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Original Article

Anthocyanin-rich tea Sunrouge upregulates expressions of heat shock proteins in the gastrointestinal tract of ICR mice: A comparison with the conventional tea cultivar Yabukita



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ABSTRACT

Sunrouge is an anthocyanin-rich, new tea cultivar that contains similar levels of catechins as Yabukita, the most popular tea cultivar consumed in Japan. Interestingly, Sunrouge preparations have previously been shown to have more pronounced acetylcholinesterase inhibitory and anticolitis activities than those of Yabukita. In this study, we examined their effects on expressions of self-defensive molecules, including heat shock proteins (HSPs), which are molecular chaperones involved in homeostasis and longevity. Hot water extract from freeze-dried Sunrouge significantly upregulated messenger RNA (mRNA) expressions of HSP40, HSP70, and HSP32 (heme oxygenase-1), with grades greater than those shown by Yabukita. Oral administration of freeze-dried preparation of Sunrouge to male ICR mice at a dose of 1% in the basal diet for 1 month resulted in marked upregulations of several HSP mRNA expressions in mucosa from the gastrointestinal tract, especially the upper small intestine. Again, its efficacy was remarkably higher than that of Yabukita. Moreover, exposure of *Caenorhabditis elegans* to Sunrouge conferred thermoresistant phenotype, and also resulted in a significant life-span elongation. Taken together, our results suggest that Sunrouge is a unique and promising tea cultivar for regulating self-defense systems.

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1. Introduction

Green tea has long been consumed as a popular beverage in many countries, and there exist increasing interest in physiological functions of green tea constituents, including green tea polyphenols (GTPs) and L-theanine (*N*-ethyl-L-glutamine) [1]. Numerous studies have so far demonstrated that GTPs exert their biological and physiological effects by scavenging or quenching reactive oxygen species (ROS) because excessive production of ROS has been implicated in the occurrence and development of a variety of ailments. However, there are a number of green tea cultivars, which are diverse in appearance of the leaves, growth phenotypes, resistance to pathogenic bacteria and viruses, and so forth. It is important to indicate that bioactivities of some cultivars are occasionally different from each other, which is presumable due to significant differences in their chemical compositions. For example, Benifuuki [2], a tea (*Camellia Sinensis* L.) cultivar in Japan, is dramatically rich in antiallergic (–)-epigallocatechin-3-O-(3-O-methyl) gallate, as compared with Yabukita (*C. sinensis* L.), the most popular green tea cultivar consumed in Japan [3]. Interestingly, drinking Benifuuki green tea, together with ginger extract, over 1 consecutive month reduced some of the symptoms from Japanese cedar pollinosis [3].

Sunrouge (*Camellia taliensis* × *C. sinensis* L.) is a new green tea cultivar that has a few unique characteristics due to its red leaves [4]. The anthocyanin content of Sunrouge is 8.4 times higher than that of Yabukita, and Sunrouge includes similar levels of catechins as Yabukita, and the red color is attributed to the anthocyanin pigments contained in the plant [4]. Anthocyanins are glycosides of anthocyanidins and universally associated with attractive, colorful, and flavorful fruits. Recently, anthocyanins have attracted attention because of their potential biological and pharmacological benefits, such as analgesic [5] and anti-inflammatory [6] activities. Chemical analysis revealed that Sunrouge contains delphinidin-3-O-β-D-(6-(*E*)-*p*-coumaroyl)galactopyranoside, delphinidin-3-O-β-D-(6-(*E*)-*p*-coumaroyl)glucopyranoside, cyanidin-3-O-β-D-(6-(*E*)-*p*-coumaroyl)galactopyranoside, cyanidin-3-O-β-D-(6-(*E*)-*p*-coumaroyl)glucopyranoside, delphinidin-(*Z*)-*p*-coumaroylgalactopyranoside, and petunidin-(*E*)-*p*-coumaroylgalactopyranoside [7]. We have recently uncovered that the acetylcholinesterase inhibitory activity of Sunrouge water extract was higher than those of Yabukita and Benifuuki, as shown in the experiments using human neuroblastoma SK-N-SH cells [8]. More recently, oral administration of Sunrouge extract resulted in marked amelioration of dextran sulfate sodium-induced mouse colitis, whereas that from Yabukita was scarcely active or rather deteriorated this pathology [8]. In addition, Fujimura et al [9] have investigated the ability of leaf extracts from 43 Japanese green tea cultivars to inhibit thrombin-induced phosphorylation of myosin regulatory light chain in human umbilical vein endothelial cells, and identified Sunrouge as one of the most potent inhibitors. Sunrouge can be characterized as a promising tea cultivar for developing physiological functional foods.

Heat shock proteins (HSPs), highly conserved families of proteins ubiquitously expressed in most types of cells, allow misfolded and unfolded proteins to achieve functional

conformation. Thus, the expression and activity status of HSPs are considered to be critical determinants of homeostasis, and thus health and longevity. In fact, maintenance of HSPs at high levels substantially contributes to an extended lifespan [10]. However, heme oxygenase (HO)-1 (also termed as HSP32) has long been shown to be involved in adaptive responses because it generates antioxidative bilirubin and carbon monoxide, the latter of which is currently emerged as a protective mediator in the regulation of several pathologies [11,12]. Along a similar line, endogenous hydrogen sulfide (H₂S), which is produced via activation of cystationine β-synthase (CBS) and cystationine γ-liase (CSE), has recently attracted attention because of its cardioprotective efficacy [13]. To the best of our knowledge, there have been only a few studies showing the effects of anthocyanins on the expressions of HSP and other self-protective genes. Thus, in the current study, we examined the effects of both Sunrouge and Yabukita on those gene expressions in both cell culture and animal models. Also, the effects of Sunrouge on thermoresistance and longevity were examined by using the nematodes *Caenorhabditis elegans*. The results clearly showed that Sunrouge has more pronounced abilities in several evaluation assays than Yabukita, suggesting that this unique anthocyanin-rich tea is a promising material for functional foods.

2. Materials and methods

2.1. Cells and animals

Hepa1c1c7 mouse hepatocytes cells (passage 15–24) were cultured in Dulbecco modified Eagle medium (DMEM) and supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. DMEM and FBS were purchased from Invitrogen (Carlsbad, CA, USA). Wild-type N2 nematodes were cultured using a standard *C. elegans* culturing method [14]. Age-synchronized populations were prepared as previously described [15]. Eggs were collected from gravid adult hermaphrodites using sodium hypochlorite and allowed to hatch at 25°C in S-basal buffer, which consisted of 100 mM NaCl and 50 mM potassium phosphate (pH 6.0). After hatching, the worms (L1-stage larvae) were transferred to fresh nematode growth medium (NGM) agar plates containing *Escherichia coli* OP50 at 25°C. Male-specific pathogen-free ICR mice (4 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and housed one per cage. All mice were fed an AIN-93G diet and given fresh tap water *ad libitum*, while being kept at 22–26°C with a relative humidity of 55–65% under a 12-hour (06:00–18:00) light/dark cycle for 6 days prior to the experiment. The mice were used for experiments after the 1st week of quarantine. The mice were treated in accordance with the Guidelines for the Treatment of Experimental Animals of Kyoto University, Kyoto, Japan and the experimental protocol was approved by the Experimentation Committee of the same institution (approval No.25-5).

2.2. Tea samples

Both Yabukita and Sunrouge leaves were collected in the National Institute of Vegetables and Tea Sciences and dried

overnight in a warm wind. The dried leaves (10 g) were soaked in water (40 mL) at room temperature for 15 minutes, and then steamed, followed by centrifugation at 10,000g. This material was frozen at -40°C and dried in a gradient condition ($5\text{--}60^{\circ}\text{C}$) for 72 hours to yield freeze-dried (FD) material (7.1 g), which was used for mouse experiments shown below. Alternatively, FD samples (1 g) were extracted with 100 mL of boiling water for 10 minutes. After being cooled, the extracts were filtered through a filter paper, followed by centrifugation at 10,000g for 10 minutes. For cellular and nematode experiments, those extracts were sterilized by using a microfilter (0.2 μm).

2.3. Cytotoxicity assay

Cell viability was determined using a Cell Counting Kit-8 (WST-8, Dojindo Molecular Technology, Kumamoto, Japan), according to the manufacturer's protocol. Cells were incubated in DMEM containing 5% WST for 1 hour, then absorbance of the cell culture medium was measured at 450 nm.

2.4. HSPs expression in mice

The mice were divided into three groups of four mice each: nontreated (Group 1), Yabukita-treated (Group 2), and Sunrouge-treated (Group 3). Groups 2 and 3 were given a diet containing 1% FD Yabukita and Sunrouge, respectively. Body weights and food and water intake of each group were recorded twice per week until the end of the experiment. After 1 month, all mice were euthanized by pentobarbital (50 mg/kg body weight, intraperitoneally) for determining the effects of tea materials on HSP mRNA expressions as shown in the next sections.

2.5. Real-time reverse transcription-polymerase chain reaction

Hepa1c1c7 cells ($1 \times 10^5/\text{mL}$) were seeded into 24-well culture plates and treated with a sample or the vehicle [0.5% dimethyl sulfoxide (DMSO), v/v] for 6 hours. Alternatively, the mucosa from the stomach, upper and lower small intestines, and large intestine were scraped off with a razor, and liver and kidney were taken from the euthanized mice, followed by storage at -80°C until use. Approximately 20 mg of each tissue were used for the total RNA extraction. Total RNA from cell cultures and animal tissues was isolated from cells using TRIzol reagent (Invitrogen), according to the manufacturer's specifications. The amount and purity of RNA were assessed by spectrophotometry using a SmartSpec 3000 Spectrophotometer (Bio-Rad Laboratories (Hercules, CA)). Complementary DNA was synthesized using 1 μg of total RNA with an RNA polymerase chain reaction (PCR) Kit (avian myeloblastosis virus (AMV)). Thermal cycling was performed with a 7300 real time PCR system using SYBR green PCR mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. PCR conditions were as follows: 95°C for 3 minutes, 95°C for 10 seconds, and 60°C for 1 minute (mouse). The primers and sequences used are summarized in Table 1.

Table 1 – Primers used for reverse transcription-polymerase chain reaction.

Gene	Primer	Sequence (5' to 3')
HSP90 α	Sense	AAAgCAGgCTgACAAgA
	Antisense	AggggAggCATTCTTCAGT
HSP90 β	Sense	gCggCAAAGACAAgAAAAg
	Antisense	gAAgTggTCCTCCCAgTCAT
HSP70	Sense	TggTgCTgACgAAgATgAAg
	Antisense	AggTCgAAgATgAgCACCGTT
HSP40	Sense	TTACAAggCgAggAgAAgAC
	Antisense	TTgACAATCTgACCTggATg
HSP27	Sense	TgCTTCACCCggAAATACAC
	Antisense	CTCgAAAgTAACCggAATgg
HO-1	Sense	TCCGAGACACgCTCCTCCAG
	Antisense	ggATTTggggCTgCTggTTTC
CBS	Sense	TGAACCAGACggAgCAAACA
	Antisense	CCAggACTgTCgggATgAAg
CSE	Sense	gATggCggTggCTCgTT
	Antisense	ggTAGCCCAggATAACAACCTT
HPRT	Sense	gTAATgATCAGTCAACGgggAC
	Antisense	CCAgCAAgCTTgCAACCTTAACCA

2.6. C. elegans motility assay

The age-synchronized Day-4 worms were incubated at 20°C for 1 hour in S-basal buffer containing various concentrations of tea samples, then subjected to heat shock at 37°C for 30 minutes. Mild heat training at 33°C for 1 hour served as a positive control. Twenty-four hours after each treatment, the motility of the worms was evaluated by counting the bending numbers per minute. Each experiment was done in triplicate, and the motility of five or more worms were counted.

2.7. Longevity evaluation

The lifespan assays were performed by using 96-well plates as previously reported [16]. In brief, the age synchronized *C. elegans* (approximately 10 worms/well) were added into an S-basal buffer with *E. coli* at 20°C on a 96-well microplate, which contained specified concentrations of tea samples or vehicle, and the survival data were recorded three times per week. The microplate was shaken to determine whether the animals were alive or dead, which was judged by movements of the worms and also those of the pharynx.

2.8. Statistical analysis

The results are presented as the means \pm standard deviations. Statistical significance was assessed using a Student t test and Wilcoxon test, and differences were considered significant at $p < 0.05$.

3. Results

3.1. Sunrouge potentially upregulated HSP expressions in Hepa1c1c7 cells

We first examined the cytotoxicity of the tea samples to explore their sublethal concentrations because HSP

expressions are known to be fundamentally increased in stressful conditions. Treatment of Hepa1c1c7 mouse hepatocytes with 2.5–10% (*v/v*) tea extract for 6 hours led to concentration-dependent decreases of cell viability (Fig. 1A). Then, we selected 5% and 10% as the putative, optimum concentrations of Sunrouge and Yabukita, respectively, where approximately 60% cell viability was seen as compared with vehicle treated control. Subsequently, mRNA expressions of the self-defensive genes (HSPs, CBS, and CSE) in the Sunrouge- and Yabukita-treated cells were quantified by quantitative reverse transcription-polymerase chain reaction (Fig. 1B and 1C). Yabukita preparation significantly upregulated mRNA expressions of HSP40 (36-fold), HSP70 (10.1-fold), and HO-1 (HSP32; 8.0-fold) [17], but not HSP90 α , HSP90 β , HSP27, CBS, and CSE, as compared with the vehicle control. More strikingly, those of HSP40, HSP70, and HO-1 were dramatically increased by Sunrouge by 230-, 19.7-, and 39-fold, respectively.

3.2. Sunrouge potently upregulated HSP expressions in mice

Given those results, we evaluated the effects of oral feeding of Sunrouge on HSP expressions in male ICR mice. All mice were fed with control diet (AIN-93G), Yabukita diet (plus 1% Yabukita FD), and Sunrouge diet (plus 1% Sunrouge FD) for 1 month.

During the experiments, there were no significant differences in intakes of the diets and water, the weights of final body, liver, and kidneys (data not shown). Then, we semiquantified mRNA expression levels of HSP90 α , HSP90 β , HSP70, HSP40, HSP27, and HO-1 (HSP32) in the mucosa of the stomach, upper small intestine, lower small intestine, and large intestine as well as whole liver and kidney (Fig. 2A–F). Of a total of 36 genes expressions from six organs, Sunrouge and Yabukita significantly increased 13 genes and six genes, respectively, whereas Yabukita, but not Sunrouge, significantly decreased HSP27 by 0.36-fold in the lower small intestinal mucosa (Fig. 2C) and HSP90 α by 0.65-fold in the liver (Fig. 2E). The largest upregulation level (28-fold increase) from the tea sample-treated two groups was observed for HSP40 expression in the stomach from Sunrouge-fed mice (Fig. 2A), and this was followed by HO-1 in the lower small intestine of Sunrouge group (23.4-fold increase, Fig. 2C). Regarding the Yabukita group, the largest induction was seen for HSP27, the increase of which was limited to 13.3-fold. It is of interest to point out that the upper small intestine was identified to be the most sensitive site for HSP induction because all six genes examined were markedly increased by Sunrouge (Fig. 2B). This is in contrast with data on the lower small intestine (only 1 gene, HO-1, Fig. 2C) and large intestine (2 genes, HSP70, HSP27, and HO-1, Fig. 2D). By contrast, Yabukita increased only two genes

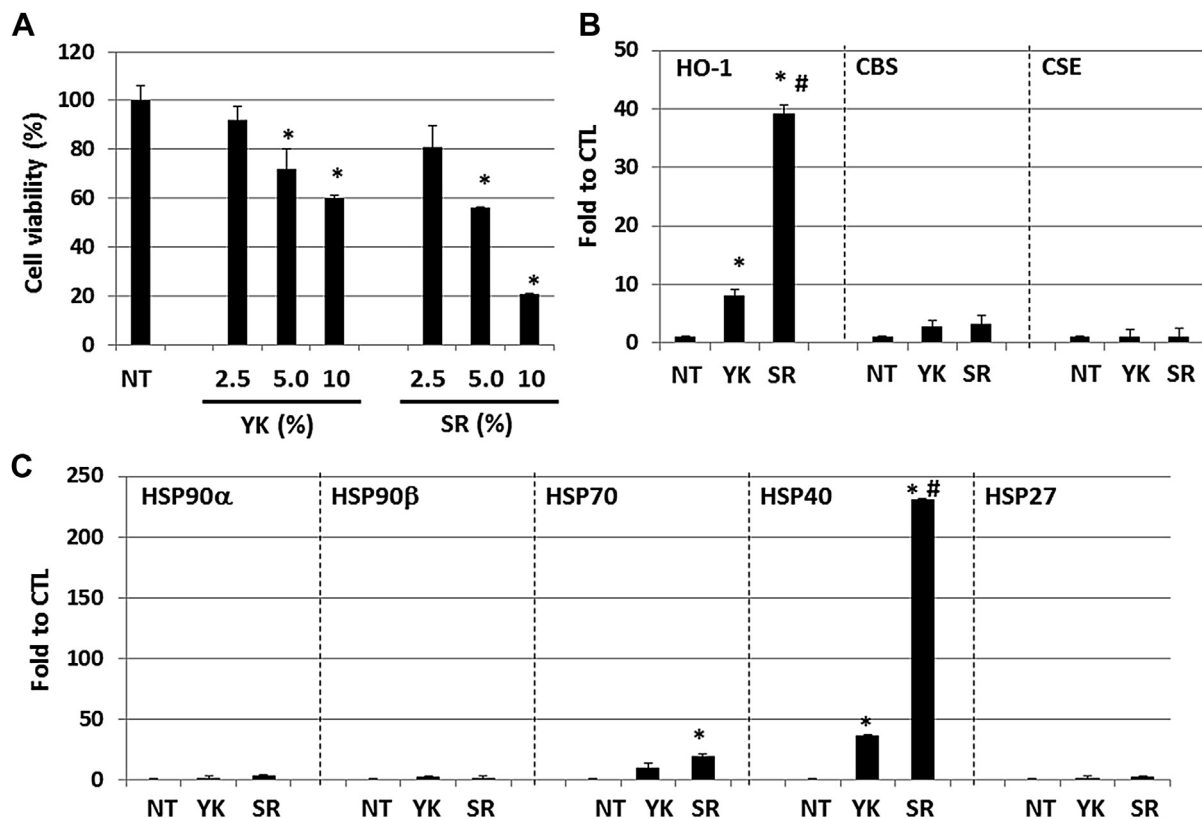


Fig. 1 – (A) Viability of Hepa1c1c7 mouse hepatoma cells exposed to Yabukita (YK) and Sunrouge (SR) preparations for 6 hours. (B,C) Effects of YK (10%, *v/v*) and SR (5%, *v/v*) on messenger RNA expressions of heme oxygenase-1 (HO-1), cystationine β -synthase (CBS), cystationine γ -liase (CSE), and heat shock protein (HSP) as quantified by reverse transcription-polymerase chain reaction analyses. **p* < 0.05 (versus non-treated control group), #*p* < 0.05 (versus YK group) in the Student *t* test.

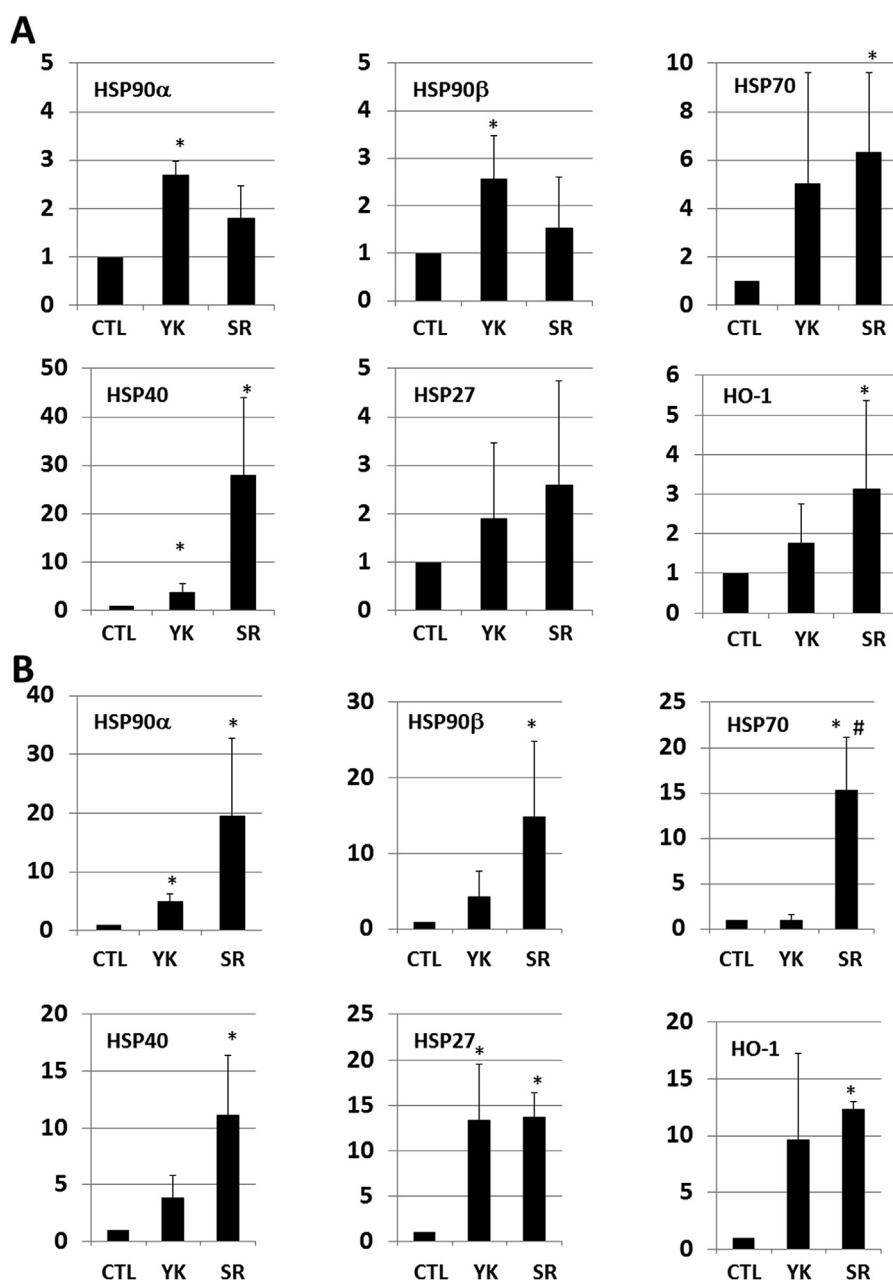


Fig. 2 – Effects of oral administrations of basal diet (CTL), Yabukita (YK) and Sunrouge (SR) (1%, w/w, in the basal diet for 1 month each) on messenger RNA expression of heat shock protein (HSP) genes and HO-1 in the mucosa of the (A) stomach, (B) upper small intestine, (C) lower small intestine, (D) large intestine, (E) liver, and (F) kidney of ICR mice. Four mice were used for each group. * $p < 0.05$ (vs. nontreated control group), # $p < 0.05$ (vs. YK group) in the Student t test.

(HSP90 α and HSP27) in those organs (Fig. 2B–D). No significant changes among the three groups were seen for the kidney samples.

3.3. Sunrouge conferred thermoresistance and extended lifespan of *C. elegans*

Heat shock treatment of nematodes at 37°C for 30 minutes, followed by 24-hour recovery, resulted in a significant decrease of the motility by 43% (Fig. 3), which was consistent with the previous findings [18]. Interestingly, mild heat

pretreatment markedly attenuated heat shock-decreased motility by 61%. When the nematodes were pretreated with increased concentrations of Yabukita (0.008–20%, v/v) for 24 hours, we found that only 5% exposure was effective to show thermoresistance, whereas Sunrouge exerted concentration-dependent, suppressive activity, and statistically significant differences were found for this unique tea preparation at the concentrations of 5% and 10%. Moreover, longevity testing revealed that Sunrouge has a significant lifespan extending activity because the average and maximum lifespan in Sunrouge group were 19.6 days and

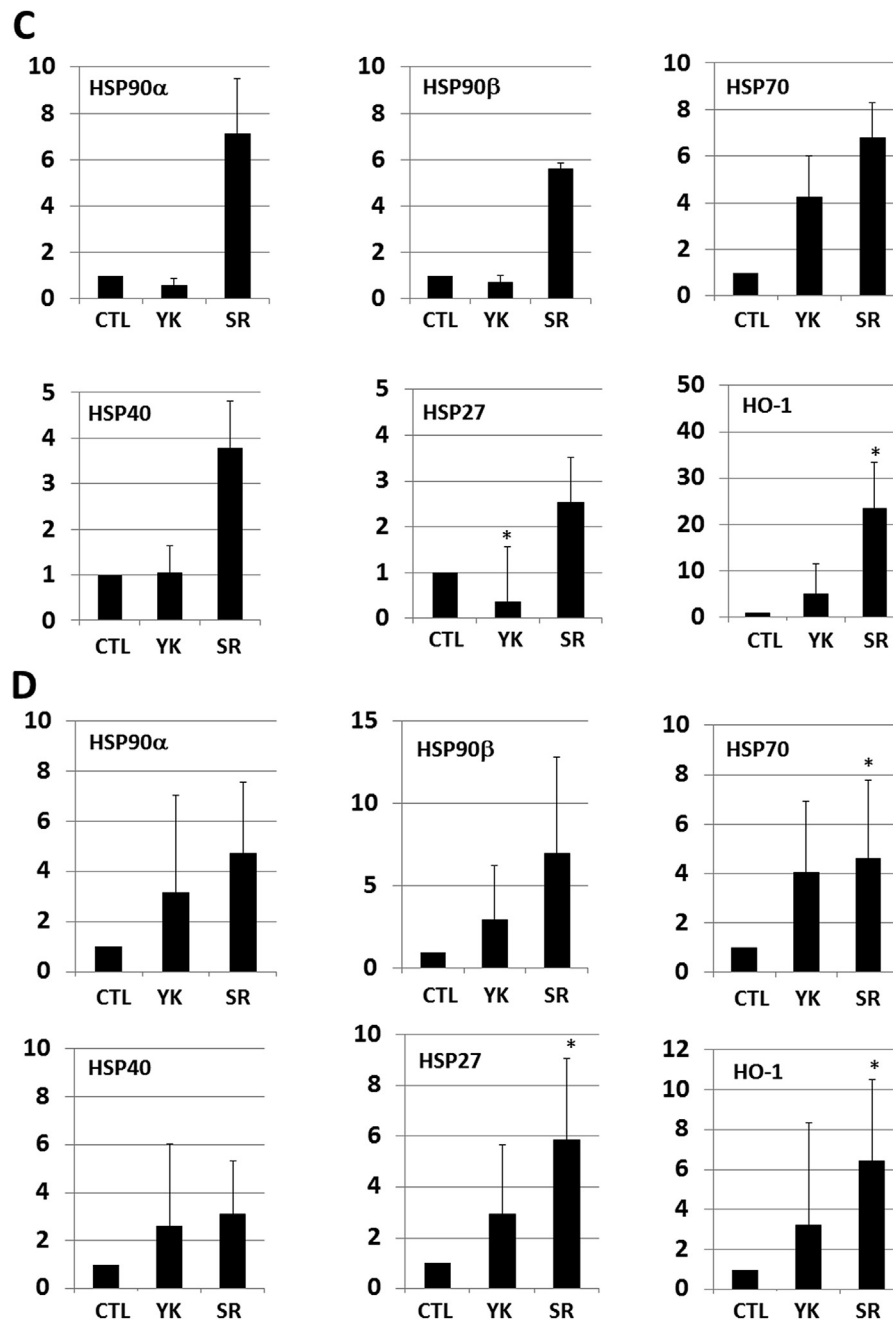


Fig. 2 – (continued).

25 days (0.2% group) and 20.5 days and 27 days (1% group), respectively, as compared with those of vehicle control group (18 days and 22 days, respectively), whereas both 1% and 5% Yabukita groups did not show any statistically significant longevity effects.

4. Discussion

There is ample evidence that green tea and its constituents have versatile physiological activities that may prevent

metabolic syndrome [19], neurodegenerative [20] and cardiovascular [21] diseases. It is also well known that green tea catechins, including (–)-epigallocatechin-3-gallate, exhibit the aforementioned bioactivities via both antioxidation-dependent [22] and -independent [23] mechanisms. Although Yabukita is one of the most popular tea cultivars in Japan, it is known that tea (*C. sinensis* L.) has a great diversity in its genetic backgrounds, and thus exhibits different chemical compositions, which presumably affect tastes, disease resistances, and bioactivities. This notion is supported by Fujimura et al [9] who did metabolomics-driven nutraceutical

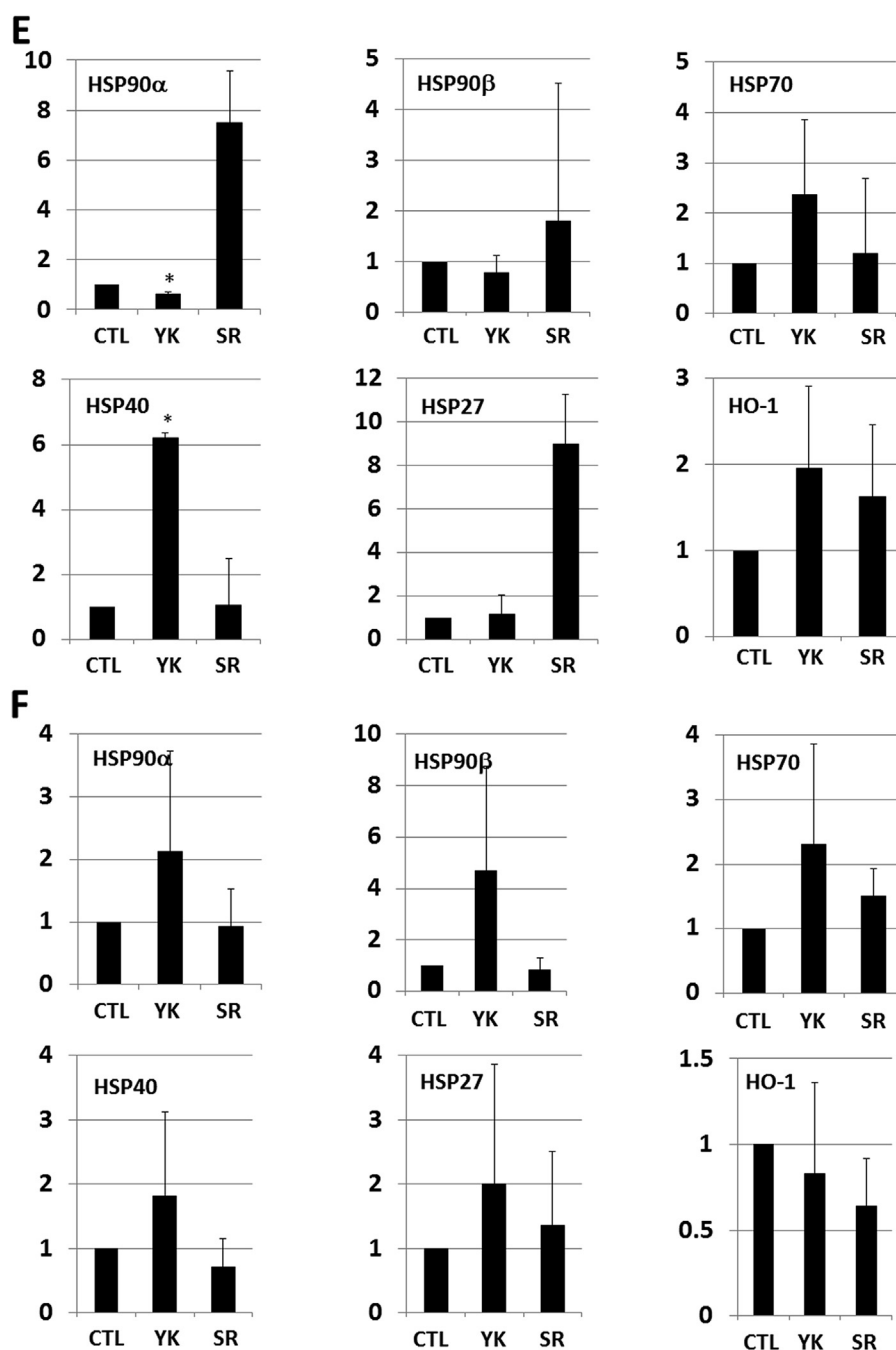


Fig. 2 – (continued).

evaluation of 43 Japanese cultivars and found that two cultivars, i.e., Sunrouge and Cha Chuukanbohon Nou-6, markedly inhibited thrombin-induced phosphorylation of myosin regulatory light chain in human umbilical vein endothelial cells, a potential hallmark of vascular endothelial dysfunction. Similarly, Sunrouge showed more pronounced acetylcholinesterase activity in human neuroblastoma SK-N-SH cells, as compared with Yabukita and another tea cultivar, Benifuuki [7]. In addition, Sunrouge effectively ameliorated mouse colitis whereas Yabukita showed either no effect or adverse effects [8]. In accordance with those observations, the current

study showed that Sunrouge is capable of upregulating several *hsp* genes in cultured hepatocytes (Fig. 1) and gastrointestinal mucosa in ICR mice (Fig. 2), and those bioactivities were consistently higher than those of Yabukita.

Molecular chaperones, including HSPs, have essential roles in protein homeostasis by refolding the denatured proteins and thereby prevent their aggregation. In 1980, McAlister and Finkelstein [24] described an essential role of HSPs in thermal resistance in yeast, and similar phenomena were found in *C. elegans* [25]. Moreover, recent studies have demonstrated that HSPs have notable preventive roles in many lifestyle-related

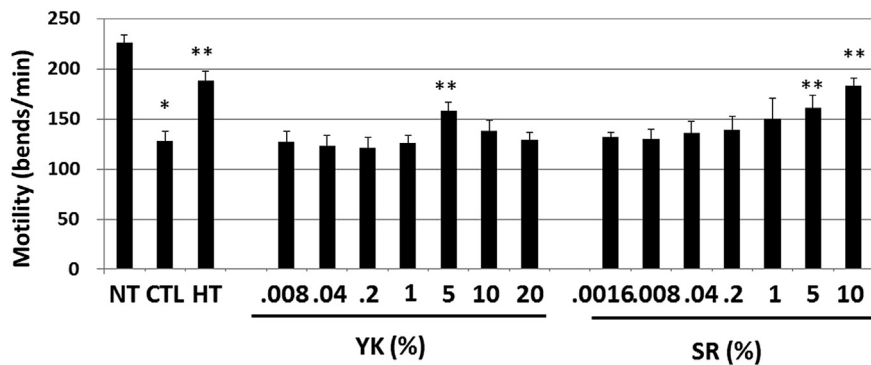


Fig. 3 – Effects of oral Yabukita (YK, 0.008–20%, *v/v*) and Sunrouge (SR, 0.0016–10%, *v/v*) on the motility of *Caenorhabditis elegans* and mild heat training. The age-synchronized Day 4 worms were incubated at 20°C for 1 hour in S-basal buffer containing tea samples, then subjected to heat shock at 37°C for 30 minutes. Mild heat training at 33°C for 1 hour served as a positive control. Twenty-four hours after each treatment, the motility of the worms was evaluated by counting the bending numbers per minute. Each experiment was done in triplicate, and the motility of five or more worms were counted. **p* < 0.05 (versus NT), ***p* < 0.05 (versus CTL) in the Student *t* test. CTL = heat shock group (37°C for 30 minutes); HT = heat training group (33°C for 1 hour); NT = nontreated group.

diseases as well as other pathologies, such as neurodegenerative disorders [26] and osteoporosis [27]. Furthermore, sustained upregulation of the *hsp* genes may contribute to the extended lifespan, as shown in many experimental models using *C. elegans* and *Drosophila melanogaster* [28]. Therefore, the capability of Sunrouge for inducing HSP expressions in cultured cells (Fig. 1) and mouse organs and tissues (Fig. 2) implies that putative HSP expression in *C. elegans* could contribute to thermoresistance (Fig. 3) and extended lifespan (Fig. 4), and those issues are now under investigation in our laboratory.

Geldanamycin, a natural benzoquinone antibiotic, has been shown to inhibit HSP90 by binding to the adenosine diphosphate-adenosine triphosphate-binding domain for

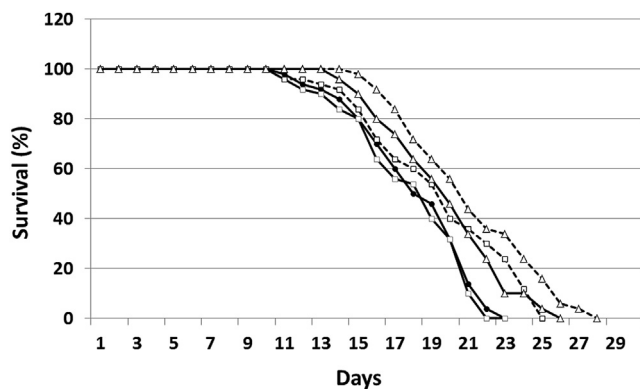


Fig. 4 – Sunrouge (SR, 0.2 or 1%, *v/v*), but not Yabukita (YK, 1 or 5%, *v/v*), significantly extended life-span of *Caenorhabditis elegans*. Solid line with closed circles = nontreated control; unbroken line with open squares = 1% YK; unbroken line with open triangles = 5% YK; broken line with open squares = 0.2% SR; broken line with open triangles = 1% SR. Both 0.2% and 1% SR, but not YK, showed significant longevity extension versus nontreated control by Wilcoxon test (*p* < 0.05).

upregulating many *hsp* genes [29]. Geranylgeranylacetone, however, has also been described as a potent antiulcer drug, which is capable of increasing the expression of inducible HSPs via binding to the C-terminal of HSP70 [30], in experimental rodents [31] and healthy volunteers [32]. In addition, Ahmed et al [33] have recently screened 80 compounds from medicinal plants for their HSP70 inducing activities in U937 human lymphoma cells. Furthermore, we have recently reported that several phytochemicals, but not nutrients, are notable HSP70 inducers in Hepa1c1c7 mouse hepatocytes [34]. To the best of our knowledge, the current study is one of the first to show that food preparation increased HSP expressions in a rodent model.

Although having not identified the active principle(s) of Sunrouge that induce HSP expressions, we recently found that delphinidin-3-O-galactoside, which is present in Sunrouge, upregulated HSP70 and heme oxygenase (HSP32) induction in Hepa1c1c7 cells [35]. Similarly, both cyanidin-3-O-glucoside and its aglycone increased HSP70 expression in Caco-2 human adenocarcinoma cells [36]. Collectively, anthocyanins may play significant roles in Sunrouge-upregulated HSPs inductions *in vivo*. Although we could not provide any mechanistic data to account for HSPs upregulation by Sunrouge, one possibility can be proposed. As shown in Fig. 3, mild heat shock at 33°C significantly suppressed severe heat shock (at 37°C)-induced motility decrease of the worms. This phenomenon can be reasoned by the concept hormesis [37], in which mild stress can activate self-defense systems, including HSP induction, for homeostasis and survival. It should be noted that mild heat stress causes denaturation stress to cellular proteins, which may activate protein homeostatic mechanisms, including HSPs induction, for adaptation. Therefore, it is reasonable to assume that phytochemicals, including those in Sunrouge, increase the HSP expressions because of their nature as xenobiotics to animals [38]. In fact, our recent findings uncovered that some phytochemicals induce “proteostress” through nonspecific interactions [34,39].

Conflicts of interest

H.Y., K.Y., M.M., T.N., and K.N. are employees of Nepuree Corporation, which sells food materials including Sunrouge.

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