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SHORT COMMUNICATION

Direct assessment of the antioxidant properties of midazolam by electron spin resonance spectroscopy

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Abstract Some antioxidant anesthetics directly inhibit lipid peroxidation mediated via the generation of reactive oxygen species (ROS). To date, the scavenging effects of midazolam on ROS have not been directly assessed. We investigated the inhibitory effect of midazolam on ROS [hydroxyl radical ($HO^{\cdot}$) and superoxide ($O_2^{\cdot-}$)] by in vitro X-band electron spin resonance with the spin-trapping agent 5,5-dimethyl-1-pyrroline-*N*-oxide. Our results indicated that $HO^{\cdot}$ and $O_2^{\cdot-}$ were not affected by midazolam at clinically relevant concentrations, but were directly scavenged by midazolam at high concentrations (i.e., >4.6 and >1.5 mM, respectively).

Keywords Reactive oxygen species · Oxidative stress · Electron spin resonance (ESR) · Antioxidants · Midazolam

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Various reports have shown that oxidative stress induced by reactive oxygen species (ROS) such as the hydroxyl radical ($HO^{\cdot}$) and superoxide ($O_2^{\cdot-}$) can contribute to the pathophysiology of brain diseases, including ischemia–reperfusion injury [1], tumor [2], aging [3], and various neurodegenerative disorders [4]. The protective effects of general anesthetics against oxidative stress are well known [5–7]. Antioxidant anesthetics, particularly propofol (2,6-diisopropylphenol), have been shown to inhibit ROS-mediated lipid peroxidation [5, 6, 8]. In a previous study using electron spin resonance (ESR), we demonstrated that propofol with a carrier emulsion consisting of medium-chain triglycerides (MCT) and long-chain triglycerides (LCT) acted as a scavenger of $HO^{\cdot}$ in the brains of stroke-prone spontaneously hypertensive rats (SHRSPs) [9].

Midazolam and propofol are commonly used as sedatives for critically ill patients [10]. Such patients usually suffer from the pathologic effects of oxidative stress, defined as an imbalance between the generation of ROS and inactivation by antioxidant defense systems. Therefore, the antioxidant activities of propofol and midazolam may be of clinical importance. Propofol pretreatment greatly attenuated the impairment in comparison with midazolam, which agrees with the concept of antioxidant activity in the presence of membranes [10]. Propofol has previously been shown to have a greater antioxidant effect than midazolam against peroxyl radicals generated in the presence of erythrocyte membranes [10]. However, to date, the direct scavenging effects of midazolam on ROS have not been examined, for example by using a direct detection method such as ESR.

Our laboratories have been developing in vitro biomedical applications for the ESR detection of ROS such as $O_2^{\cdot-}$ [11], H_2O_2 [12], $^1O_2$ [13], and $HO^{\cdot}$ [14]. We have successfully applied this method to assess the antioxidant

properties of drugs in biological systems [9]. In the present study, we used the same technique to investigate the antioxidant effects of midazolam. We found that midazolam scavenged ROS at high concentrations, but that the antioxidant effect of midazolam was less potent than that of propofol.

Midazolam was purchased from Astellas Pharma Inc. (Tokyo, Japan). Xanthine oxidase [XO; (grade III: from bovine milk, chromatographically-purified suspension in 2.3 M $(NH_4)_2SO_4$–10 mM sodium phosphate buffer, pH 7.8, containing 1 mM EDTA and 1 mM sodium salicylate)], xanthine, and superoxide dismutase were obtained from Sigma (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) and $FeSO_4$ were obtained from Wako Chemical (Osaka, Japan). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Labotec (Tokyo, Japan).

$HO^{\cdot}$ was generated by the Fenton reaction (H_2O_2 plus $FeSO_4$) and by ultraviolet (UV) irradiation of H_2O_2, as described previously [9]. O_2^{-} was generated from xanthine–XO by methods also described previously [9]. All solutions were prepared in 0.1 M phosphate-buffered saline (pH 7.2). ESR spin-trapping was conducted with an ROS generating system containing DMPO. ESR observations were performed with a JES-RE 1X X-band spectrometer (JEOL, Tokyo, Japan) connected to a WIN-RAD ESR Data Analyzer (Radical Research, Tokyo, Japan) at the following instrument settings: microwave power 8.00 mW, magnetic field 335.0 ± 5 mT, field modulation width 0.079 mT, receiver gain 400–500, sweep time 1 min, and time constant 0.03 s. To quantify the spin adducts detected, we obtained ESR spectra for manganese oxide standards. After the ESR spectra had been recorded, the signal intensity (expressed as relative height) was normalized against the signal intensity of the manganese oxide standard marker [9]. All experiments were repeated a minimum of three times. The scavenging effect was considered to correspond to the signal intensity (percent of control), as described previously [15, 16].

Statistical analyses were performed using Ekuseru-Toukei 2008 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Results are presented as the mean ± SD. The Dunnett test was used for multiple comparisons. A p value of less than 0.05 was considered to be statistically significant.

In the present study, we used ESR spin-trapping with DMPO to assess the effect of midazolam on $HO^{\cdot}$ generated by the Fenton reaction. As reported previously [9], following the addition of H_2O_2 to $FeSO_4$ a characteristic DMPO–OH spin adduct with hyperfine splitting giving rise to four resolved peaks was observed (Fig. 1a; control). Midazolam at high concentrations (7.7–10 mM) reduced the DMPO–OH signal in a dose-dependent manner (Fig. 1).

In the next study, ESR spin-trapping with DMPO was used to assess the effect of midazolam on $HO^{\cdot}$ generated by

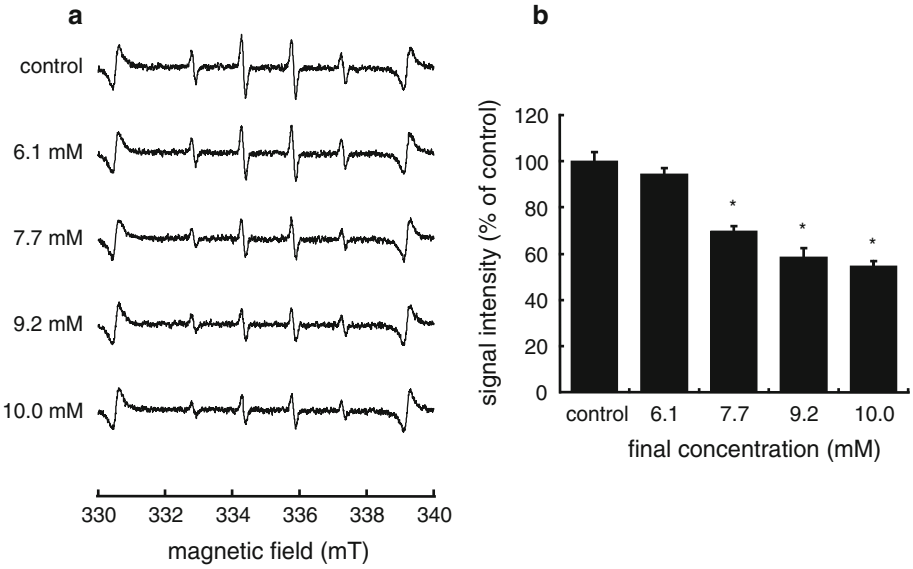

Fig. 1 Scavenging effects of midazolam on hydroxyl radicals ($HO^{\cdot}$) generated from the Fenton reaction. **a** Electron spin resonance (ESR) spin-trapping measurement of $HO^{\cdot}$ generated from H_2O_2 (20 μM) and $FeSO_4$ (20 μM) in 0.1 M phosphate-buffered saline (pH 7.2) with the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (50 mM) in the absence of midazolam (control) and with midazolam pretreatment at 6.1, 7.7, 9.2, and 10.0 mM. Signals appearing on either side of the ESR spectrum correspond to manganese oxide (MnO), used as a reference. **b** Dose–response showing the scavenging effect of midazolam (*closed column* 6.1–10.0 mM) on $HO^{\cdot}$ generated from the Fenton reaction. The signal intensity of the second peak of the spectrum was normalized to the signal intensity of the MnO standard. Data represent mean ± SD of three identical experiments. Values annotated with *asterisks* were significantly different ($p < 0.05$) from the corresponding control value

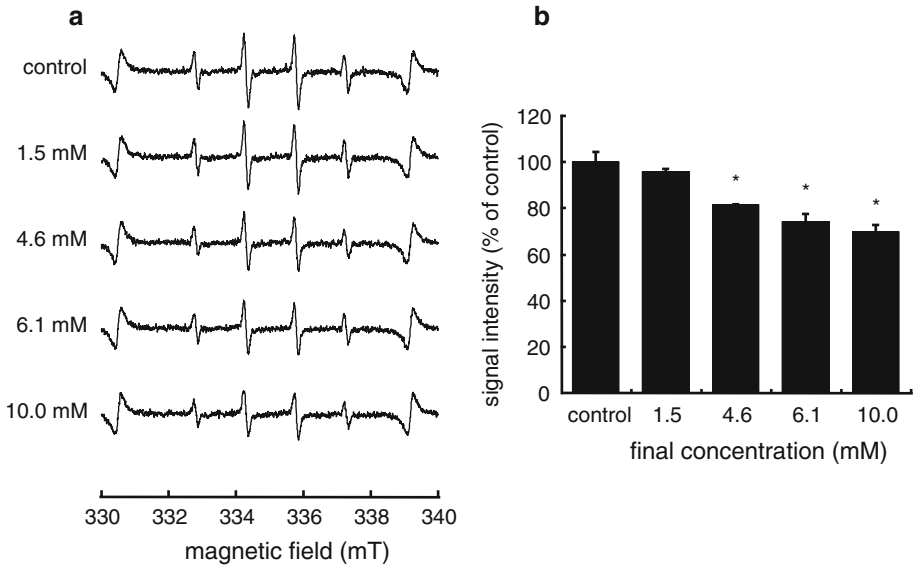

Fig. 2 Scavenging effects of midazolam on hydroxyl radicals ($HO^{\cdot}$) generated from the ultraviolet (UV) irradiation of H_2O_2. **a** Electron spin resonance (ESR) spin-trapping measurement of $HO^{\cdot}$ generated from the UV irradiation (365 nm, 40 mW) of H_2O_2 (20 mM) in 0.1 M phosphate-buffered saline (pH 7.2) with the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (50 mM) in the absence of midazolam (control) and with midazolam pretreatment at 1.5, 4.6, 6.1, and 10.0 mM. Signals appearing on either side of the ESR spectrum correspond to manganese oxide (MnO), used as a reference. **b** Dose–response showing the scavenging effect of midazolam (*closed column* 1.5–10.0 mM) on $HO^{\cdot}$ generated from the UV irradiation of H_2O_2. The signal intensity of the second peak of the spectrum was normalized to the signal intensity of the MnO standard. Data represent mean ± SD of three identical experiments. Values annotated with *asterisks* were significantly different ($p < 0.05$) from the corresponding control value

UV irradiation of H_2O_2. Consistent with previous studies [14, 17, 18], a characteristic DMPO–OH spin adduct spectrum with hyperfine splitting giving rise to four resolved peaks (Fig. 2a; control) was observed following the UV irradiation of H_2O_2. The DMPO–OH spin adduct was significantly reduced at high concentrations (>4.6 mM) (Fig. 2a). Pretreatment with midazolam (4.6–10.0 mM) reduced the DMPO–OH signal in a dose-dependent manner (Fig. 2b). These data suggest that high concentrations of midazolam directly scavenged $HO^{\cdot}$ by a mechanism that did not involve iron chelation (Fig. 1).

We also investigated the scavenging effects of midazolam on $O_2^{\cdot-}$, as measured by ESR spin-trapping with DMPO, in the presence of midazolam. As reported previously [9, 18], after the addition of xanthine to XO, a characteristic DMPO–OOH spin adduct with hyperfine splitting giving rise to 12 resolved peaks was observed (Fig. 3a; control). The addition of high concentrations of midazolam (1.5–6.1 mM) to the xanthine–XO system led to a concentration-dependent decrease in the intensity of the DMPO–OOH signal (Fig. 3).

When given intravenously for sedation, midazolam has been shown to decrease ROS production under conditions of surgical stress [19]. The potential neuroprotective effect of intravenous anesthetics such as propofol may be mediated by their antioxidant properties, which have been shown to inhibit apoptosis, ischemia–reperfusion injury, and inflammatory-induced neuronal damage [20, 21]. Several prior studies have suggested that midazolam does not have antioxidant effects, based on its inability to inhibit lipid peroxidation [22, 23]. Previous studies have shown that midazolam can inhibit lipid peroxidation in vitro [24]; however, it was not clear whether this was due to a direct scavenging effect on ROS. We therefore undertook the present ESR study to directly assess the effect of midazolam on $HO^{\cdot}$ and $O_2^{\cdot-}$. Our results provide direct evidence that high concentrations of midazolam scavenge $HO^{\cdot}$ as well as $O_2^{\cdot-}$ generated from xanthine–XO (Figs. 1, 2, 3).

First, we showed that midazolam dose-dependently scavenged $HO^{\cdot}$ generated by the Fenton reaction (Fig. 1). This type of iron-dependent generation of $HO^{\cdot}$ has been implicated in the pathophysiologies of various disease states; for example, DMPO–OH spin adducts can be detected in the synovial fluid of patients with temporomandibular disease [25], and in gingival fibroblasts from patients with Down's syndrome [26].

We also investigated the effect of midazolam on $HO^{\cdot}$ generated by the UV irradiation of H_2O_2, which is known to be an Fe^{2+}-independent reaction [14, 17]. Interestingly, high concentrations of midazolam dose-dependently inhibited the $HO^{\cdot}$ generated via the UV irradiation of H_2O_2 (Fig. 2). Propofol MCT/LCT was previously shown to scavenge $HO^{\cdot}$ in a dose-dependent manner in this system [9], and the inhibitory effects of propofol MCT/LCT were observed at

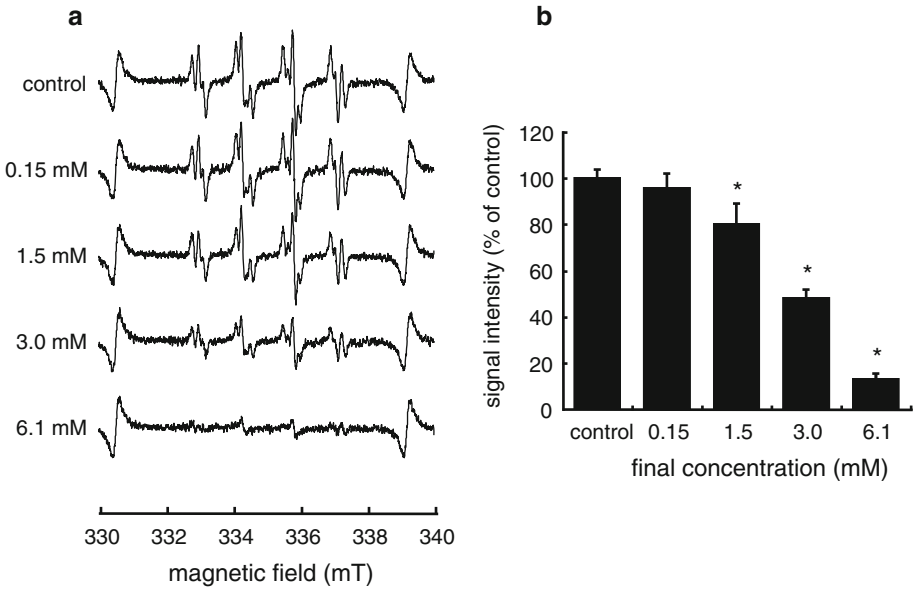

Fig. 3 Scavenging effects of midazolam on superoxide ($O_2^{\cdot-}$) generated by the addition of xanthine to XO. **a** Electron spin resonance (ESR) spin-trapping measurement of $O_2^{\cdot-}$ generated from xanthine oxidase (XO; 0.1 U/ml) and xanthine (362 μM) in 0.1 M phosphate-buffered saline (pH 7.2) with the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (440 mM) in the absence of midazolam (control) and with midazolam pretreatment at 0.15, 1.5, 3.0, and 6.1 mM. Signals appearing on either side of the ESR spectrum correspond to manganese oxide (MnO), used as a reference. **b** Dose–response showing the scavenging effect of midazolam (*closed column* 0.15–6.1 mM) on $O_2^{\cdot-}$ generated from XO and xanthine. The signal intensity of the second peak of the spectrum was normalized to the signal intensity of the MnO standard. Data represent mean ± SD of three identical experiments. Values annotated with *asterisks* were significantly different ($p < 0.05$) from the corresponding control value

concentrations lower than those required with midazolam in the Fenton system (>7.7 mM; Fig. 1) and H_2O_2/UV system (>4.6 mM; Fig. 2). Midazolam at high concentrations (>1.5 mM) was also found to be a scavenger of $O_2^{\cdot-}$ generated by xanthine–XO (Fig. 3), but it did not scavenge $O_2^{\cdot-}$ treated with the propofol MCT/LCT (original liquid 5.609 mM) [9].

These results indicate that midazolam and propofol MCT/LCT differ in their ROS-scavenging properties and that this difference may be clinically significant. In the case of propofol, antioxidant activity seems to be mediated by hindered phenolic structures [27], as in the case of butylated hydroxytoluene, butylated hydroxyanisole, and tocopherols [28], whereas for midazolam, the structure responsible is unclear. However, in a previous study on stobadine, a pyridoindole derivative that possesses potent antioxidant activity, Kagan et al. [29] noted the possibility that intrinsic nitrogen atoms might act as an antioxidant by donating electrons to free radicals. This might also be the case with midazolam.

The lowest concentration of midazolam used clinically was about 0.3 μM; the minimum concentrations required for inhibition ranged from 1.5 mM for $O_2^{\cdot-}$ to 4.6–7.7 mM for $HO^{\cdot}$. This finding contrasts with our previous ESR study, which showed ROS-scavenging effects of propofol MCT/LCT at concentrations below 112 μM [9]. These results suggest that the antioxidant activity of propofol MCT/LCT may be therapeutically beneficial in patients with diseases associated with ROS. Propofol was also shown to be more effective than midazolam at reducing oxidative stress in a homogeneous solution containing erythrocyte membranes [24]. Interestingly, $O_2^{\cdot-}$ scavenging was observed with high concentrations of midazolam in our study (Fig. 3), but not with propofol MCT/LCT in a previous study [9]. This finding may be clinically relevant because $O_2^{\cdot-}$ has been shown to play a role in ischemia–reperfusion injury [30].

In conclusion, this in vitro ESR study showed that the $O_2^{\cdot-}$ and $HO^{\cdot}$ can be scavenged by midazolam, but this effect occurs only at high rather than clinically relevant concentrations.

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References

1. Saito A, Maier CM, Narasimhan P, Nishi T, Song YS, Yu F, Liu J, Lee YS, Nito C, Kamada H, Dodd RL, Hsieh LB, Hassid B, Kim EE, Gonzalez M, Chan PH. Oxidative stress and neuronal death/

survival signaling in cerebral ischemia. Mol Neurobiol. 2005;31:105–16.
2. Ajamieh H, Merino N, Candelario-Jalil E, Menendez S, Martinez-Sanchez G, Re L, Giuliani A, Leon OS. Similar protective effect of ischaemic and ozone oxidative preconditionings in liver ischaemia/reperfusion injury. Pharmacol Res. 2002;45:333–9.
3. Poon HF, Calabrese V, Scapagnini G, Butterfield DA. Free radicals and brain aging. Clin Geriatr Med. 2004;20:329–59.
4. Emerit J, Edeas M, Bricaire F. Neurodegenerative diseases and oxidative stress. Biomed Pharmacother. 2004;58:39–46.
5. Wilson JX, Gelb AW. Free radicals, antioxidants, and neurologic injury: possible relationship to cerebral protection by anesthetics. J Neurosurg Anesthesiol. 2002;14:66–79.
6. Chikutei K, Oyama TM, Ishida S, Okano Y, Kobayashi M, Matsui H, Horimoto K, Nishimura Y, Ueno SY, Oyama Y. Propofol, an anesthetic possessing neuroprotective action against oxidative stress, promotes the process of cell death induced by H_2O_2 in rat thymocytes. Eur J Pharmacol. 2006;540:18–23.
7. Cuadrado A, Solares G, Gonzalez S, Sanchez B, Armijo JA. Propofol concentrations in whole blood: influence of anticoagulants and storage time. Methods Find Exp Clin Pharmacol. 1998;20:297–300.
8. De La Cruz JP, Villalobos MA, Sedeno G, Sanchez De La Cuesta F. Effect of propofol on oxidative stress in an in vitro model of anoxia-reoxygenation in the rat brain. Brain Res. 1998;800:136–44.
9. Kobayashi K, Yoshino F, Takahashi SS, Todoki K, Maehata Y, Komatsu T, Yoshida K, Lee MC. Direct assessments of the antioxidant effects of propofol medium chain triglyceride/long chain triglyceride on the brain of stroke-prone spontaneously hypertensive rats using electron spin resonance spectroscopy. Anesthesiology. 2008;109:426–35.
10. Tsuchiya H. Structure-specific membrane-fluidizing effect of propofol. Clin Exp Pharmacol Physiol. 2001;28:292–9.
11. Lee C, Miura K, Liu X, Zweier JL. Biphasic regulation of leukocyte superoxide generation by nitric oxide and peroxynitrite. J Biol Chem. 2000;275:38965–72.
12. Kiyose M, Lee CI, Okabe E. Inhibition of skeletal sarcoplasmic reticulum Ca^{2+}-ATPase activity by deferoxamine nitroxide free radical. Chem Res Toxicol. 1999;12:137–43.
13. Yoshino F, Shoji H, Lee MC. Vascular effects of singlet oxygen ($^1O_2$) generated by photo-excitation on adrenergic neurotransmission in isolated rabbit mesenteric vein. Redox Rep. 2002;7:266–70.
14. Ogasawara Y, Namai T, Yoshino F, Lee MC, Ishii K. Sialic acid is an essential moiety of mucin as a hydroxyl radical scavenger. FEBS Lett. 2007;581:2473–7.
15. Guo Q, Zhao B, Li M, Shen S, Xin W. Studies on protective mechanisms of four components of green tea polyphenols against lipid peroxidation in synaptosomes. Biochim Biophys Acta. 1996;1304:210–22.
16. Ohsugi M, Fan W, Hase K, Xiong Q, Tezuka Y, Komatsu K, Namba T, Saitoh T, Tazawa K, Kadota S. Active-oxygen scavenging activity of traditional nourishing-tonic herbal medicines and active constituents of *Rhodiola sacra*. J Ethnopharmacol. 1999;67:111–9.
17. Sakurai K, Sasabe H, Koga T, Konishi T. Mechanism of hydroxyl radical scavenging by rebamipide: identification of mono-hydroxylated rebamipide as a major reaction product. Free Radic Res. 2004;38:487–94.
18. Buettner GR. Spin trapping: ESR parameters of spin adducts. Free Radic Biol Med. 1987;3:259–303.
19. Cheng YJ, Wang YP, Chien CT, Chen CF. Small-dose propofol sedation attenuates the formation of reactive oxygen species in tourniquet-induced ischemia–reperfusion injury under spinal anesthesia. Anesth Analg. 2002;94:1617–20.
20. Engelhard K, Werner C, Eberspacher E, Pape M, Stegemann U, Kellermann K, Hollweck R, Hutzler P, Kochs E. Influence of propofol on neuronal damage and apoptotic factors after incomplete cerebral ischemia and reperfusion in rats: a long-term observation. Anesthesiology. 2004;101:912–7.
21. Yasuda T, Takahashi S, Matsuki A. Tumor necrosis factor-alpha reduces ketamine- and propofol-induced anesthesia time in rats. Anesth Analg. 2002;95:952–5.
22. Sayin MM, Ozatamer O, Tasoz R, Kilinc K, Unal N. Propofol attenuates myocardial lipid peroxidation during coronary artery bypass grafting surgery. Br J Anaesth. 2002;89:242–6.
23. Erol U, Gurdal M, Erol A, Aslan R, Konukoglu D, Onmus H. Is midazolam effective as an antioxidant in preventing reperfusion injury in rat kidney? Int Urol Nephrol. 2002;34:121–7.
24. Tsuchiya M, Asada A, Maeda K, Ueda Y, Sato EF, Shindo M, Inoue M. Propofol versus midazolam regarding their antioxidant activities. Am J Respir Crit Care Med. 2001;163:26–31.
25. Lee MC, Kawai Y, Shoji H, Yoshino F, Miyazaki H, Kato H, Suga M, Kubota E. Evidence of reactive oxygen species generation in synovial fluid from patients with temporomandibular disease by electron spin resonance spectroscopy. Redox Rep. 2004;9:331–6.
26. Komatsu T, Lee MC, Miyagi A, Shoji H, Yoshino F, Maehata Y, Maetani T, Kawamura Y, Ikeda M, Kubota E. Reactive oxygen species generation in gingival fibroblasts of Down syndrome patients detected by electron spin resonance spectroscopy. Redox Rep. 2006;11:71–7.
27. Murphy PG, Myers DS, Davies MJ, Webster NR, Jones JG. The antioxidant potential of propofol (2,6-diisopropylphenol). Br J Anaesth. 1992;68:613–8.
28. Kagan VE, Serbinova EA, Packer L. Generation and recycling of radicals from phenolic antioxidants. Arch Biochem Biophys. 1990;280:33–9.
29. Kagan VE, Tsuchiya M, Serbinova E, Packer L, Sies H. Interaction of the pyridoindole stobadine with peroxyl, superoxide and chromanoxyl radicals. Biochem Pharmacol. 1993;45:393–400.
30. Chan PH. Role of oxidants in ischemic brain damage. Stroke. 1996;27:1124–9.