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Assessment of the effect of fermented papaya preparation on oxidative damage in spontaneously hypertensive rat brain using electron spin resonance (ESR) imaging and L-band ESR spectroscopy

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ABSTRACT

Oxidative and nitrosative stress mechanisms are widely implicated in the biological and pathological processes involved in aging, cardiovascular and neurodegenerative diseases. Although this has continued to fuel suggestions of the benefits of antioxidant functional foods, *in vivo* methods for assessing the integrity of this remain limited. A novel electron spin resonance (ESR) technique for evaluating oxidative stress and location of its damage in the brain of spontaneously hypertensive rats (SHR) has been described [Lee, M.-C., et al. (2004). Assessment of oxidative stress in the SHR brain using electron spin resonance (ESR) imaging and in vivo L-Band ESR. *Hypertension Research*, *27*, 485–492]. The reconstructed 2D ESR images of the distribution of a blood brain barrier-permeable nitroxyl spin probe, 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (MC-PROXYL) was used to investigate the ability of fermented papaya preparation (FPP, a product of yeast fermentation of *Carica papaya* Linn.) to modulate oxidative stress of SHR brain. Supplementation (5–7 months) with FPP (50 mg/rat/day) significantly increased the decay of the ESR images of the MC-PROXYL, suggesting that FPP may have up-regulated the redox defense activity in the SHR brain. Herein is an *in vivo* noninvasive technique for the study of oxidative stress and its modulation by dietary factors (that may be intended for applications as neuroprotectants in chronic degenerative disease involving loss of brain function).

1. Introduction

Inflammation, cellular and redox signaling mechanisms have been implicated in the pathophysiology of neurodegenerative disorders such as stroke, Alzheimer's disease, and Parkinson's disease prompting the suggestion that the disease pathology can potentially be modulated by treatment with free-radical scavengers and antioxidant (Aruoma et al., 2006; Halliwell and Aruoma, 1991; Olanow, 1993; Behl, 1999; Wellen and Hotamisligil, 2005; Marchetti and Abbracchio, 2005). Strategies

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for such intervention and prevention require an understanding of the basic molecular mechanism(s) of prophylactic agents (including dietary antioxidant factors from food plants and medicinal plants) that may potentially prevent or reverse the promotion or progression of the diseases.

Fermented papaya preparation (FPP, composition analysis shown in Table 1) is a product of yeast fermentation of *Carica papaya* Linn. FPP is rich in amino acids and carbohydrates. Many reports on papaya are either based on the papaya fruit (Melo et al., 2006; Simirgiotis et al., 2009; Kondo et al., 2005; Mahattanatawee et al., 2006; Balasundram et al., 2006) and the leaf (Canini et al., 2007; Seigler et al., 2002) where reference to their phenolic, allosides and glucosides composition have been reported. FPP has a different composition due to the production process involving yeast fermentation. The composition identified in Table 1 has been authenticated by the Japanese Food Research Laboratories. The nature of the carbohydrates identified in FPP is the subject of ongoing research. Previous studies *in vitro* and *in vivo* have demonstrated antioxidant functions for FPP. The ability to inhibit lipid peroxidation and protect supercoiled plasmid DNA against ferric nitrilotriacetate (Fe-NTA) plus H_2O_2-induced single and double strand breaks and protecting human T-lymphocytes challenged with Fe-NTA/H_2O_2 was reported by Rimbach et al. (2000a). FPP could act as a macrophage activator as a result of its augmentation of nitric oxide synthesis and the secretion of TNF-α (a central regulatory cytokine in macrophage antimicrobial activity) (Rimbach et al., 2000b). Marotta et al. (2003) have demonstrated that FPP modulates atrophic and metaplastic changes of gastric mucosa in chronic atrophic gastritis patients. Aruoma et al. (2006) have shown that FPP modulates the H_2O_2-induced cytotoxicity in PC12 cells and could modulate the oxidative stress in these cells through the inhibition of the intracellular accumulation of reactive oxygen species induced by the activation of the MAPKs gene cascade.

Table 1 – Composition of FPP. FPP® is prepared using a biotechnology process adhering to the ISO 9001 and 14001 using non genetically modified papayas. The composition analysis was carried out and authenticated by the Japan Food Research Laboratories, Fukuoka, Japan.

Component	Level of component per 100 g FPP
Moisture [vacuum oven method]	8.4 g
Protein[a]	0.3 g
Fat	<0.1 g
Ash	<0.1 g
Carbohydrates[b]	91.3 g
Energy[c]	366 kcal
Sodium	0.5 mg
Amino acids	
Arginine	16 mg
Lysine	6 mg
Histidine	6 mg
Phenylalanine	12 mg
Tyrosine	8 mg
Leucine	18 mg
Isoleucine	10 mg
Methionine	5 mg
Valine	14 mg
Alanine	13 mg
Glycine	11 mg
Proline	12 mg
Glutamic acid	40 mg
Serine	11 mg
Threonine	8 mg
Aspartic acid	23 mg
Tryptophan	2 mg
Cysteine	Not detected

a The nitrogen to protein conversion factor was 6.25.
b The formula used was 100 − (moisture + protein + fat + ash).
c Energy conversion factors were in accordance with Notification No. 176 (2003) Standards for Nutrition Labeling, Ministry of Health, Labour and Welfare, Japan.

The pathology in beta-hemoglobinopathies (beta-thalassemia and sickle cell anemia) involves oxidative stress mechanisms. The study of Amer et al. (2008) found that oral administration of FPP to beta-thalassemic mice (50 mg/mouse/day for 3 months) and to patients (3 g × 3 times/day for 3 months) afforded important antioxidant potential contributing to the alleviation symptoms associated with oxidative stress in severe forms of thalassemia. Increased oxidative stress burden in the brain can arise given that the brain has low levels of antioxidant enzymes, a high and constant oxygen requirement, contains membrane lipids rich in oxidizable polyunsaturated fatty acids and non-protein-bound Fe^{3+} in the cerebrospinal fluid and in brain tissues. Studies of Aruoma and colleagues reported elevated catalytic metal ions particularly copper ions in the cerebellum, median putamen and substantia nigra of post-mortem PD brains (Spencer et al., 1994). The normal vs. PD values of 21 ± 10 vs. 29 ± 7 nmol/g tissue (cerebellum), 21 ± 16 vs. 56 ± 30 nmol/g tissue (median putamen) and 53 ± 48 vs. 63 ± 48 nmol/g tissue (substantia nigra) based on the assessment of 20–30 mg of post-mortem human brain tissues from the various regions were reported. The increased iron loading in the substantia nigra may participate in free-radical production and neuronal damage (Olanow, 1993; Ren et al., 2001).

An accepted hypothesis of the mechanism of hypertension is that excess superoxide radical ($O_2^{\cdot-}$), by interacting with endothelial nitric oxide ($^{\cdot}NO$), could contribute to increased vascular smooth muscle contraction (in a reaction mediated by peroxynitrite, $ONOO^-$) and hence cause the elevated total peripheral resistance (Lee et al., 2000a, 2000b; Ignarro et al., 1999; Cuzzocrea et al., 2004; Miyazaki et al., 2002). It is widely suggested that $O_2^{\cdot-}$ contributes to increased systemic vascular tone in SHR (Suzuki et al., 1995; Schnackenberg et al., 1998). Nitroxyl radicals are very useful as exogenous spin probes for measuring free-radical distribution, oxygen concentration, and redox metabolism by *in vivo* ESR in biological systems. Given that the nitroxyl radicals lose their paramagnetism through a redox reaction when exposed to a reducing agent in biological systems, the signal decay rate of the nitroxyl radical gives evidence of free-radical generation and changes of redox status in biological systems. This has led to the description of the technique involving the blood brain barrier-permeable nitroxyl spin probe 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (MC-PROXYL) for the assessment of oxidative stress in the brain (Miyazaki et al., 2002; Miura et al., 1997; Lee et al., 2003, 2004).

The spontaneously hypertensive rat (SHR), a model of essential hypertension, has several characteristics of increased oxidative stress. The ability of long supplementation (5–7 months) of FPP to modulate oxidative stress as the ESR images of the MC-PROXYL spin probe in the brain was investigated to illustrate the potential application of the technique in mapping oxidative injury in the brain. It is worth mentioning that molecular imaging techniques are being looked at for the evaluation of the pathobiology of diseases at cellular and molecular levels coupling information on organ function and morphology (Lindner, 2009; Razzouk and Farkouh, 2009). The ESR imaging methodology is presented with the view that it may facilitate future assessment of the neuroprotective potentials directed at functional foods (essentially viewing functional food's ability to modulate oxidative stress burden in the brain).

2. Materials and methods

2.1. Spin probes, chemicals and animals

MC-PROXYL was synthesized from 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carboxy-PROXYL; Tokyo Kasei, Tokyo, Japan) and diazomethane. MC-PROXYL was purified by column chromatography and characterized as described previously (Miyazaki et al., 2002; Miura et al., 1997; Lee et al., 2003). 3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL) was purchased from Sigma Chemical Co. (St. Louis, USA). Fermented papaya preparation (FPP) (Osato Research Institute, Gifu, Japan; Table 1) was dissolved in sterilized and filtered (<0.22 μm) double distilled water (the dose tested was 50 mg/rat/day for the duration of the study). Male SHR weighing about 350–450 g, were purchased from Japan SLC (Hamamatsu, Japan). The procedures used in this study were in accordance with the guidelines of the US National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication NO. 85-23, revised 1985) and the protocols were approved by our Institutional Animal Care Committees.

2.2. Animal preparation for ESR studies

ESR imaging of the isolated rat brain was performed as follows. The rats were anesthetized with 40 mg/kg (i.p.) pentobarbital (Dinabot, Osaka, Japan), then received 10 mg/kg of 140 mmol/l MC-PROXYL solution *i.v. via* the tail vein, and the brain was isolated 30 s after the treatment. To ensure that microwave radiation inside the ESR resonator did not alter the brain core temperature, the temperature of isolated brains was measured with a needle type (0.46 mm o.d.) thermal probe (MT-26; BRC, Tokyo, Japan). It was observed that the core temperature of the brain (3 mm posterior to the bregma and 5 mm below the surface parallel with the sagittal plane) remained stable at 34 ± 1 °C during a 20 min interval, which was the typical duration of the experiments (Lee et al., 2003, 2004).

2.3. ESR imaging analysis

An ESR imaging system at the Kanagawa Dental College constructed in conjunction with the JEOL ESR Application Laboratory was used in this study. The system consists of a commercially available electromagnet (modi-fied RE3X; JEOL, Akishima, Japan), a pair of field scan coils, power supplies, a personal computer (a modified Hewlett–Packard KAYAK XW/400 computer with laboratory-developed software, Hewlett–Packard, Palo Alto, USA) and a 1 GHz microwave unit containing a four-window loop-gap resonator (Lee et al., 2003, 2004). The system was equipped with four different coil sets, three for the gradients and one for rapid scanning. A setting at 0.9 mT/cm produced a field gradient with maximum strength. The gradient field was controlled by a current stabilizer, which was controlled by a personal computer, as described above. The ESR images were constructed on the basis of Lauterbur's method (Lee et al., 2003, 2004; Lauterbur, 1980), a procedure known as 3D zeugmatography (Lauterbur, 1980) with application of the linear magnetic field gradients along the *x*-, *y*-, and *z*-axes produced by the magnetic field gradient coils. For 2D imaging, 18 projections were acquired in 55 s. Each projection required 1024 points of acquisition data for imaging. The mid-field hyperfine line in the spectrum was separated from the triplet signal of the nitroxyl radicals. Each signal data set was convoluted with Shepp's filter function into the Fourier domain before performing the inverse Fourier transformation to the spatial domain (Woods et al., 1991). Two-dimensional images (512 × 512 pixels) were obtained from 18 projections at gradient steps of 10° in the spatial domain. Instrument settings for ESR detection of MC-PROXYL were as follows: microwave power, 1 mW; magnetic field, 31.0–34.0 ± 5 mT; field modulation width, 0.1 mT; receiver gain, 63–100; time constant, 0.03 s.

2.4. Statistical analysis

Results are expressed as the means ± SD. Statistical analysis was performed using Student's *t*-test or one-way analysis of variance. A p value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of supplementation with FPP on oxidative stress in the isolated brain of SHR

The effect of FPP on SHR-induced oxidative stress in the brain was investigated using the spin probe MC-PROXYL with the resulting spectra analyzed with an ESR imaging system. Supplementation of SHR rats with FPP for 5–7 months significantly increased decay of the 2D ESR image of MC-PROXYL in the isolated SHR brain (Figs. 1 and 2). The metabolism of the MC-PROXYL ESR signal in isolated rat brain occurred in two phases, phase I and phase II, according to a two-compartment model of distribution (Fig. 3A) (Lee et al., 2003, 2004). The increased decay rate constant (K_1) of ESR signal intensity of MC-PROXYL in the isolated SHR brain of long supplementation (5–7 months) with FPP was also observed (Fig. 3B) under same conditions of ESR imaging experiment (Figs. 1 and 2). Thus supplementation with FPP led to a rapid decay of MC-PROXYL in the SHR brain (Figs. 1–3).

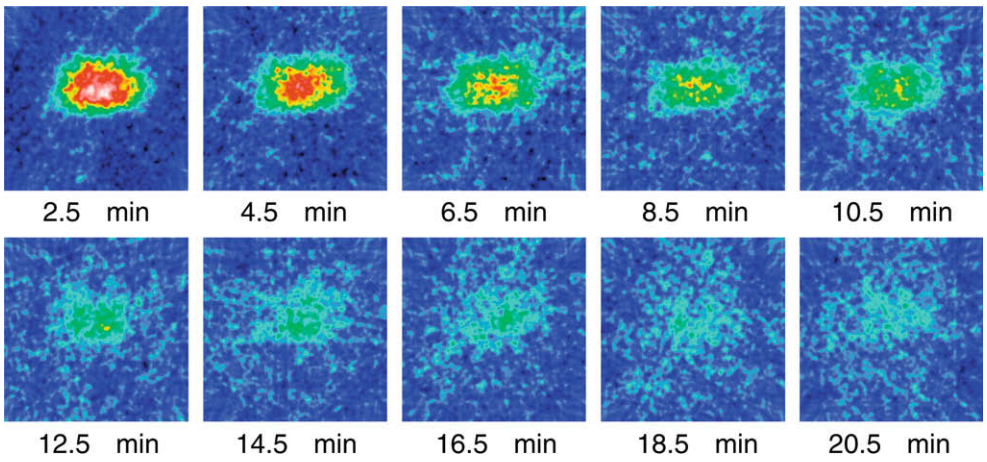

Fig. 1 – Typical 2D ESR images (*y–z* plane) of MC-PROXYL distribution in isolated brain of spontaneously hypertensive rats (SHR). ESR was measured at 2.5, 4.5, 6.5, 8.5, 10.5, 12.5, 14.5, 16.5, 18.5 and 20.5 min after *i.v.* treatment with MC-PROXYL (isolated 30 s after the treatment). As indicated by the attached color scale (16 colors; white and 100 being the maximum ESR signal), ESR images were reproduced in 16 colors and signals lower than 10% of the maximal signal intensity detected in all slices were regarded as noise. Experimental conditions are as described in Section 2.

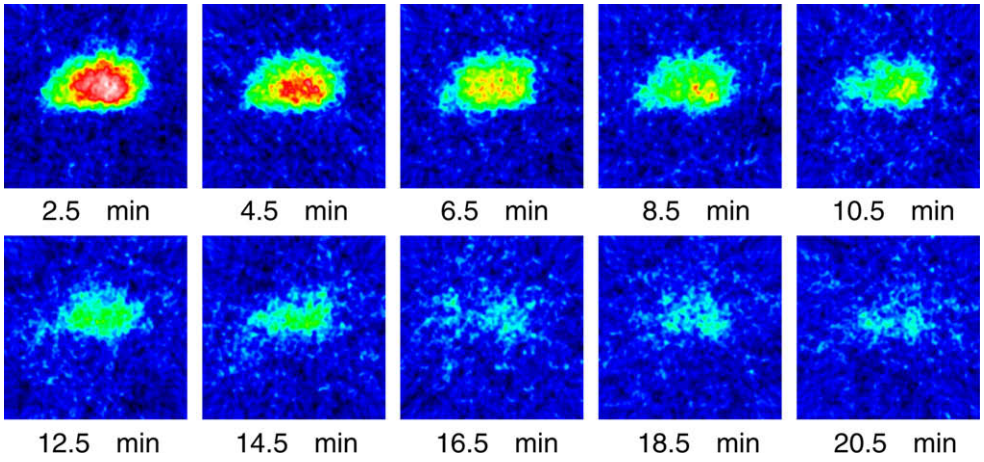

Fig. 2 – Effects of long supplementation with fermented papaya preparation on 2D ESR images (*y–z* plane) of MC-PROXYL distribution in isolated brain of spontaneously hypertensive rats (SHR). ESR was measured at 2.5, 4.5, 6.5, 8.5, 10.5, 12.5, 14.5, 16.5, 18.5 and 20.5 min after *i.v.* treatment with MC-PROXYL (isolated 30 s after the treatment). The ESR images are reproduced in 16 colors with signals lower than 10% of the maximal signal intensity detected in all slices were regarded as noise. Experimental conditions were as described under Section 2.

4. Discussion

Defining if the presence of oxidative stress in various diseases and how these could be attenuated by the administration of antioxidant compounds in food plants coupled with establishing the relationship to the presence of particular genetic polymorphism and modulation of the complex cell signaling cascades involving gene transcription remains a major future scientific challenge (Aruoma et al., 2006; Marchetti and Abbracchio, 2005; Gallou-Kabani and Junien, 2005; Smith et al., 2005). Novel therapeutic approaches must rely on potentiation of endogenous anti-inflammatory pathways and a combination of treatment involving immune modulation and anti-inflammatory therapies require the use of biomarkers.

In the context of the methodology described here, a nitroxyl radical loses its paramagnetism when exposed to a reducing agent in biological systems (Miura et al., 1992; Takeshita et al., 1999). The signal decay rate of the nitroxyl radical gives evidence of free-radical generation and changes of redox status in biological systems (Gomi et al., 1993; Miura and Ozawa, 2000). The application of ESR imaging for 2D isolated rat brain and 3D or ESR-computed tomography (CT) of the head regions of mice or rats, clearly showed that large amounts of MC-PROXYL were distributed to the brain compared to the BBB-impermeable carbamoyl-PROXYL. Thus the *in vivo* ESR technique/nitroxyl spin probe technique employed herein could represent a powerful tool to selectively detect free radicals and to monitor free-radical reactions *in vivo*. Quantitative ESR analysis using MC-PROXYL would enable an understand-

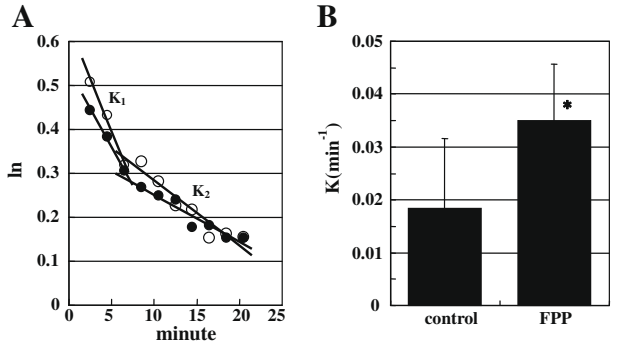

Fig. 3 – (A) Typical L-band ESR signal decay of MC-PROXYL in the isolated spontaneously hypertensive rats (SHR) brain after *i.v.* injection of MC-PROXYL (●), and the effects of long supplementation (5–7 months) with fermented papaya preparation (FPP) (○). ESR was measured 2.5 min after *i.v.* injection of MC-PROXYL (brains isolated 30 s after the treatment). ESR was measured 2.5 min after *i.v.* injection of MC-PROXYL (brains isolated 30 s after treatment). The logarithmic signal intensity of the second peak of the ESR spectrum of MC-PROXYL was plotted against time. Linearity was observed in phase I and phase II of the corresponding semilogarithmic plots. (B) The logarithmic signal intensity of the second peak of the ESR spectrum of the MC-PROXYL was plotted against time. K_1 indicate the decay rate constant (min^{-1}) in phase I as shown in (A). Each K_1 indicates the decay rate constant (min^{-1}) for the control and effects of long supplementation (7 months) with FPP. Each column represents the mean ± SEM (n = 3–6). $^*P < 0.05$ vs. corresponding value for controls.

ing of the redox status under conditions of oxidative stress in the rodent brain (Miyazaki et al., 2002; Lee et al., 2003, 2004; Miura and Ozawa, 2000).

The SHR rat is a model of essential hypertension that shows several characteristics of increased oxidative stress (Suzuki et al., 1995; Schnackenberg et al., 1998). The decay rate constant of MC-PROXYL in the isolated brains of SHR is increased in the brain of normal male Wistar Kyoto rats. Supplementation with FPP increased the decay rate constant of MC-PROXYL in the isolated brains of SHR. This appears to indicate that FPP presumably up-regulated antioxidant activity in the SHR brain. Increased oxidative stress can initiate specific adaptive responses including stimulation of the activation of antioxidant enzymes, thiols, and enhanced oxidative damage repair (Radak et al., 2001). Regular exercise causes adaptation of the antioxidant and repair systems, which could result in a decreased base level of oxidative damage and increased resistance to oxidative stress (Radak et al., 2001). Imao et al. (1998) showed that FPP was able to increase superoxide activity in the cortex and hippocampus of rats. Thus that FPP may have up-regulated antioxidant defenses in the SHR brain correlates well with the pattern of the decay rate constant of MC-PROXYL reported here. The molecular changes in inflammatory markers and antioxidant enzymes gene transcription are currently under investigation. It can however be concluded that FPP has protective effects against oxidative injury, supporting the view that prophylactic potentials in chronic degenerative diseases and in particular diseases of overt inflammation. Thus *in vivo* ESR as described by Lee et al. (2004) can be envisioned to provide a noninvasive technique for the study of oxidative stress and its modulation by dietary factors.

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