Free Radical Research, April 2008; 42(4): 305–311

informa
healthcare

Effect of anaesthesia-induced alterations in haemodynamics on *in vivo* kinetics of nitroxyl probes in electron spin resonance spectroscopy

TAKAKI TSUTSUMI[1], TOMOMI IDE[1], MAYUMI YAMATO[2], MAKOTO ANDOU[1], TAKESHI SHIBA[2], HIDEO UTSUMI[3], & KENJI SUNAGAWA[1]

[1]*Department of Cardiovascular Medicine, Graduate School of Medical Sciences,* [2]*Department REDOX Medicinal Science, and* [3]*Department of Biofunctional Science, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan*

Accepted by Dr E. Niki

(Received 13 November 2007; in revised form 11 February 2008)

Abstract

Although the advent of *in vivo* electron spin resonance (ESR) spectroscopy has allowed analysis of the redox status of living animals, whether the haemodynamic condition affects the signal decay rate remains unknown. Three kinds of haemodynamic conditions were generated by changing the anaesthetic dosage in mice. Haemodynamics was analysed ($n = 6$ each) and *in vivo* ESR was performed to measure the signal decay rates of three nitroxyl spin probes (carbamoyl-, carboxy- and methoxycarbonyl-PROXYL) at the chest and head regions ($n = 6$ for each condition and probe). Haemodynamic analysis revealed negative inotropic and chronotropic effects on the cardiovascular system depending on the depth of anaesthesia. Although signal decay rates differed among three probes, they were not affected by heart rate alteration. In this study we report the haemodynamics-independent signal decay rate of nitoxyl probes.

Keywords: *ESR, nitroxide, reactive oxygen species*

Abbreviations: *BP, blood pressure; 3-carbamoyl-PROXYL, 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl; 3-carboxy-PROXYL, 3-carboxy-2,2,5,5-tetramethylpyrrolidine-l-oxyl; CmP, 3-carbamoyl-PROXYL; CxP, 3-carboxy-PROXYL; ESR, electron spin resonance; FS, fractional shortening; HR, heart rate; LV, left ventricle; LVEDD, left ventricular end-diastolic dimension; LVEDP, left ventricular end-diastolic pressure; LVESD, left ventricular end-systolic dimension; 3-methoxycarbonyl-PROXYL, 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl; MCP, 3-methoxycarbonyl-PROXYL; ROS, reactive oxygen species.*

Introduction

Free radicals and reactive oxygen species (ROS) are important mediators in the pathogenesis of several diseases. Experimental studies have shown that free radicals and ROS cause lipid peroxidation [1], protein oxidation [2] and DNA damage [3], resulting in cellular damage. The evaluation of ROS generation and/or redox status *in vivo* is important in understanding the pathogenic mechanisms of oxidative stress.

In the last few decades, the development of *in vivo* electron spin resonance (ESR) spectroscopy has made it possible to measure exogenously administered paramagnetic species in living animals [4,5]. Furthermore, nitroxyl radicals are used as spin probes in a variety of biological experiments in which ESR spectroscopy was used to detect ROS [6–9] and redox status [10]. The *in vivo* ESR signal decay rates of spin probes are enhanced by ROS such as hydroxy radical [3] and superoxide [11,12].

Correspondence: Tomomi Ide, MD PhD, Departmentt of Cardiovascular Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582 Japan. Tel: +81-92-642-5359. Fax: +81-92-642-5374. Email: tomomi_i@cardiol.med.kyushu-u.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760801986542

The signal decay rate also depends on kinetic factors such as the distribution of the spin probe from blood to tissues and vice versa [13], urinary excretion via the kidney [14,15], faecal excretion via the liver and bile and transport into specific tissues/organs [15]. These factors are affected by anaesthesia-related physiological responses such as the haemodynamic changes and anaesthetics are often required in experiments using living animals. Anaesthetics are known to have significant negative inotropic effects on cardiovascular parameters [16–19]. They depress the cardiac function and decrease the peripheral vascular resistance, causing bradycardia, hypotension and decreased cardiac output. However, little is known about the effect of haemodynamic change on signal decay rates of spin probes *in vivo*.

In the present study, we evaluated whether the haemodynamic change affects the signal decay rates of spin probe *in vivo*. For this purpose, mice were anaesthetized by varying doses of pentobarbital sodium and divided into three groups of low-, intermediate- and high-heart rate (HR), which was used as an index of haemodynamic parameter.

Materials and methods

Animals and experimental protocol

Male CD-1 mice weighing 30–35 g were purchased from Kyudo Ltd. (Fukuoka, Japan). The mice were housed in a temperature- and humidity-controlled room. They were fed by a commercial diet and provided water *ad libitum*. Mice were randomly assigned to three groups: high-HR group, intermediate-HR group and low-HR group, and then anaesthetized by intraperitoneal injection of pentobarbital sodium at doses of 30, 40 and 50 mg/kg body weight, respectively ($n = 6$ in each group for *in vivo* ESR) (Figure 1. After 15 min of anaesthesia, we measured rectal temperature quickly and performed ESR analysis. We also measured their rectal temperature during ESR analysis (at 3 min after the injection of spin probe). Although rectal temperature tended to be $\sim 1^{\circ}C$ lower during EPR measurement than before, there was no statistical significance among the groups (Table I). At 30 min after the injection of pentobarbital, blood samples were taken from LV for blood gas analysis. There was no difference in blood pH, pCO_2 or pO_2 among groups. All of the procedures were done within 40 min.

All procedures and animal care were approved by the Committee on Ethics of Animal Experiment, Kyushu University Graduate School of Medical and Pharmaceutical Sciences and carried out in accordance with the Guideline for Animal Experiment, Kyushu University and the Law (No.105) and Notification (No.6) of the Government. The investigations conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Chemicals

3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (3-carbamoyl-PROXYL) and 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl (3-carboxy-PROXYL) were purchased from Aldrich Chemical Co. (Milwaukee, WI). 3-Methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-l-oxyl (3-methoxycarbonyl-PROXYL) was synthesized as described previously [20]. Each nitroxyl probe was dissolved in physiological saline to a final concentration of 100 mmol/L. The three isotonic probe solutions were analysed by X-band ESR and adjusted to contain the same concentration of nitroxyl radicals. All other reagents used were of the highest commercially available purity.

In vivo ESR spectroscopy

For the *in vivo* ESR measurements, 3 μL/g body weight of an isotonic PROXYL solution (100 mmol/L) was administered intravenously. ESR spectra were recorded every minute at the chest or head region using a L-band ESR spectrometer (JEOL Co. Ltd., Akishima, Japan) with a loop-gap resonator (33 mm i.d. and 30 mm in length). The power of the 1.1 GHz microwave was 10 mW. The amplitude of the 100-kHz field modulation was 0.063 mT. Signal intensity was estimated from the height of the first positive peak in the spectrum. The signal decay rate was determined from the semi-logarithmic plot of signal intensity vs time after probe injection.

ECG acquisition

Needle electrodes were inserted subcutaneously into each of the four limbs. HR was calculated from electrocardiogram that was amplified and digitized online at 200 Hz by a 12-bit analogue-to-digital converter (AIO ADA 12-32/2(CB)F; Contec Co., CA). The digitized data and HR were stored on a hard disk for subsequent off-line analysis.

Echocardiography and haemodynamics measurements

Physiological evaluation with echocardiography and left heart catheterization was conducted as an independent experiment ($n = 6$, each group). At 15 min after anaesthesia, a 7.5-MHz transducer connected to a dedicated ultrasonographic system (SSD-5500, Aloka Co., Tokyo) was applied to the left hemithorax. Two-dimensional targeted M-mode imaging was obtained from the short-axis view at a level showing the greatest left ventricular (LV)

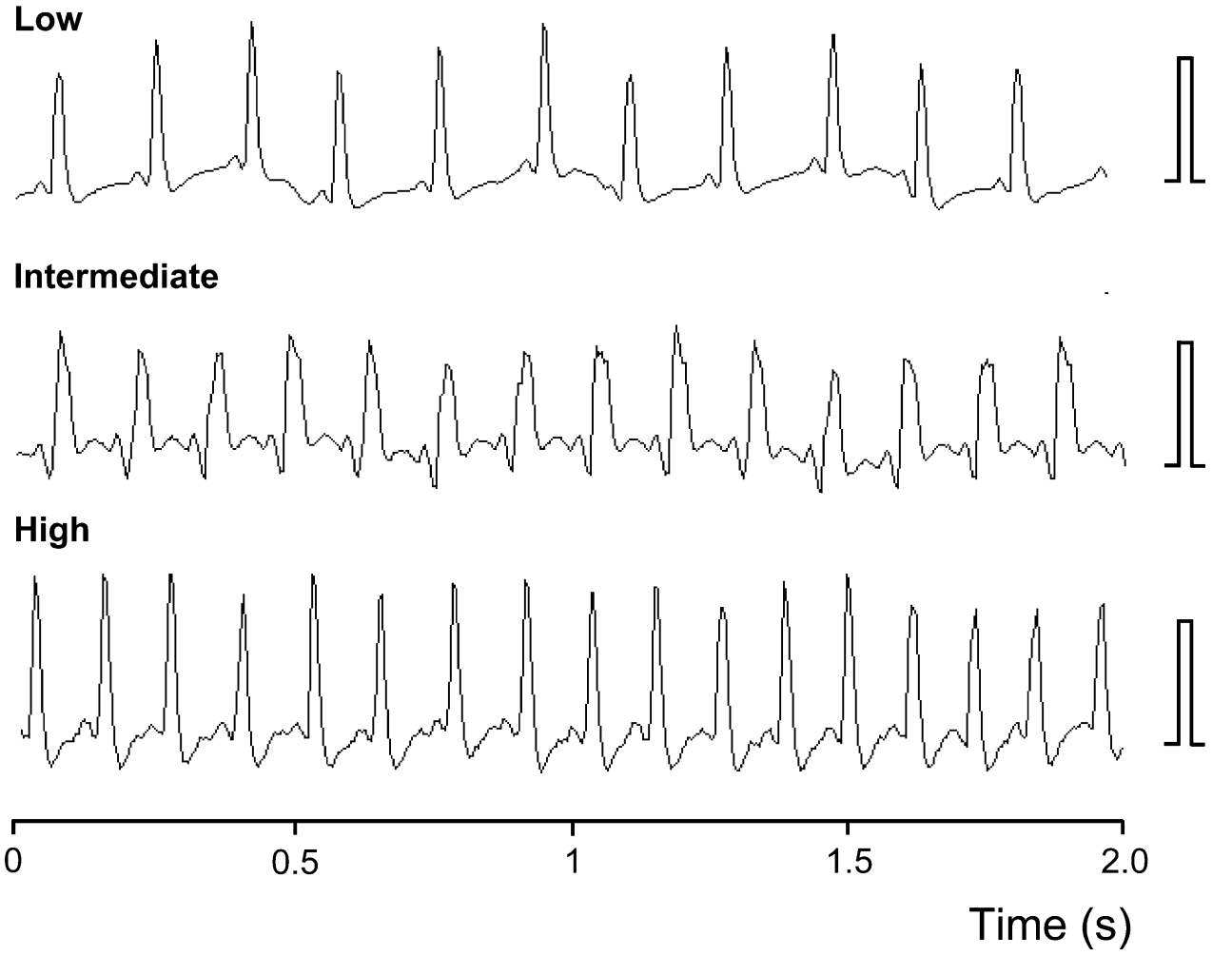

Figure 1. Typical electrocardiograms recorded from mice in the low-HR (HR: 330 bpm), intermediate-HR (HR: 420 bpm) and high-HR (HR: 510 bpm) groups. The height of the square on the right represents 1 mV.

dimension and end-diastolic (LVEDD) and end-systolic LV dimension (LVESD) were measured. Fractional shortening (FS) was calculated as follows.

$$\text{FS}\ (\%) = (\text{LVESD} - \text{LVEDD})/\text{LVEDD} \times 100$$

After echocardiographic recording, a 1.4 F micro-manometer-tipped catheter (Millar Instruments, Houston, TX) was inserted into the right common carotid artery and advanced into the ascending aorta and LV for pressure measurement. All of the procedures ended within 40 min for each mouse.

Statistical analysis

Each value represents the mean ± SEM. Inter-group differences were analysed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Values of P less than 0.05 were accepted as statistically significant.

Results

HR and cardiac function

Echocardiography and left heart catheterization were performed to evaluate haemodynamics under different HR conditions induced by anaesthesia. As summarized in Table II, a marked negative inotropic and chronotropic effect on the cardiovascular system was observed dependent on the depth of anaesthesia. The HR ranged from 287–359 bpm in low-, from

Table I. Physiological variables.

	Low-HR	Intermediate-HR	High-HR
n	6	6	6
pH	7.15 ± 0.11	7.17 ± 0.09	7.19 ± 0.06
P_{CO2}, mmHg	38.5 ± 1.9	40.3 ± 8.7	40.4 ± 7.6
P_{O2}, mmHg	113.9 ± 15.8	119.6 ± 17.0	113.1 ± 28.3
Temperature, °C (before ESR)	36.1 ± 1.7	35.5 ± 1.6	36.6 ± 1.0
Temperature, °C (after ESR)	35.0 ± 1.9	34.5 ± 1.4	35.6 ± 1.0

Each value represents the mean ± SEM.

Table II. Characteristics of animal models.

	Low-HR	Intermediate-HR	High-HR
n	6	6	6
Heart rate, bpm	317±9	434±6*	538±8*†
Echocardiographic data			
LVEDD, mm	4.1±0.1	3.7±0.1	3.5±0.1*
LVESD, mm	2.2±0.1	1.9±0.1	1.6±0.1*
Fractional shortening, %	45±1	49±3	54±2
Haemodynamic data			
Mean BP, mmHg	65.2±4.0	83.1±4.8	101.2±2.6*†
Systolic BP, mmHg	83.1±4.1	102.0±5.4	122.0±2.3*†
Diastolic BP, mmHg	48.9±3.4	62.4±5.1	79.1±2.5*†
LVEDP, mmHg	3.5±0.4	3.1±0.7	3.2±0.7
dP/dt_{max}, mmHg/s	5890±670	8730±650	13 500±870*†
dP/dt_{min}, mmHg/s	−4510±430	−6640±310	−9 900±1190*†

LV indicates left ventricular; EDD, end-diastolic diameter; ESD, end-systolic diameter; BP, blood pressure; EDP, end-diastolic pressure. Value are mean±SEM. *$P<0.05$ vs low-HR. †$P<0.05$ vs intermediate-HR.

426–451 bpm in intermediate- and from 510–545 bpm in high-HR group. LVESD and LVEDD were significantly larger and FS was significantly lower in low-HR mice compared with high-HR mice. Aortic pressure and the first derivative of LV pressure (dP/dt_{max}) were also depressed in low-HR group compared to high-HR group. There were no significant differences in left ventricular end-diastolic pressure (LVEDP) among three groups.

In vivo *ESR spectroscopy*

ESR spectra of three spin probes (Figure 2A) were recorded at the chest or head region in mice with different haemodynamic conditions. The *in vivo* spectrum (Figure 2B) consisted of sharp triplet lines, the intensity of which decreased gradually with time after probe injection (Figure 2C). Although the rate of signal decay recorded at the chest differed among

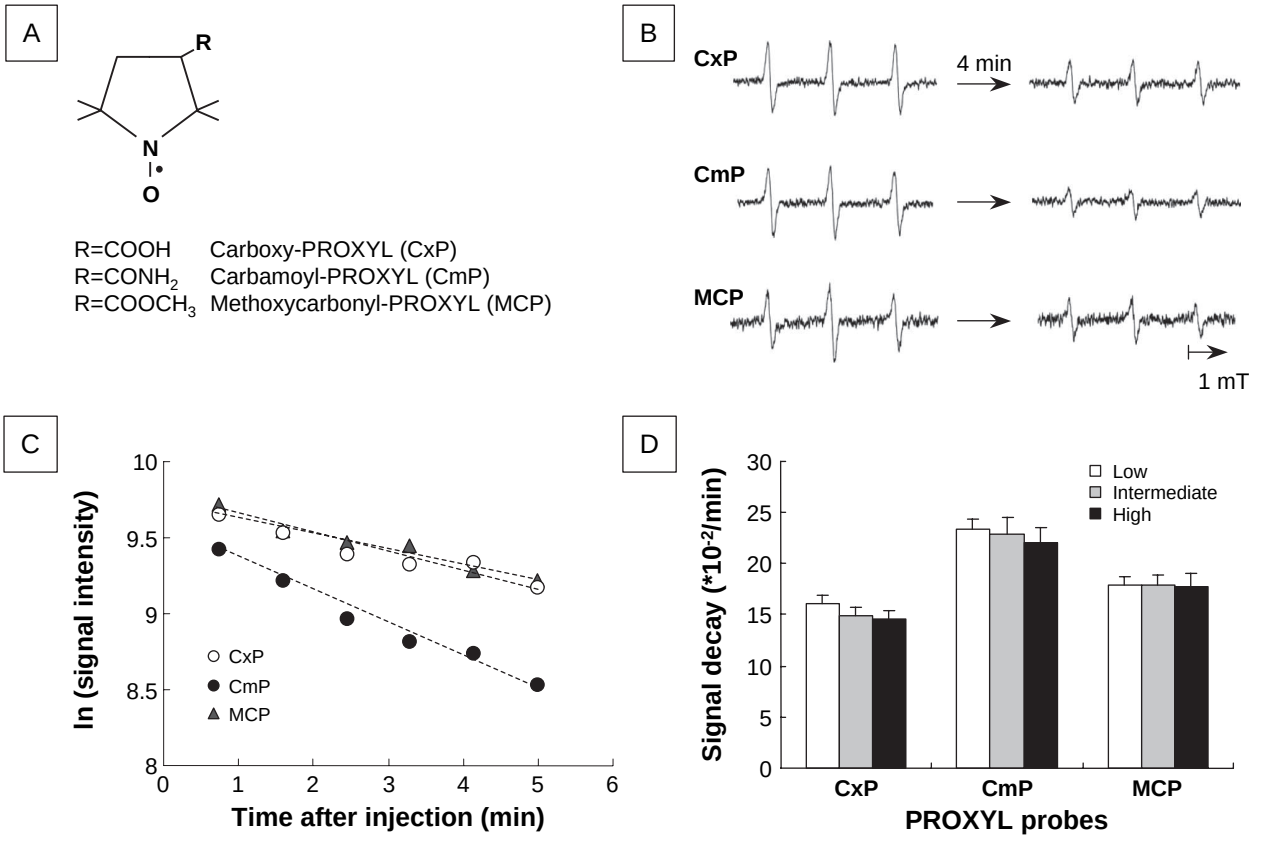

Figure 2. The chemical structure of nitroxide spin probes (A); typical ESR spectra of three spin probes (B); *in vivo* ESR signal decay curves of three spin probes from spectra recorded at chest region in the high-HR group (C); and signal decay rates of spin probes in low-HR, intermediate-HR and high-HR groups (D). Values are means±SEM. The data of (C) and (D) were obtained from six animals.

Table III. Signal deay rate at the head region ($\star 10^{-2}$/min).

	Low-HR	Intermediate-HR	High-HR
Carboxy-PROXYL	5.2±0.9	6.2±0.3	5.7±1.7
Carbamoyl-PROXYL	9.5±1.0	9.3±1.6	10.3±2.2
Methoxycarbonyl-PROXYL	17.5±1.3	17.0±1.3	17.6±1.5

Values are mean±SEM. No statistical significant differences among low-HR, intermediate-HR and high-HR were observed in each probe.

probes, they were not affected by the HR alteration (Figure 2D). Similarly, the signal decay is the highest with Methoxycarbonyl-PROXYL compared with the other two probes at the head level; however, HR did not affect the decay rate at all (Table III).

Discussion

Although *in vivo* ESR spectroscopy is a powerful tool to determine the free radical generation and redox status in living animals, the effect of haemodynamic change associated with the depth of anaesthesia on the decay rate of ESR signals remains unclear. In this study, we demonstrated that the signal decay rate of particular nitroxyl probes was independent of the haemodynamic condition.

The kinetics of the intravenously administered spin probes are influenced by several factors [13–15]. However, in performing *in vivo* ESR spectroscopy, spin probe has to be administered intravenously and its transport to the target organs is dependent on the blood flow. Therefore, the haemodynamic condition is considered to have considerable effect on the decay kinetics of spin probes. In this study, for the purpose of generating a broad range of haemodynamic states, we chose pentobarbital sodium among many anaesthetics, because of its wide use in experiments using rodents and its profound cardiac depressive effect compared with other intravenous or inhalation anaesthetics [19,21]. Mice were divided into three HR groups by changing the pentobarbital dosage. The average HR and dp/dt_{max} in high-HR mice were ~550 bpm and 13500 mmHg/s, respectively, which were close to the values reported in conscious mice [22]. In contrast, in mice of the low-HR group, HR and dp/dt_{max} were profoundly depressed, supporting the success of producing various haemodynamic conditions by varying the anaesthetic dose. Among these three groups, there were no significant differences in signal decay rate when each spin probe was used to acquire ESR spectra at chest and head levels.

Several mechanisms may be proposed to explain the haemodynamics-independent signal decay rate. The first factor is the acquisition of ESR spectra 30–50 s after injection of the probe. In general, the circulation time of the probes ranges from a few seconds to 1 min at the longest. Therefore, it probably takes a few seconds to a few minutes for the nitroxyl probes to reach an equilibrium state at the target organ. Takeshita et al. [23] have reported that the ESR signal obtained from mouse skin increased up to 2–3 min after injection of carbamoyl-PROXYL and then began to decrease in a first-order kinetics. However, such lag before decay was not observed in ESR spectra recorded at the chest and head levels in this study. Therefore, the time to reach equilibrium probably depends on the amount of blood supply and the vasculature structure in each organ. However, once equilibrium is achieved, the nitroxyls are reduced to reaction adduct such as secondary amine and their corresponding hydroxylamine without being affected by haemodynamics, consequently demonstrating loss of paramagnetism in a first order kinetics [24]. As we started ESR spectroscopy a few minutes after spin probe injection, the equilibrium condition has already been achieved in all groups. The second factor is the potential tissue transfer of the nitroxyl probes. Methoxycarbonyl-PROXYL is a lipophilic agent that passes through cell membrane [20,25,26]. Although carboxy-PROXYL are relatively hydrophilic [20,25,26], it was reported to be promptly taken up into tissues through the organic anion transporter [15]. Thus, a high potential of tissue transfer is also considered to be associated with the haemodynamics-independent signal decay.

There are a couple of unresolved questions in this study. First, there is a possibility that haemodynamic effects may induce biochemical alteration in longer period. Since we observed only a short-term change, further investigation is necessary for the long-time assessment. Secondly, we observe a slight tendency of the HR dependent decrease of the signal decay in carboxy-PROXYL and carbamoyl-PROXYL but not in MC-PROXYL. This might be due to the hydrophobicity of the spin probes. To clear this, we need to repeat a similar experiment in animals with more extreme conditions, which may be technically difficult in small animals.

On the other hand, there are some interesting observations in this study. After intravenous administration, the spin probes distribute from the blood to

the tissues [13], where they are reduced by enzymatic reaction [27–30]. There might be organ-specific differences in the enzymatic reduction of each probe. The order of clearance constant at the chest was as follows: carboxy- < methoxycarbonyl- < carbamoyl-PROXYL. In contrast, methoxycarbonyl-PROXYL showed the fastest signal decay rate at the head. Although the mechanisms of these differences were not elucidated in this study, the determinants of signal decay rate of each probe may depend not only on the target organs but also on the characteristics of the probe. Further studies are required to clarify these differences between spin probes.

In Conclusion, the haemodynamic condition does not affect the decay rate of ESR signal when using the *in vivo* ESR spectroscopy spin probe method. The variation in HR by anaesthetics may be compensated by homeostatic control *in vivo*, resulting in unaltered signal decay of spin probes.

Acknowledgements

This study was supported in part by grants from the Ministry of Education, Science and Culture, Health and Labor Sciences Research Grant for Comprehensive Research in Aging and Health Labor and Welfare of Japan, Japan Cardiovascular Research Foundation and Uehara Memorial Foundation. A part of this study was conducted in Kyushu University Station for Collaborative Research II.

References

[1] Kato N, Yanaka K, Hyodo K, Homma K, Nagase S, Nose T. Stable nitroxide Tempol ameliorates brain injury by inhibiting lipid peroxidation in a rat model of transient focal cerebral ischemia. Brain Res 2003;979:188–193.

[2] Hayashi T, Suda K, Imai H, Era S. Simple and sensitive high-performance liquid chromatographic method for the investigation of dynamic changes in the redox state of rat serum albumin. J Chromatogr B Analyt Technol Biomed Life Sci 2002;772:139–146.

[3] Offer T, Samuni A. Nitroxides inhibit peroxyl radical-mediated DNA scission and enzyme inactivation. Free Radic Biol Med 2002;32:872–881.

[4] Berliner LJ, Wan XM. *In vivo* pharmacokinetics by electron magnetic resonance spectroscopy. Magn Reson Med 1989;9:430–434.

[5] Ferrari M, Colacicchi S, Gualtieri G, Santini MT, Sotgiu A. Whole mouse nitroxide free radical pharmacokinetics by low frequency electron paramagnetic resonance. Biochem Biophys Res Commun 1990;166:168–173.

[6] Yamato M, Egashira T, Utsumi H. Application of *in vivo* ESR spectroscopy to measurement of cerebrovascular ROS generation in stroke. Free Radic Biol Med 2003;35:1619–1631.

[7] Yamada K, Nakamura T, Utsumi H. Enhanced intraarticular free radical reactions in adjuvant arthritis rats. Free Radic Res 2006;40:455–460.

[8] Kasazaki K, Yasukawa K, Sano H, Utsumi H. Non-invasive analysis of reactive oxygen species generated in NH4OH-induced gastric lesions of rats using a 300 MHz *in vivo* ESR technique. Free Radic Res 2003;37:757–766.

[9] Phumala N, Ide T, Utsumi H. Noninvasive evaluation of in vivo free radical reactions catalyzed by iron using *in vivo* ESR spectroscopy. Free Radic Biol Med 1999;26:1209–1217.

[10] Kuppusamy P, Li H, Ilangovan G, Cardounel AJ, Zweier JL, Yamada K, Krishna MC, Mitchell JB. Noninvasive imaging of tumor redox status and its modification by tissue glutathione levels. Cancer Res 2002;62:307–312.

[11] Krishna MC, Grahame DA, Samuni A, Mitchell JB, Russo A. Oxoammonium cation intermediate in the nitroxide-catalyzed dismutation of superoxide. Proc Natl Acad Sci USA 1992;89:5537–5541.

[12] Samuni A, Krishna CM, Mitchell JB, Collins CR, Russo A. Superoxide reaction with nitroxides. Free Radic Res Commun 1990;9:241–249.

[13] Takechi K, Tamura H, Yamaoka K, Sakurai H. Pharmacokinetic analysis of free radicals by *in vivo* BCM (Blood Circulation Monitoring)-ESR method. Free Radic Res 1997;26:483–496.

[14] Daryani A, Basu S, Becker W, Larsson A, Riserus U. Antioxidant intake, oxidative stress and inflammation among immigrant women from the Middle East living in Sweden: associations with cardiovascular risk factors. Nutr Metab Cardiovasc Dis 2007;17:748–756.

[15] Ichikawa K, Sato Y, Kondo H, Utsumi H. An ESR contrast agent is transported to rat liver through organic anion transporter. Free Radic Res 2006;40:403–408.

[16] Smith TL, Hutchins PM. Anesthetic effects on hemodynamics of spontaneously hypertensive and Wistar-Kyoto rats. Am J Physiol 1980;238:H539–H544.

[17] Shimosato S, Etsten BE. Effect of anesthetic drugs on the heart: a critical review of myocardial contractility and its relationship to hemodynamics. Clin Anesth 1969;3:17–72.

[18] Yang XP, Liu YH, Rhaleb NE, Kurihara N, Kim HE, Carretero OA. Echocardiographic assessment of cardiac function in conscious and anesthetized mice. Am J Physiol 1999;277:H1967–H1974.

[19] Janssen BJ, De Celle T, Debets JJ, Brouns AE, Callahan MF, Smith TL. Effects of anesthetics on systemic hemodynamics in mice. Am J Physiol Heart Circ Physiol 2004;287:H1618–H1624.

[20] Sano H, Matsumoto K, Utsumi H. Synthesis and imaging of blood-brain-barrier permeable nitroxyl-probes for free radical reactions in brain of living mice. Biochem Mol Biol Int 1997;42:641–647.

[21] Oguchi T, Kashimoto S, Yamaguchi T, Nakamura T, Kumazawa T. Is pentobarbital appropriate for basal anesthesia in the working rat heart model? J Pharmacol Toxicol Methods 1993;29:37–43.

[22] Kass DA, Hare JM, Georgakopoulos D. Murine cardiac function: a cautionary tail. Circ Res 1998;82:519–522.

[23] Takeshita K, Takajo T, Hirata H, Ono M, Utsumi H. *In vivo* oxygen radical generation in the skin of the protoporphyria model mouse with visible light exposure: an L-band ESR study. J Invest Dermatol 2004;122:1463–1470.

[24] Utsumi H, Muto E, Masuda S, Hamada A. *In vivo* ESR measurement of free radicals in whole mice. Biochem Biophys Res Commun 1990;172:1342–1348.

[25] Sano H, Naruse M, Matsumoto K, Oi T, Utsumi H. A new nitroxyl-probe with high retention in the brain and its application for brain imaging. Free Radic Biol Med 2000; 28:959–969.

[26] Yamato M, Egashira T, Utsumi H. Application of *in vivo* ESR spectroscopy to measurement of cerebrovascular ROS generation in stroke. Free Radic Biol Med 2003;35:1619–1631.

[27] Iannone A, Tomasi A, Vannini V, Swartz HM. Metabolism of nitroxide spin labels in subcellular fraction of rat liver. I. Reduction by microsomes. Biochim Biophys Acta 1990; 1034:285–289.

[28] Iannone A, Tomasi A, Vannini V, Swartz HM. Metabolism of nitroxide spin labels in subcellular fractions of rat liver. II. Reduction in the cytosol. Biochim Biophys Acta 1990; 1034:290–293.

[29] Quintanilha AT, Packer L. Surface localization of sites of reduction of nitroxide spin-labeled molecules in mitochondria. Proc Natl Acad Sci USA 1977;74:570–574.

[30] Utsumi H, Shimakura A, Kashiwagi M, Hamada A. Localization of the active center of nitroxide radical reduction in rat liver microsomes: its relation to cytochrome P-450 and membrane fluidity. J Biochem (Tokyo) 1989;105:239–244.