 295 K. Tunneling reaction in biological system. Radiat. Phys. Chem. 49 (5), 547–551.

Mesenge, C., Verrecchia, C., Allix, M., Boulu, R.G., Plotkine, M., 1997. Protective effects of phenyl-tert-butyl-nitrone (PBN) and melatonin in a brain injury model in mice. J. Cereb. Blood Flow Metab. 17 (1), 676.

Miki, M., Tamai, H., Mino, M., Yamamoto, Y., Niki, E., 1987. Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. Arch. Biochem. Biophys. 258 (2), 373–380.

Miyajima, T., Kotake, Y., 1997. Optimal time and dosage of phenyl *N*-tert-butyl nitrone (PBN) for the inhibition of nitric oxide synthase induction in mice. Free Radical Biol. Med. 22 (3), 463–470.

Miyazaki, T., Yoshimura, T., Mita, K., Suzuki, K., Watanabe, M., 1995. Rate constant for reaction of vitamin C with protein radicals in γ-irradiated aqueous albumin solution at 295 K. Radiat. Phys. Chem. 45 (2), 199–202.

Niki, E., Kawakami, A., Saito, M., Yamamoto, Y., Tsuchiya, J., Kamiya, Y., 1985. Effect of phytyl side chain of vitamin E on its antioxidant activity. J. Biol. Chem. 260 (4), 2191–2196.

Ohno, S., 1991. Radiation chemistry of aqueous systems. In: Tabata, Y., Ito, Y., Tagawa, S. (Eds.), CRC Handbook of Radiation Chemistry. CRC Press, Boca Raton, Ann Arbor, Boston, pp. 321–394.

Pazos, A.J., Green, E.J., Busto, R., Mccabe, P.M., Baena, R.C., Ginsberg, M.D., Globus, M.Y.-T., Schneiderman, N., Dietrich, W.D., 1999. Effects of combined postischemic hypothermia and delayed *N*-tert-butyl-α-pheylnitrone (PBN) administration on histopathological and behavioral deficits associated with transient global ischemia in rats. Brain Res. 846 (2), 186–195.

Prost, M., 1989. Utilisation de générateur de radicaux libres dans le domaine des dosages biologiques, Vol. 642, France: 2, p. 526.

Prost, M., 1992. Process for the determination by means of free radicals of the antioxidant properties of a living organism or potentially aggressive agents, Vol. 135, US: 5, p. 850.

Richards, J., Adams, F., 1987. Study of reaction rates of the antioxidants gallic acid, BHT and BHA using the technique of pulse radiolysis. Int. J. Food Sci. Technol. 22 (5), 501–508.

Sawa, T., Nakao, M., Akaike, T., Ono, K., Maeda, H., 1999. Alkylperoxyl radical scavenging activity of various flavonoids and other phenolic compounds: implications for the anti-tumor-promoter effect of vegetables. J. Agric. Food Chem. 47 (2), 397–402.

Senter, S.D., Horvat, R.J., Forbus, W.R., 1983. Comparative GLC–MS analysis of phenolic acids of selected tree nuts. J. Food Sci. 48 (3), 798–799.

Thomas, C.E., Kalyanaraman, B., 1997. Oxygen Radicals and the Disease Process. Harwood Academic Publishers, Amsterdam.

Yamashita, T., Ohshima, H., Asanuma, T., Inukai, N., Miyoshi, I., Kasai, N., Kon, Y., Watanabe, T., Sato, F., Kuwabara, M., 1996. The effects of α-phenyl-tertbutyl nitrone (PBN) on copper-induced rat fulminant hepatitis with jaundice. Free Radical Biol. Med. 21 (6), 755–762.

Yoshimura, T., Matsumoto, K., Miyazaki, T., Suzuki, K., Watanabe, M., 1993. Electron spin resonance studies of free radicals in gamma-irradiated golden hamster embryo cells: radical formation at 77 K and 295 K, and radioprotective effects of vitamin C at 295 K. Radiat. Res. 136 (3), 361–365.

Zimmer, K.G., 1961. Studies on Quantitative Radiation Biology. Oliver & Boyd, Edinburgh.