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Kangen-karyu, a prescription containing six herbs, has been shown to achieve its pharmacological effect through oxidative stress-dependent pathways in animal models. The aim of this study is to investigate the relationship between the antioxidative effect and pharmacological mechanisms of Kangen-karyu, specifically its body temperature elevating effect in humans. Healthy human volunteers, age  $35 \pm 15$  years old, were enrolled in this study. Surface body temperature, serum nitrite, reactive oxygen species (ROS) scavenging activities, and inflammatory cytokines were investigated before and 120 min after Kangen-karyu oral intake. Kangen-karyu significantly increased the surface-body temperature of the entire body; this effect was more remarkable in the upper body and continued for more than 120 min. Accompanying this therapeutic effect, serum nitrite levels were increased 120 min after oral administration. Serum ROS scavenging activities were enhanced against singlet oxygen and were concomitantly decreased against the alkoxyl radical. Serum nitrite levels and superoxide scavenging activities were positively correlated, suggesting that Kangen-karyu affects the  $O_2^{\cdot-}$ -NO balance *in vivo*. Kangen-karyu had no effect on IL-6, TNF- $\alpha$  and adiponectin levels. These results indicate that the therapeutic effect of Kangen-karyu is achieved through NO- and ROS-dependent mechanisms. Further, this mechanism is not limited to ROS production, but includes ROS-ROS or ROS-NO interactions.

**Key Words:** antioxidant capacity, hydroxyl radical, alkoxyl radical, nitric oxide, traditional herbal prescription

Traditional herbal prescriptions have attracted much attention due to their beneficial effects, observed in the course of long-term clinical experience. Kangen-karyu, a prescription consisting of six herbs (*Paeoniae Radix*, *Cnidii Rhizoma*, *Carthami Flos*, *Cyperii Rhizoma*, *Saussureae Radix* and *Salviae miltiorrhizae Radix*) is known to have antihypertensive effects and has been clinically used for cardiovascular diseases such as angina pectoris and cerebrovascular disorders.<sup>(1)</sup> Recent studies demonstrated the therapeutic effects of Kangen-karyu including antihypertension, anti-platelet aggregation, neuroprotection and anti-dementia in animal models.<sup>(2-4)</sup> While the underlying biological reactions of these therapeutic effects are varied, an oxidative stress-controlling effect is currently suggested as a key mechanism in animal studies.<sup>(4-6)</sup> Although these reports revealed important results, little is known about the pharmacological mechanisms of Kangen-karyu in human subjects.

Currently, oxidative stress is recognized as an important pathological factor in many diseases. Ongoing investigations are revealing cellular reactions after oxidative stimuli, such as the

NF- $\kappa$ B and Nrf2-Keap1 pathways.<sup>(7-9)</sup> However, upper stream reactions of oxidative stress that evoke the following responses, including the identification of reactive oxygen species (ROS) that act as the responsible stimulator of oxidative stress reactions, or interactions among ROS that generate oxidative stimulators, remain unclear.<sup>(10,11)</sup> Because ROS are not uniform and individual ROS have specific characteristics during *in vivo* reactions,<sup>(12)</sup> analysis of multiple ROS is necessary. Using electron spin resonance (ESR) technology, we have reported changes in the hydroxyl radical and superoxide scavenging activity in patients with various diseases.<sup>(13,14)</sup> Recently, we developed this ESR-based method for the analysis of multiple ROS scavenging activities in biological samples, allowing us to describe details of oxidative stress-related reactions.<sup>(15)</sup>

This study aimed to reveal the relationship between the pharmacological effect of Kangen-karyu and oxidative stress in human subjects. Changes in the body surface temperature induced by vasodilation were the targeted pharmacological effect. Oxidative stress was evaluated by measuring multiple free radical scavenging activities in serum and analyzing the evoked radical chain reactions. The results show that Kangen-karyu elevated the body surface temperature through alternations of singlet oxygen, alkoxyl radical, ROS interactions among specific ROS, and the ROS-nitric oxide (NO) balance. Our study provides the first evidence of the oxidative stress-controlling effect of Kangen-karyu in human subjects.

## Materials and Methods

**Subjects and informed consent.** Eight healthy volunteers, four males and four females, mean age  $35 \pm 15$  years old, were employed in the study. All human subject procedures were conducted with individual written consent and followed a protocol that had been approved by Tsukuba University of Technology Committee.

**Study design.** The study was conducted with a single oral administration of Kangen-karyu (5 g). Blood and urinary samples were obtained before and 120 min after oral administration of Kangen-karyu. Serum samples were separated by centrifugation and stored at  $-80^\circ\text{C}$  until used. All subjects fasted at least 6 h before the oral administration of Kangen-karyu.

**Thermography.** Body surface temperature was measured using an infrared thermograph (JTG4310S; JEOL, Tokyo, Japan) placed in a temperature-controlled chamber. The room tempera-

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ture and humidity of the chamber were set at  $25 \pm 1^\circ\text{C}$  and 50%, respectively. The subjects entered into the chamber 30 min prior to the Kangen-karyu administration to void any outside effect. The thermographic measurements were performed before and 30, 60 and 120 min after Kangen-karyu administration. Body surface temperatures were measured on the face, frontal neck, abdomen, frontal lower limb, back of the neck, back, hand and dorsum of the hand. Female subjects were measured wearing undershirts.

**Measurements of multiple free radical scavenging activity.** Multiple free radical scavenging activities (MULTIS method) were measured by an ESR based method developed by one of our co-authors.<sup>(15)</sup> The ROS employed in this system were five free radicals including superoxide ( $\text{O}_2^-$ ), hydroxyl radical ( $\text{OH}$ ), alkoxy radical ( $\text{RO}$ ,  $t\text{-BuO}$ ), peroxy radical ( $\text{ROO}$ ,  $t\text{-BuOO}$ ), alkyl radical ( $\text{R}$ ,  $\text{CH}_3$ ), and singlet oxygen ( $^1\text{O}_2$ ). Each ROS was produced via *in situ* illumination with UV/visible light from an illuminator (RUVF-203SR UV illuminator; Radical Research Inc., Tokyo, Japan). 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO) was used as an ESR-spin trapping reagent. Light sources, illumination times, precursors and photo-sensitizers used to produce ROS are summarized in Table 1. The UV light source was a 200 W medium-pressure mercury/xenon arc lamp, where UV-visible light was guided through a quartz light-guide into the resonator cavity. The ESR spectrometer employed was a JEOL FR-80 equipped with 100 kHz field modulation and WIN-RAD operation software (Radical Research Inc.). Typical spectrometer settings were: field modulation width 0.1 mT; microwave power 10 mW; field scan width and rate  $\pm 7.5$  mT/2 min; time constant 0.1 s. ROS scavenging activities were calculated according to the previously described method,<sup>(15)</sup> and expressed as the unit equivalent to known pure scavengers; GSH for  $\text{OH}$  and  $^1\Delta\text{O}_2$ , superoxide dismutase (SOD) for  $\text{O}_2^-$ , 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX) for  $\text{RO}$ , and  $\alpha$ -lipoic acid ( $\alpha\text{LA}$ ) for  $\text{ROO}$ . Because the alkyl radical has no specific scavenger, scavenging activity against  $\text{R}$  was expressed as CYPMPO equivalents.

The MULTIS method was applied to the measurement of the *in vitro* profiles of prescriptions and the changes of antioxidative activities after the administration of Kangen-karyu. The *in vitro* profiles were obtained for Kangen-karyu and Tsudosan, a Kampo formula also reported to possess high antioxidative activity.<sup>(16)</sup> *In vitro* scavenging activities of the prescriptions were measured using 10% aqueous solutions and expressed in terms of their daily doses. Serum scavenging activities were determined in 10% buffer solution of serum or pure water and the total volume of the sample solution was approximately 0.2 ml.

**Measurements of NO metabolites and inflammatory cytokines.** Serum NO metabolites were measured as  $\text{NO}_2$  by colorimetric measurement based on the Griess reaction described elsewhere.<sup>(17)</sup> IL-6 and adiponectin were measured using a chemiluminescent enzyme immunoassay, TNF- $\alpha$  was measured using

an enzyme-linked immunoassay, both at SRL laboratory (Tokyo, Japan).

**Materials.** CYPMPO was obtained from Radical Research Inc.; hydrogen peroxide, riboflavin, 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH), tert-butyl hydroperoxide, dimethyl sulfoxide (DMSO), rosebengal, and 4-hydroxy-2,2,6,6-tetramethylpiperidine (4-OH-TEMP) were purchased from Tokyo Chemical Industry (Tokyo, Japan) and used without modification. Buffers and biochemical reagents were obtained from Wako Chemical Co. (Osaka, Japan).

**Statistical analysis.** Statistical analysis was performed using computer software (Prism 6; GraphPad Software Inc., La Jolla). Comparison of data from two groups was analyzed with a Student's paired *t* test. Multiple data comparisons were performed using the one-way factorial analysis of variance (ANOVA). Repeated measures of ANOVA with the Dunnett's test were used for the time-course measurements of body surface temperature. Multiple regression analysis results are expressed as the mean  $\pm$  SEM.

## Results

**Kangen-karyu raises body surface temperature.** Before Kangen-karyu administration, the body surface temperature of subjects was  $35.59 \pm 0.06^\circ\text{C}$  in the facial area. Thirty, 60 and 120 min after Kangen-karyu administration, the corresponding temperature rose to  $35.90 \pm 0.09$ ,  $36.01 \pm 0.12$  and  $36.08 \pm 0.14^\circ\text{C}$ , respectively (Table 2). These significant elevations in surface body temperature were also observed in the areas of the shoulder, forehead, abdomen, occiput, posterior shoulder and back, and continued until 90 to 120 min after Kangen-karyu administration. A typical sequential change in body temperature is shown in Fig. 1. This effect on temperature was predominantly observed in the upper-half of the subjects, and was not significant in the area of hands, palms and lower legs. No remarkable changes in blood pressure were observed during the study period (data not shown).

***In vitro* ROS scavenging activities of Kangen-karyu.** The ESR spectra of the spin adducts for  $\text{OH}$ ,  $\text{O}_2^-$ ,  $\text{RO}$ ,  $\text{ROO}$ ,  $\text{R}$  and  $^1\text{O}_2$  agreed with the previously reported spectra of corresponding radical adducts, confirmed by hyperfine coupling constants (Supplemental Fig. 1\*<sup>(15)</sup>). In the presence of the Kangen-karyu solution, the signal amplitude decreased from that from which the scavenging activity was calculated.

The *in vitro* profiles of scavenging activity converted into equivalent units of known pure scavengers are summarized in Table 3. The obtained scavenging activities were compared to those of Tsudosan, which was reported to possess a high oxygen radical absorbance capacity (ORAC) value.<sup>(16)</sup> The reported ORAC value of Tsudosan is 5913.27 ORAC unit (formula/day), and the  $\text{RO}$  scavenging activity in our method, which may relate to ORAC in principle, was 8665.62  $\mu\text{M}$ -TROLOX equivalent (formula/day). The scavenging activities of Kangen-karyu were

**Table 1.** Photolytic production methods of multiple reactive oxygen species

Free radical	Spin trap	Precursor/Sensitizer	UV/VL	Irradiation period	Antioxidant equivalent
$\text{OH}$	CYPMPO	$\text{H}_2\text{O}_2$ 10 mM	UV	5 s	GSH
$\text{O}_2^-$	CYPMPO	Riboflavin 20 $\mu\text{M}$	VL	60 s	SOD
$\text{RO}$	CYPMPO	AAPH 10 mM	UV	5 s	Trolox
$\text{ROO}$	CYPMPO	$t\text{BHP}$ 10 mM	UV	5 s	$\alpha$ -lipoic acid
$\text{R}$	CYPMPO	$\text{H}_2\text{O}_2$ 100 mM DMSO 10 mM	UV	30 s	CYPMPO
$^1\text{O}_2$	TMPO	Rosebengal 200 $\mu\text{M}$	VL	30 s	GSH

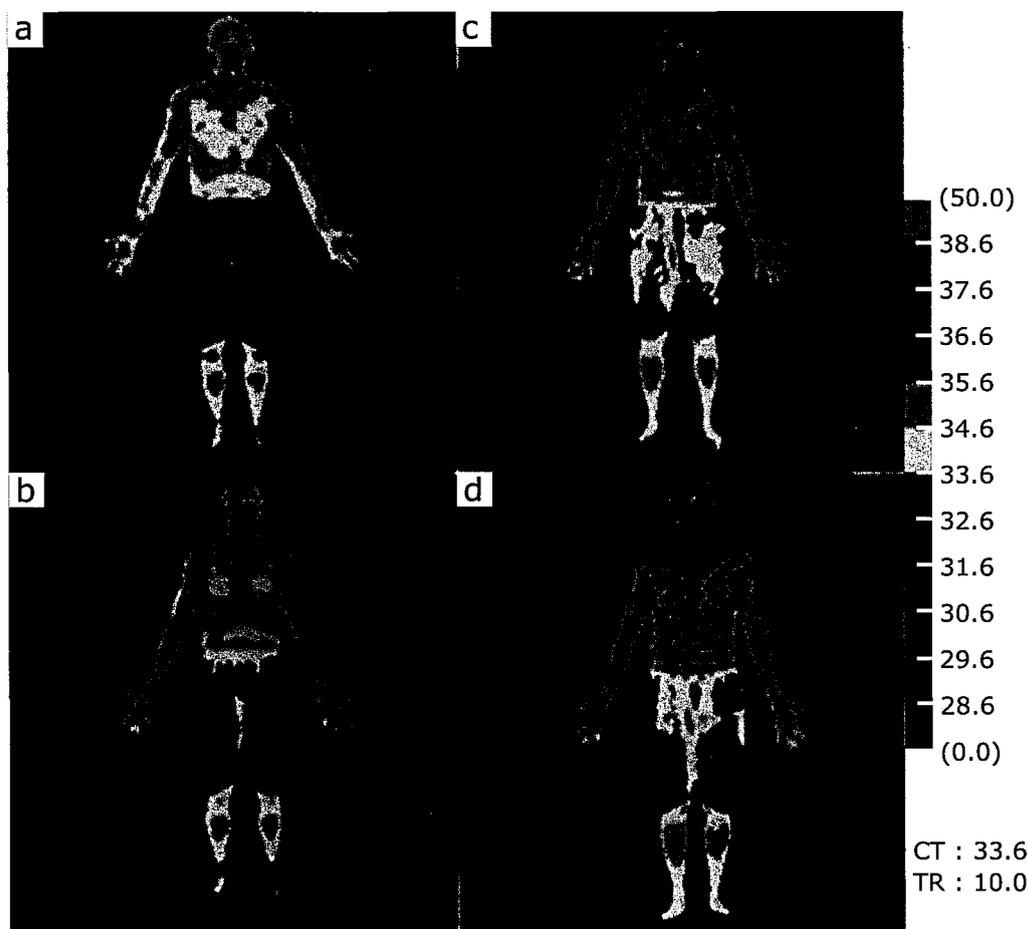
UV, ultraviolet (300–400 nm); VL, visual light (500–600 nm); AAPH, 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride,  $t\text{BHP}$ ; tert-butyl hydroperoxide; GSH, glutathione; SOD, superoxide dismutase; CYPMPO, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide; TMPO, 4-hydroxy-2,2,6,6-tetramethylpiperidine.

\*See online. [https://www.jstage.jst.go.jp/article/jcfn/58/3/58\\_15-135/\\_article/supplement](https://www.jstage.jst.go.jp/article/jcfn/58/3/58_15-135/_article/supplement)

**Table 2.** Effect of Kangen-karyu administration on changes in body surface temperatures

	Before	30 min	60 min	120 min
Face	35.59 ± 0.06	35.9 ± 0.09*	36.01 ± 0.12*	36.08 ± 0.15*
Shoulder	35.2 ± 0.12	35.58 ± 0.1*	35.89 ± 0.1*	36.01 ± 0.09*
Forehead	35.58 ± 0.13	35.85 ± 0.13*	36.13 ± 0.08*	36.29 ± 0.08*
Abdomen	34.14 ± 0.26	34.55 ± 0.21*	34.96 ± 0.22*	35.3 ± 0.21*
Occiput	34.76 ± 0.1	35.15 ± 0.1*	35.54 ± 0.13*	35.73 ± 0.15*
Posterior shoulder	34.53 ± 0.17	34.84 ± 0.21	35.06 ± 0.24*	35.31 ± 0.18*
Back	34.81 ± 0.17	35.34 ± 0.27*	35.55 ± 0.29*	35.74 ± 0.24*
Hand	34.73 ± 0.11	35.19 ± 0.14*	35.25 ± 0.19	34.85 ± 0.2
Palm	35.3 ± 0.08	35.44 ± 0.15	35.5 ± 0.18	34.81 ± 0.24
Lower thigh	33.99 ± 0.1	34.14 ± 0.06	34.38 ± 0.11*	34.45 ± 0.07*

Body surface temperatures were measured before and 30, 60, and 120 min after Kangen-karyu administration. Data represent means ± SEM. \**p*<0.05 vs before administration.



**Fig. 1.** Image of a representative whole body thermograph. (a) Before Kangen-karyu administration. (b), (c) and (d) 30, 60 and 120 after Kangen-karyu administration, respectively. See color figure in the on-line version.

higher against  $O_2^{\cdot-}$  and lower against  $ROO^{\cdot}$  and  $R^{\cdot}$  than those of Tsudosan. The scavenging activities against  $^{\cdot}OH$ ,  $RO^{\cdot}$  and  $^1O_2$  were of the same level in the two formulae.

**Effect of Kangen-karyu oral administration on serum ROS scavenging activities.** Decreases in signal amplitudes in the presence of serum and scavenging activities were calculated. Table 4 and Fig. 2 show the serum radical scavenging activities before and 120 min after Kangen-karyu administration. Before Kangen-karyu administration, the serum ROS scavenging activities

were  $10.0 \pm 2.63$  mM-GSHeq for  $^{\cdot}OH$ ,  $12.6 \pm 1.2$  U/ml-SODEq for  $O_2^{\cdot-}$ ,  $1.7 \pm 0.3$  mM-TROLOXeq for  $RO^{\cdot}$ ,  $1.05 \pm 0.05$  mM- $\alpha$ LAeq for  $ROO^{\cdot}$ ,  $0.24 \pm 0.02$  mM-CYPMPOeq for  $R^{\cdot}$  and  $22.2 \pm 2.5$   $\mu$ M-GSHeq for  $^1O_2$ .

The serum ROS scavenging activities before administration generally agreed with the previously reported values of healthy adults.<sup>(15)</sup> After Kangen-karyu administration, the serum ROS scavenging activities were  $8.32 \pm 2.22$  mM-GSHeq for  $^{\cdot}OH$ ,  $11.9 \pm 2.5$  U/ml-SODEq for  $O_2^{\cdot-}$ ,  $1.0 \pm 0.2$  mM-TROLOXeq for  $RO^{\cdot}$ ,

**Table 3.** *In vitro* scavenging activities of Kangen-karyu and Tsudosan as measured for multiple ROS converted into the equivalent units to specific scavengers

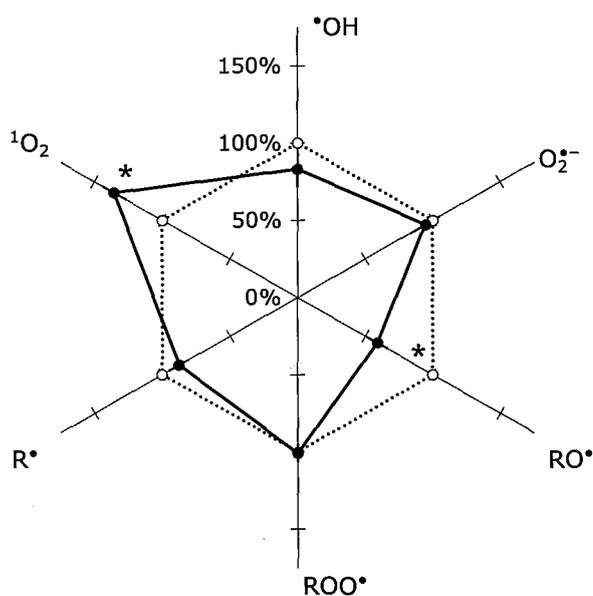
	Free radical					
	$\cdot\text{OH}$	$\text{O}_2^{\cdot-}$	$\text{RO}\cdot$	$\text{ROO}\cdot$	$\text{R}\cdot$	$^1\Delta\text{O}_2$
	mM-GSHeq	U/ml-SODeq	mM-TROLOXeq	mM- $\alpha$ LAeq	mM-CYPMPOeq	mM-GSHeq
Kangen-karyu	275.1 $\pm$ 8.3	2789.9 $\pm$ 88.5	9.39 $\pm$ 1.13	42.4 $\pm$ 5.6	212.7 $\pm$ 27.1	392.0 $\pm$ 26.8
Tsudosan	308.5 $\pm$ 10.9	833.5 $\pm$ 52.8	8.67 $\pm$ 0.76	53.4 $\pm$ 5.2	308.6 $\pm$ 37.9	350.5 $\pm$ 22.4

Both values of Kangen-karyu and Tsudosan are expressed as those of daily doses.

**Table 4.** *In vivo* scavenging activity of serum before and 120 min after Kangen-karyu administration, as measured for multiple ROS converted into the equivalent units to specific scavengers

	Free radical					
	$\cdot\text{OH}$	$\text{O}_2^{\cdot-}$	$\text{RO}\cdot$	$\text{ROO}\cdot$	$\text{R}\cdot$	$^1\Delta\text{O}_2$
	mM-GSHeq	U/ml-SODeq	mM-TROLOXeq	mM- $\alpha$ LAeq	mM-CYPMPOeq	mM-GSHeq
Before	10.0 $\pm$ 2.63	12.6 $\pm$ 1.2	1.7 $\pm$ 0.3	1.05 $\pm$ 0.05	0.24 $\pm$ 0.02	22.2 $\pm$ 2.5
After	8.32 $\pm$ 2.22	11.9 $\pm$ 2.5	1.0 $\pm$ 0.2*	1.06 $\pm$ 0.07	0.21 $\pm$ 0.03	30.1 $\pm$ 4.0*

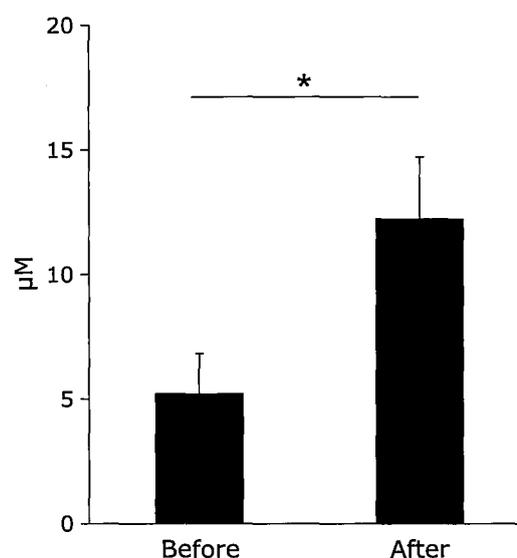
Data represent means  $\pm$  SEM. \* $p$ <0.05 vs before administration.



**Fig. 2.** A radar chart of serum scavenging activity before and after Kangen-karyu administration. Data are listed in Table 3. Percent changes in the scavenging activity after Kangen-karyu administration (dashed lines) are shown with respect to that of before the administration (solid line). Error bars shown for each free radical species are SEM. \* $p$ <0.05 vs before administration.

1.06  $\pm$  0.07 mM- $\alpha$ LAeq for  $\text{ROO}\cdot$ , 0.21  $\pm$  0.03 mM-CYPMPOeq for  $\text{R}\cdot$  and 30.1  $\pm$  4.0  $\mu\text{M}$ -GSHeq for  $^1\text{O}_2$ . Serum scavenging activity against  $^1\text{O}_2$  was significantly increased and that against  $\text{RO}\cdot$  was significantly decreased. Scavenging activities against  $\text{O}_2^{\cdot-}$ , in which the *in vitro* activity of Kangen-karyu was remarkable, showed no significant increase. The scavenging activities against  $\cdot\text{OH}$ ,  $\text{ROO}\cdot$  and  $\text{R}\cdot$  also showed no remarkable changes.

**Kangen-karyu effects on NO metabolites and ROS-NO balance.** The serum  $\text{NO}_2$  concentration was 5.22  $\pm$  1.60  $\mu\text{M}$  before the test. Two hours after Kangen-karyu administration, serum  $\text{NO}_2$  value was significantly increased to 12.2  $\pm$  2.46  $\mu\text{M}$  (Fig. 3). The effects of Kangen-karyu on ROS-ROS or ROS-NO interactions were evaluated by measuring ROS scavenging

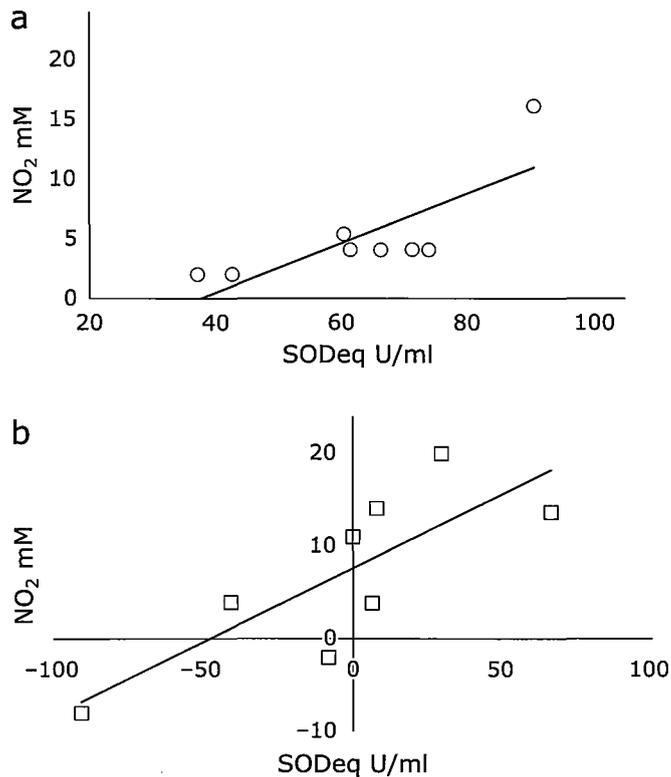


**Fig. 3.** Serum  $\text{NO}_x$  concentration before and 120 min after Kangen-karyu administration. Data represent means  $\pm$  SEM. \* $p$ <0.05.

activities. Before Kangen-karyu administration, the serum  $\text{O}_2^{\cdot-}$  scavenging activity significantly correlated with serum  $\text{NO}_2$  concentrations, suggesting the existence of an  $\text{O}_2^{\cdot-}$ -NO balance that regulates the redox status in serum (Fig. 4a). After Kangen-karyu administration, the  $\text{O}_2^{\cdot-}$ -NO balance became indefinite, while a significant correlation between  $\text{O}_2^{\cdot-}$  scavenging activity and  $\text{NO}_2$  concentration appeared (Fig. 4b).

**Kangen-karyu does not alter inflammatory cytokines.**

Effects of Kangen-karyu on non-radical pathways were evaluated by measuring adiponectin, IL-6 and  $\text{TNF-}\alpha$ . Before Kangen-karyu administration, adiponectin, IL-6 and  $\text{TNF-}\alpha$  levels were 8.42  $\pm$  1.04  $\mu\text{g/ml}$ , 1.95  $\pm$  0.54  $\text{pg/ml}$  and 1.40  $\pm$  0.15  $\text{pg/ml}$ , respectively (Fig. 5). After administration, the values were 8.65  $\pm$  1.08  $\mu\text{g/ml}$ , 2.20  $\pm$  0.55  $\text{pg/ml}$  and 1.31  $\pm$  0.10  $\text{pg/ml}$ , respectively, not significantly different. No remarkable correlations were observed between inflammatory cytokine levels and ROS scavenging activities.

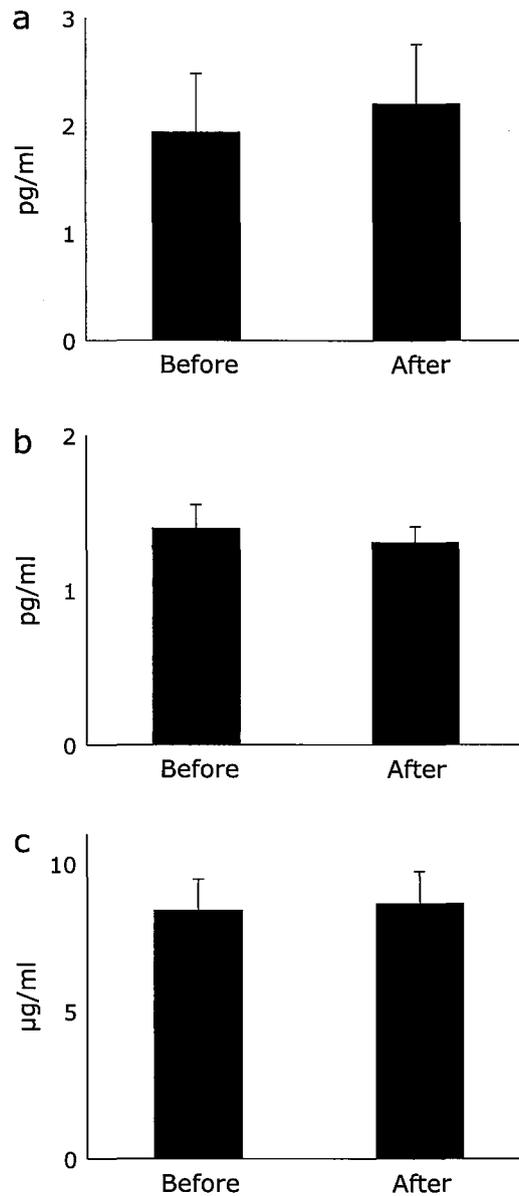


**Fig. 4.** Correlation between serum superoxide scavenging activity and  $\text{NO}_2$  concentration (a) before Kangen-karyu administration, (b) variation between before and after Kangen-karyu administration. Significant correlations were observed in (a) ( $R = 0.78$ ,  $p < 0.05$ ) and (b) ( $R = 0.80$ ,  $p < 0.05$ ).

## Discussion

Oxidative stress is one of the most critical and universal pathological factors impacting various diseases. A series of studies revealed the roles of oxidative stress during Kangen-karyu treatment in fructose-induced metabolic syndrome, diet-induced hypercholesterolemic rats, hyperlipidemia in streptozotocin-induced type 1 diabetic rats and type 2 diabetic *db/db* mice.<sup>(1,4,18,19)</sup> Though these reports revealed important results, little is known about the pharmacological mechanisms of Kangen-karyu in humans. Here, we reveal details of Kangen-karyu induced oxidative stress in human subjects.

First, we evaluated the antioxidative profile of Kangen-karyu *in vitro*. Many Kampo medicines are known to possess beneficial effects against oxidative stress. Prescriptions containing *Rheum rhizoma*, such as Tsudosan or Dajoukito, exhibit high ORAC values.<sup>(16)</sup> ORAC values are measured using TROLOX as the antioxidant, and mainly relate to reactions toward  $\text{RO}^\bullet$ .<sup>(20)</sup> Differences were noted between the reported ORAC value and the  $\text{RO}^\bullet$  scavenging activity of Tsudosan, due in part to methodological differences. A previous study applying MULTIS for food chemistry found correlations between ORAC and MULTIS for  $\text{O}_2^{\bullet-}$  and  $\text{RO}^\bullet$ , but not  $\text{OH}^\bullet$ .<sup>(21)</sup> Since the United States Department of Agriculture withdrew ORAC data from their website, alternative methods that are more reliable for oxidative stress measurements are required.<sup>(22)</sup> Consequently we employed an ESR-based MULTIS method for this study. Although ESR has methodological demerits, such as dielectric loss, ESR is able to specify the target ROS and has high sensitivity for the detection of oxidative stress.<sup>(13,14,23,24)</sup>



**Fig. 5.** Serum concentrations of (a) interleukin-6 and (b) tumor necrosis factor- $\alpha$  and (c) adiponectin before and 120 min after Kangen-karyu administration. Data represent means  $\pm$  SEM. No significant differences were observed.

Currently, only some reports specify the target radical(s) in the measurement of antioxidative activity. A series of reports from Kohno and colleagues made an extensive screening of  $\text{O}_2^{\bullet-}$  scavenging activity of herbal extracts.<sup>(25,26)</sup> They found that extracts such as *Rheum palmatum*, *Ephedra sinica*, *Punica granatum* and *Caesalpinia sappan* have high  $\text{O}_2^{\bullet-}$  scavenging activity. However, the  $\text{O}_2^{\bullet-}$  scavenging activity of the main contents of Kangen-karyu, including *Paeoniae Radix*, *Cnidii Rhizoma*, *Carthami Flos*, *Cyperii Rhizoma*, *Saussureae Radix* and *Salviae miltiorrhizae Radix*, are relatively low.<sup>(26)</sup> In contrast, the superoxide scavenging activity of Kangen-karyu measured using our method was remarkable, and was higher than that of Tsudosan, which contains *Rheum palmatum*.

The changes of *in vivo* ROS scavenging activities produced by Kangen-karyu were completely different from its *in vitro* antioxidative profile. In contrast, with its high  $\text{O}_2^{\bullet-}$  scavenging activity

*in vitro*, the remarkable *in vivo* changes showed an increase against  $^1\text{O}_2$  and a decrease against  $\text{RO}^\cdot$ . Compared to  $\text{O}_2^{\cdot-}$  or  $^{\cdot}\text{OH}$ , the detailed roles of  $\text{RO}^\cdot$ ,  $\text{ROO}^\cdot$ ,  $\text{R}^\cdot$ ,  $^1\text{O}_2$  and other oxygen radicals in human diseases remains unknown.<sup>(10)</sup> A previous report from one of our co-authors revealed remarkable shifts in MULTIS-measured ROS scavenging activities in patients with chronic kidney disease.<sup>(15)</sup> However, the *in vivo* role of  $\text{RO}^\cdot$  was not clear in the study. On the other hand,  $^1\text{O}_2$  is reportedly produced through myeloperoxidase, prostaglandin hydroperoxidase and the interaction between  $\text{O}_2^{\cdot-}$  and  $\text{H}_2\text{O}_2$  during the Haber–Weiss reaction.<sup>(27,28)</sup> Among ROS,  $^1\text{O}_2$  has relatively high toxicity *in vivo*, provokes lipid peroxidation, inducing further  $\text{RO}^\cdot$  and  $\text{ROO}^\cdot$ , and diminishes radical chain reactions.<sup>(29)</sup> In ischemia-reperfusion injury,  $^1\text{O}_2$  is thought to play a crucial role and may relate to the protective effects of Kangen-karyu.<sup>(30)</sup>

On the other hand, Kangen-karyu also affected the internal NO and NO-ROS balance. Kangen-karyu remarkably increased the surface body temperature in the face and trunk and was accompanied by an increase in serum  $\text{NO}_2$ , suggesting that Kangen-karyu causes direct and/or indirect stimulation of NO production. A previous animal study detected an inhibition of inducible nitric oxide synthase (iNOS) after Kangen-karyu treatment in a diabetic model, a result that is not in agreement with our present study.<sup>(31)</sup> The cause of this disagreement is not clear, although our finding of increasing  $^1\text{O}_2$  scavenging activity may be related. A previous

report revealed that quenching of  $^1\text{O}_2$  increased NO synthase (NOS) activity in synaptosomes isolated from rabbit brain.<sup>(32)</sup> Also,  $^1\text{O}_2$  deactivating activity was reduced in serum from patients with diabetes mellitus, in which a major complication was endothelial injury.<sup>(33)</sup> Considering these results, the increased scavenging activity of  $^1\text{O}_2$  by Kangen-karyu may increase NO production. Moreover, we also observed the relation between alterations in serum  $\text{O}_2^{\cdot-}$  scavenging activity and  $\text{NO}_2$  concentration, suggesting Kangen-karyu modifies the  $\text{O}_2^{\cdot-}$ -NO balance. The pathophysiological role of *in vivo*  $\text{O}_2^{\cdot-}$ -NO balance is investigated mainly in hypoxia, though the detailed mechanism remains unclear.<sup>(34–36)</sup> Further studies analyzing the roles of specific ROS are required.

In conclusion, our results show that Kangen-karyu raises surface-body temperature and is accompanied by a significant increase in serum  $^1\text{O}_2$  scavenging activity and serum NO and amelioration of  $\text{O}_2^{\cdot-}$ -NO balance. Therefore, Kangen-karyu may alter the *in vivo* oxidative balance.

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