Journal of Radiation Industry 5 (3) : 221~225 (2011)

Application of Chemical Probes to Detect Superoxide Anion and Singlet Oxygen in Biological Systems during Gamma Irradiation

Min Hee Lee †, Eun Ju Cho †, Ji Hong Kim, Ji Eun Kim, Byung Yeoup Chung, Jae-Young Cho[1], Kang-Soo Lee[2] and Jin-Hong Kim*

Advanced Radiation Research Institute, Korea Atomic Energy Research Institute, Jeongeup 580-185, Korea
[1]*Bio-environmental Science Major, Chonbuk National University, Jeonju 561-756, Korea*
[2]*Crop Production and Technology Major, Chonbuk National University, Jeonju 561-756, Korea*

Abstract - To detect superoxide anion ($O_2\cdot^-$) or singlet oxygen ($^1O_2$) in biological systems during gamma irradiation, specific chemical probes, 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron) or 2,2,6,6-tetramethyl-piperidine (TEMP), were evaluated. Tiron or TEMP spin adducts was structurally stable in aqueous solution during gamma irradiation up to 500 or 1,000 Gy, respectively. The signal of Tiron semiquinone radical, a spin adduct of Tiron upon reaction with $O_2\cdot^-$, was slightly increased by gamma irradiation. This trend was dose-dependently manifested in O_2-saturated aqueous solution using nitro blue tetrazolium (NBT), a common probe for both hydrated electron (e^-_{aq}) and $O_2\cdot^-$. In contrast, a spin adduct of TEMP, was never inducible by gamma irradiation, while its signal was substantially enhanced by photosensitization of riboflavin. These results suggest that Tiron and NBT or TEMP could be utilized to detect $O_2\cdot^-$ or $^1O_2$ in biological systems during gamma irradiation, although $O_2\cdot^-$ or $^1O_2$ are not the main reactive oxygen species produced by water radiolysis.

Key words : Gamma radiation, Reactive oxygen species, Singlet oxygen, Superoxide anion

INTRODUCTION

Water radiolysis indicates the dissociation of water molecules by ionizing radiation. Its intermediates and products include water radical cation ($H_2O\cdot^+$), excited water (H_2O^*), hydronium ion (H_3O^+), hydroxyl radical ($\cdot OH$), hydrogen ion (H^+), hydrated electron (e^-_{aq}), hydrogen radical ($H\cdot$), hydrogen peroxide (H_2O_2), hydroxyl ion (OH^-), superoxide anion ($O_2\cdot^-$), and molecular hydrogen (H_2) (Spinks and Woods 1990; Zielonka *et al*. 2006). Among them, $\cdot OH$, $O_2\cdot^-$, and H_2O_2 are biologically toxic and damaging to cellular components (Cadenas 1989; Lidon and Henriques 1993). In addition, photosynthesis of plants produces another damaging agent, singlet oxygen ($^1O_2$), which is enhanced by gamma irradiation due to the inhibition of zeaxanthin-dependent thermal energy dissipation (Moon *et al*. 2008; unpublished data). Accordingly, $^1O_2$ also belongs to the reactive oxygen species (ROS) responsible for the radiation-induced biological damages.

The primary difficulty in most of ROS detecting is due to the short lifetime and reactivity (Bačić and Mojovič 2005). To assay ROS, it is necessary to introduce into the biological system under investigation of a trapping compound that specifically reacts with a ROS, forming a more stable compound. Previously, we reported the stability and application of chemical probes for detection of $\cdot OH$ and H_2O_2 in bio-

† These authors contributed equally.
* Corresponding author: Jin-Hong Kim, Tel. +82-63-570-3333, Fax. +82-63-570-3390, E-mail. jhongkim@kaeri.re.kr

logical systems during gamma irradiation (Lee *et al*. 2009). However, we lacked information about the practical use of a chemical probe specific for $O_2\cdot^-$ or $^1O_2$ under gamma irradiation.

The objective of this study is to test the stability and application of trapping chemical probes to detect $O_2\cdot^-$ or $^1O_2$ in aqueous solution during gamma irradiation using nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR), and spectrophotometric analyses.

MATERIALS AND METHODS

1. Chemical treatments and gamma irradiation

Chemical probes for oxygen free radicals or reactive oxygen species (ROS) are 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron) for superoxide anion ($O_2\cdot^-$), 2,2,6,6-tetramethylpiperidine (TEMP) for singlet oxygen ($^1O_2$), and nitro blue tetrazolium (NBT) for both hydrated electron (e^-_{aq}) and $O_2\cdot^-$ (Hideg *et al*. 1994; Peskin *et al*. 1998; Kovács *et al*. 1999b; Fryer *et al*. 2002). These probes were directly added to aqueous solution, achieving the final concentrations for Tiron, TEMP, and NBT of 200, 50, and 6 mM, respectively. Additionally, riboflavin was added and illuminated for photo-generation of $^1O_2$ (Huang *et al*. 2004).

Gamma irradiation was performed at dose rates of 25～250 Gy h^{-1} for 4 h (100～1,000 Gy) using a ^{60}Co gamma irradiator (IR-222, MDS Nordion Inc., Kanata, Canada) in the Advanced Radiation Technology Institute (ARTI).

2. ^{1}H-NMR analysis of chemical probes

For ^{1}H-NMR analysis, chemical probes and their spin adducts (about 10 mg) in aqueous solution after gamma irradiation were freeze-dried and re-dissolved in 1 ml of D_2O or CD_3OD. NMR spectra were recorded on a NMR spectrometer (JNM-ECA 500, Jeol Ltd., Tokyo, Japan).

3. EPR analysis of $O_2\cdot^-$ and $^1O_2$

The production of $O_2\cdot^-$ or $^1O_2$ was detected by the EPR spectroscopy with a specific chemical probe, Tiron or TEMP, respectively (Hideg *et al*. 1994; Hideg and Vass 1996; Peskin *et al*. 1998). EPR-active spin adducts, Tiron semiquinone radical or a stable nitroxide radical 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), are formed from Tiron or TEMP upon reaction with $O_2\cdot^-$ or $^1O_2$, respectively. EPR spectra were measured with an ESR spectrometer (JES-FA200, Jeol Ltd., Tokyo, Japan). Spectra were recorded at room temperature with 9.4 GHz microwave frequency, 16 mW or 10 mW microwave power, 0.2 mT modulation amplitude, 100 kHz modulation frequency and 2.0×10 or 5.0×10^2 amplification for the Tiron or TEMP, respectively. The analyses were conducted in three individual experiments and the data analyzed by SigmaPlot10.0 (San Jose, CA, USA).

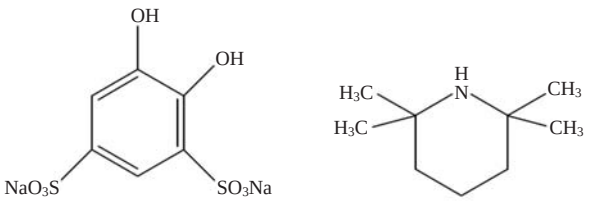
4. Colorimetric analysis of $O_2\cdot^-$

The production of $O_2\cdot^-$ by water radiolysis was indirectly estimated by the difference in reduction of NBT in N_2- and O_2-saturated aqueous solutions during gamma irradiation. The reduction of NBT was measured as increase in absorbance at 560 nm using a spectrophotometer (UVICON 923, Bio-tek Kontron Instruments, Winooski, VM) (Lee *et al*. 2009). Since e^-_{aq} produced by water radiolysis reacts with O_2 to generate $O_2\cdot^-$, the reduction of NBT should be kept lower in the O_2-saturated solution than in the N_2-saturated one. The analyses were performed in three individual experiments and the data analyzed using SigmaPlot10.0 (San Jose, CA, USA).

RESULTS AND DISCUSSION

1. Chemical probes for $O_2\cdot^-$ and $^1O_2$

For several decades, diverse chemical probes have been used to trap reactive oxygen species (ROS) in biological systems. Previous reports have shown that Tiron, TEMP, or NBT is a sensitive and selective chemical probe for $O_2\cdot^-$, $^1O_2$, or e^-_{aq} and $O_2\cdot^-$, respectively (Hideg *et al*. 1994; Peskin *et al*. 1998; Fryer *et al*. 2002; Liszkay *et al*. 2003). These



Tiron TEMP

Fig. 1. Structures of the chemical probes specific for detection of $O_2\cdot^-$ and $^1O_2$.

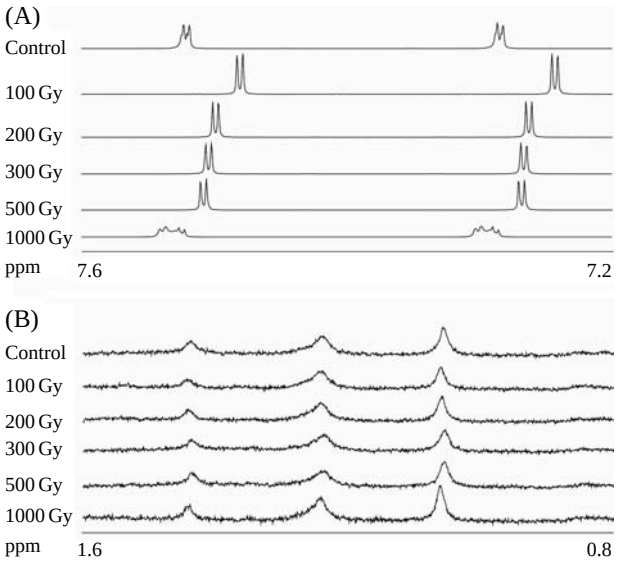


Fig. 2. NMR spectra of the probes for detection of $O_2 \cdot^-$ and $^1O_2$ during gamma radiation. (A) Tiron and/or its spin adducts; (B) TEMPO.

probes are not terribly toxic, so they are useful to *in vitro*/*in vivo* trapping of $O_2 \cdot^-$ or $^1O_2$. The resultant adducts of Tiron or TEMP in reaction with $O_2 \cdot^-$ or $^1O_2$ are detectable by spin trapping (Fig. 1).

2. Stability of chemical probes for $O_2 \cdot^-$ or $^1O_2$ to gamma irradiation

As mentioned above, the chemical probes may also be good candidates to detect such ROS produced in biological systems by gamma irradiation. This application necessitates the stability of the probes to gamma radiation. Therefore, we investigated the structural stability of Tiron and TEMP spin adducts to gamma radiations of 100 ~ 1,000 Gy, which have been used to industrial irradiation for the food and agricultural products. NMR analyses showed that the structure of TEMPO was unaffected by gamma irradiation up to 1,000 Gy (Fig. 2). Previously, it was also reported that NBT was stable to gamma radiations up to 1,000 Gy (Lee *et al*. 2009). In contrast, Tiron and its spin adducts were broken down at 1,000 Gy, implying that it can not work as a sensitive and selective probe for $O_2 \cdot^-$ with gamma radiations of 1,000 Gy or above.

3. Detection of $O_2 \cdot^-$ in aqueous solution during gamma irradiation, but not $^1O_2$

Tiron forms a Tiron semiquinone radical upon reaction

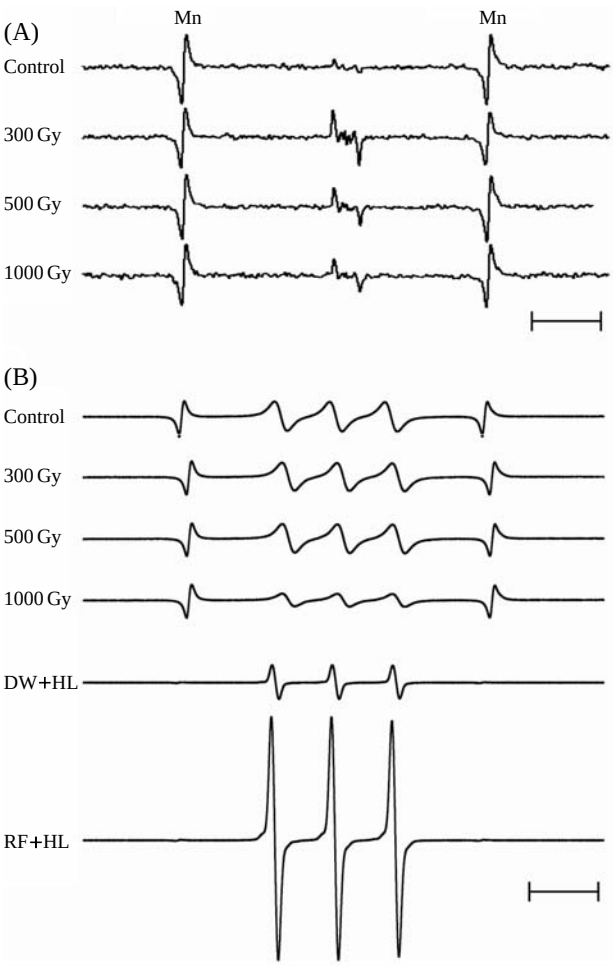


Fig. 3. EPR spectra of Tiron and TEMP spin adducts in aqueous solution during gamma irradiation. (A) Tiron semiquinone radicals; (B) TEMPO. Riboflavin (RF) was used to produce $^1O_2$ by photosensitization under the high light (HL). Bars: (A) and (B)=2 mT.

with $O_2 \cdot^-$ (Peskin *et al*. 1998). The Tiron radical is an EPR-active spin adduct. When an aqueous solution including 200 mM Tiron was irradiated with different doses of gamma radiation, the EPR signal from the Tiron radicals was slightly increased (Fig. 3A). These experiments demonstrate that $O_2 \cdot^-$ is the major ROS produced in aqueous solution by gamma irradiation. Actually, the production of $O_2 \cdot^-$ by gamma irradiation depends on the amount of O_2 dissolved in aqueous solution (Zielonka *et al*. 2006).

EPR analysis for ROS, $O_2 \cdot^-$, using Tiron (Hideg *et al*. 1994; Hideg and Vass 1996) revealed that the ROS are not mainly produced in aqueous solution by gamma irradiation (Fig. 3A). TEMP can react with another ROS, $^1O_2$, by forming TEMPO, a stable nitroxide radical yielded in the reaction

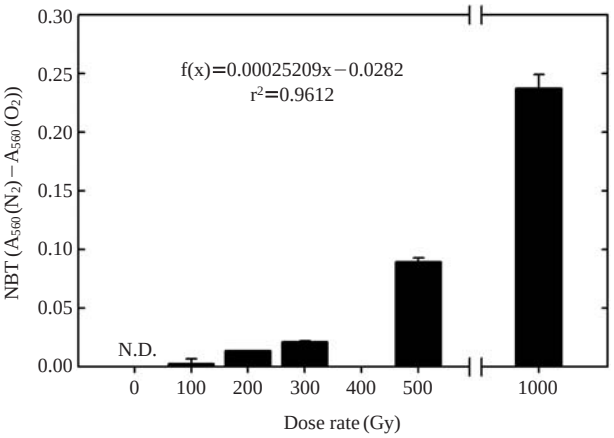


Fig. 4. Differential reduction of NBT in N_2- and O_2-saturated aqueous solution during gamma irradiation. The NBT reduction was measured as increase in absorbance at 560 nm. Bars represent means ± S.E. ($3 \leq n \leq 9$ from three independent experiments).

of singlet oxygen (Hideg *et al*. 1994). This ROS is a critical damaging factor in plants exposed to excessively high light. However, the EPR signal from the TEMPO radicals was not changed in aqueous solution by gamma irradiation, while it was remarkably increased by photosensitization of riboflavin (Fig. 3B). This result suggests that $^1O_2$ cannot be substantially produced in biological systems by gamma irradiation.

4. Estimation of $O_2 \cdot^-$ production depending on the radiation dose

NBT has been used as a practical probe for both e^-_{aq} and $O_2 \cdot^-$ (Kovács *et al*. 1999b; Fryer *et al*. 2002). When the pale yellow NBT reacts with $O_2 \cdot^-$, a dark blue insoluble diformazan compound is produced (Beyer and Fridovich 1987). Otherwise, the radiation-dependent reduction of NBT in aqueous solution, which is mediated by e^-_{aq}, can result in the formation of monoformazan and then diformazan (Kovács *et al*. 1999b). Since the production rate of $O_2 \cdot^-$ by gamma irradiation is generally very low because of the limited amount of O_2 dissolved in the air-saturated aqueous solution (Zielonka *et al*. 2006). The radiation-dependent reduction of NBT in aqueous solution has been explained by the increasing production of e^-_{aq} rather than $O_2 \cdot^-$ (Lee *et al*. 2009). Therefore, the production of $O_2 \cdot^-$ by gamma irradiation as shown in Fig. 3A was further confirmed by using the reactivity of NBT to both e^-_{aq} and $O_2 \cdot^-$ in aqueous solution. The production rate of $O_2 \cdot^-$ is expected to be higher in the O_2-saturated aqueous solution during gamma irradiation than in the N_2-saturated one (Fig. 4). Instead, since the formation of NBT monoformazan upon reaction with e^-_{aq} is much faster than that of NBT diformazan upon reaction with $O_2 \cdot^-$ from the initial reaction of O_2 and e^-_{aq}, the radiation-dependent reduction of NBT should be much higher in the N_2-saturated aqueous solution than the O_2-saturated one (Fig. 4). Accordingly, the difference in the radiation-dependent reduction of NBT between the N_2- and O_2-saturated aqueous solutions was dose-dependently increased during gamma irradiation, implying that the production of $O_2 \cdot^-$ would be similarly enhanced depending on the radiation dose as well as the concentration of O_2.

CONCLUSION

In the present study, we demonstrated that $O_2 \cdot^-$ or $^1O_2$ are not the main reactive oxygen species produced by water radiolysis. In addition, considering the structural stability and specificity of chemical probes tested, we suggest that Tiron and NBT or TEMP could be utilized as semi-quantitative chemical probes to estimate the level of $O_2 \cdot^-$ or $^1O_2$ in biological systems during gamma irradiation.

ACKNOWLEDGMENT

This research was supported by the Nuclear R & D Program of the Ministry of Education, Science and Technology (MEST), Republic of Korea.

REFERENCES

Bačić G and Mojovič M. 2005. EPR spin trapping of oxygen radicals in plants: A methodological overview. *Ann. N.Y. Acad. Sci.* **1048**:230-243.

Beyer WF and Fridovich I. 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Biochem.* **161**:559-566.

Cadenas E. 1989. Biochemistry of oxygen toxicity. *Annu. Rev. Biochem.* **58**:79-110.

Fryer MJ, Oxborough K, Mullineaux PM and Baker NR. 2002. Imaging of photo-oxidative stress responses in leaves. *J. Exp. Bot.* **53**(372):1249-1254.

Hideg É and Vass I. 1996. UV-B induced free radical production in plant leaves and isolated thylakoid membranes. *Plant*

Sci. **115**:251-260.

Hideg É, Spetea C and Vass I. 1994. Singlet oxygen production in thylakoid membranes during photoinhibtion as detected by EPR spectroscopy. *Photosynth. Res.* **39**:191-199.

Huang R, Choe E and Min DB. 2004. Kinetics for singlet oxygen formation by riboflavin photosensitization and the reaction between riboflavin and singlet oxygen. *J. Food Sci.* **69**(9):726-732.

Kovács A, Baranyai M, Wojnárovits L, Moussa A, Othman I and McLaughlin WL. 1999a. Aqueous-ethanol nitro blue tetrazolium solutions for high dose dosimetry. *Radiat. Phys. Chem.* **55**:799-803.

Kovács A, Wojná rovits L, Baranyai M, Moussa A, Othman I and McLaughlin WL. 1999b. Radiolytic reactions of nitro blue tetrazolium under oxidative and reductive conditions: a pulse radiolysis study. *Radiat. Phys. Chem.* **55**:795-798.

Lee MH, Moon YR, Chung BY, Kim JS, Lee KS, Cho JY and Kim JH. 2009. Practical use of chemical probes for reactive oxygen species produced in biological systems by γ-irradiation. *Radiat. Phys. Chem.* **78**:323-327.

Lidon FC and Henriques FS. 1993. Oxygen metabolism in higher plant chloroplasts. *Photosynthetica.* **29**(2):249-279.

Liszkay A, Kenk B and Schopfer P. 2003. Evidence for the involvement of cell wall peroxidase in the generation of hydroxyl radicals mediating extension growth. *Planta* **217**: 658-667.

Moon YR, Kim JH, Lee MH, Kim JS and Chung BY. 2008. Thermal dissipation of excess light in Arabidopsis leaves is inhibited after gamma-irradiation. *J. Plant Biol.* **51**(1):52-57.

Peskin AV, Labas YA and Tikhonov AN. 1998. Superoxide radical production by sponges *Sycon* sp. *FEBS Lett.* **434**: 201-204.

Spinks JWT and Woods RJ. 1990. Water and inorganic aqueous systems. pp. 243-313. *In*: An Introduction to Radiation Chemistry (Spinks JWT and Woods RJ *eds.*), John Wiley and Sons, New York, NY.

Thordal-Christensen H, Zhang Z, Wei Y and Collinge DB. 1997. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.* **11**:1187-1194.

Zielonka J, Sarna T, Roberts JE, Wishart JF and Kalyanaraman B. 2006. Pulse radiolysis and steady-state analyses of the reaction between hydroethidine and superoxide and other oxidants. *Arch. Biochem. Biophys.* **456**(1):39-47.

Manuscript Received: August 12, 2011
Revised: August 16, 2011
Revision Accepted: August 19, 2011