Extension of lifespan and protection against oxidative stress by an antioxidant herb mixture complex (KPG-7) in *Caenorhabditis elegans*

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Excessive generation of reactive oxygen species within cells results in oxidative stress. Furthermore, accumulation of reactive oxygen species has been shown to reduce cell longevity. Many dietary supplements are believed to have anti-aging effects. The herb mixture KPG-7 contains several components with antioxidant activity. We aim to clarify the mechanisms responsible for the antioxidant activity of KPG-7 and to establish whether KPG-7 has an anti-aging effect. We examined whether dietary supplementation with KPG-7 could provide protection against oxidative stress, extend lifespan, and delay aging in *Caenorhabditis elegans* (*C. elegans*). We found that KPG-7 extended lifespan and delayed aging in adult *C. elegans*. The expression of oxidation resistance 1 protein was induced by juglone and this effect was significantly suppressed in KPG-7-treated. In addition, the amount of oxidized protein was significantly lower in KPG-7-treated worms than untreated worms. Furthermore, locomotive activity was increased in *C. elegans* at 3 days of age following the treatment with KPG-7. On the other hand, the level of cellular ATP was lower at 3 days of age in worms treated with KPG-7 than in untreated worms. KPG-7 increases lifespan and delays aging in *C. elegans*, well corresponding to its activity to protect against oxidative stress.

***Key Words*: oxidative stress, *C. elegans*, aging, herb mixture, antioxidant**

Reactive oxygen species (ROS) such as the superoxide anion radical ($O_2^{\bullet-}$), the hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) are generated continually in living cells during normal cellular metabolism.[1] ROS are also generated by exogenous sources such as ionizing radiation and various chemical oxidants.[2] Excessive accumulation of ROS within cells results in oxidative stress that causes oxidative damage to DNA, proteins and lipids.[3,4] If such damage is not eliminated or repaired, it can lead to cellular dysfunction, cell death, mutations and cancer.[5] ROS also cause cardiovascular and neurodegenerative diseases.[5–7] Furthermore, excessive accumulation of ROS has been shown to reduce lifespan in mice, yeast and *Caenorhabditis elegans* (*C. elegans*).[8–12] To overcome these deleterious effects, organisms have evolved strong defense mechanisms against oxidative stress.[13,14]

Cells are equipped with several antioxidant enzymes; superoxide dismutase, catalase, glutathione peroxidase, thioredoxin and glutaredoxin.[15–18] These enzymes play important roles in removing ROS and maintaining the redox state in cells.[15–18] In addition, many hydrophilic radical scavengers such as ascorbate (vitamin C) and lipophilic radical scavengers like α-tocopherol (vitamin E) protect cells from oxidative damage caused by ROS.[19,20] Cellular oxidative stress occurs through an imbalance between the production of ROS and their removal by antioxidant enzymes and scavengers.[21,22]

Many dietary supplements are thought to protect against oxidative stress.[23–26] For example, vegetables and fruits are rich in flavonoid-related compounds with antioxidant activity.[26,27] Recently, herbal medicines have gained attention because of their antioxidant and anti-aging activities.[23–29] For example, blueberry polyphenols increase lifespan and thermotolerance in *C. elegans*.[30] Dietary supplementation with antioxidants has become more and more popular. However, their biochemical mechanisms of protection against oxidative stress and anti-aging effects are not fully understood.

KPG-7 is a commercially available herb mixture containing *Thymus vulgaris*, *Rosmarinus officinalis, Curcuma longa Linn., Foeniculum vulgare, Vitis vinifera* (polyphenol), silk protein, *Taraxacum officinale Weber* and *Eleutherococcus senticosus*. Rosemary leaves, *Rosmarinus officinalis*, *Curcuma longa Linn.*, and *Thymus vulgaris*, have been reported to include a variety of antioxidant, anti-tumoral and anti-inflammatory bioactivities.[31–33]

C. elegans is a useful model animal for studying the mechanisms of aging, because of its relatively short lifespan, rapid generation time and simple body plan.[34,35] In this study, we examined whether dietary supplementation with KPG-7 could provide protection against oxidative stress and extend lifespan in *C. elegans*. We tried to understand whether traditional herbal formulas protecting against age-related disorders could be evaluated in animal models of aging. We found that treatment with KPG-7 results in a longer lifespan and delayed aging in *C. elegans*. We showed that KPG-7 suppresses the oxidation of proteins in cells and the expression of the oxidative stress-inducible oxidation resistance 1 (OXR1) protein. Furthermore, we found that locomotive activity (bending frequency) was higher in KPG-7-treated worms, while the amount of ATP was lower. From these results it is concluded that KPG-7 protects *C. elegans* against oxidative stress and exerts an anti-aging effect.

Materials and Methods

C. elegans strains and culture conditions. The wild-type (Bristol N2) strain, *fem-3* (*q20*) mutant (JK816) and GFP reporter strain for the *oxr1* gene (BC10281) of *C. elegans* were supplied by the Caenorhabditis Genetics Center (Minneapolis, MN). The worms were cultured at 20°C, unless otherwise stated, on 90-mm NGM plates containing 0.3% (w/v) NaCl, 0.25% (w/v) polypeptone, 0.005% (w/v) cholesterol, 1 mM $MgSO_4$, 1 mM $CaCl_2$,

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25 mM potassium phosphate, pH 6.0 and 0.17% (w/v) agar with a lawn of *E. coli* OP50.

KPG-7. KPG-7 is a commercially available herb complex (KIPPO Sci. Ltd. Tokyo, Japan). Standardized KPG-7 tablets (0.375 g) contain 10% (w/w) *Thymus vulgaris*, 10% (w/w) *Rosmarinus officinalis*, 10% (w/w) *Curcuma longa Linn.*, 10% (w/w) *Foeniculum vulgare*, 5% (w/w) *Vitis vinifera* [4.25% (w/w) polyphenol], 5% (w/w) silk protein, 5% (w/w) *Taraxacum officinale Weber* and 45% (w/w) *Eleutherococcus senticosus*. Ten tablets were dissolved in distilled water (total 40 ml) and centrifuged at 12,000 × g for 15 min. The supernatant was taken and added to the plates at a final concentration of 10%.

Measurement of lifespan. Wild-type N2 and mutant *fem-3* worms on NGM plates were harvested by rinsing with distilled water and resuspended in alkaline hypochlorite [500 mM NaOH and 1.2% (v/v) hypochlorite] until their bodies were dissolved completely (5–10 min). Eggs were then recovered and washed three times with S basal (50 mM potassium phosphate, pH 6.0, containing 100 mM NaCl). The eggs were hatched at 20°C overnight without food. L1 larvae were then transferred to NGM plates and allowed to develop into adults (about 3 days). For determining lifespan, worms were transferred to 60-mm NGM plates with a lawn of *E. coli* OP50 in the presence of 10 μg/ml of 5-fluoro-2'-deoxyuridine.[36] Worms failing to move spontaneously or to respond to touch were counted as dead. Living worms were counted every 2 days. To determine the lifespan of the *fem-3* mutant, adult worms were transferred to NGM plates with a lawn of *E. coli* OP50 and cultured at 25°C. The experimental procedures are shown in Fig. 1.

Measurement of the frequency of body bends. To measure the frequency of body bends, worms were placed in a dish containing a drop of M9 buffer and observed by microscopy. Movement was recorded by a digital camera, and the frequency of sinusoidal movements (for 10 s) was counted. Experiments were typically done with more than 50 worms.

Determination of carbonylated proteins. Worms were harvested from NGM plates and living worms were separated with 30% sucrose, washed several times with M9 buffer and then homogenized on ice. Extracts were subsequently incubated with 2,4-dinitrophenylhydrazine (DNPH) as described by Levine *et al.*[37] with some modifications.[37,38] After transfer to a Hybond-C Extra membrane by a slot blot method, carbonylated proteins were detected with anti-DNPH antibody and a Chemiluminescence Detection Kit (ECL 34080, Thermo Scientific, Rockford, IL).

ATP assay. Worms were collected from the NGM plates. Living worms were separated with 30% sucrose and washed several times with 10 mM HEPES-KOH (pH 7.5) containing 0.25 M sucrose. The worms were homogenized on ice and then subjected to ATP extraction using a LL100-2 kit (Toyo INK, Tokyo, Japan). After determination of the protein concentration using a protein BCA assay kit, an Kinshiro ATP luminescence kit (LL100-1, Toyo INK) was used to determine the amount of ATP.

Microscopy. The expression of the human OXR1 homolog was assayed in adult worms raised at 20°C for 3 days from L1 larvae. The worms were incubated in M9 buffer containing 0 or 10 μM of juglone at 20°C for 6 h. After the treatment, the worms were fixed with 4% paraformaldehyde for 20 min at room temperature. Fluorescence images were obtained using a fluorescence microscope (Olympus IX70, Olympus, Tokyo, Japan).

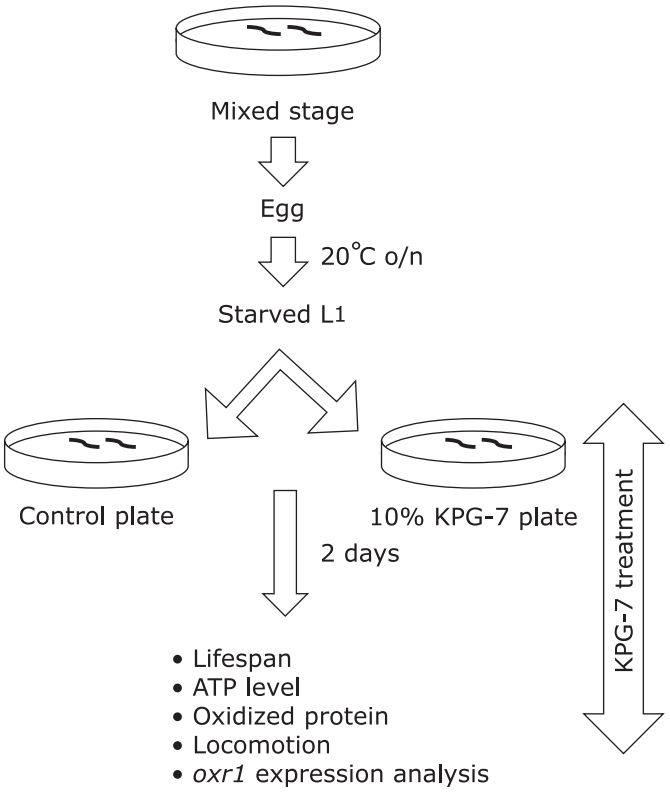

Fig. 1. Experimental procedures. Worms were placed on NGM plates. The eggs were collected and incubated in M9 buffer for overnight at 20°C until the worms hatched completely. Then starved L1 worms were transferred to NGM plates containing KPG-7 and cultured for 2 days. Then these worms were used for analysis.

Results

Decrease in the amount of oxidized protein in *C. elegans* with KPG-7 treatment. KPG-7 is a mixture of commercially available herbal supplements which contains extracts from thyme, rosemary, curcumin, fennel, grape seed, silk protein, dandelion and eleuthero. KPG-7 includes several compounds that possess antioxidant activity.[31–33] In this study, we examined whether supplementation with KPG-7 has antioxidant effects in *C. elegans*. We first compared the amount of oxidized protein using the Oxyblot™ kit. *C. elegans* has a short lifecycle; the worms hatch 12 h after eggs are laid, change to L1 larvae and 3 days later change into adults. In this study, to avoid the influence of eggs, the *fem-3* (*q20*) mutant worms[39] were cultured, synchro-

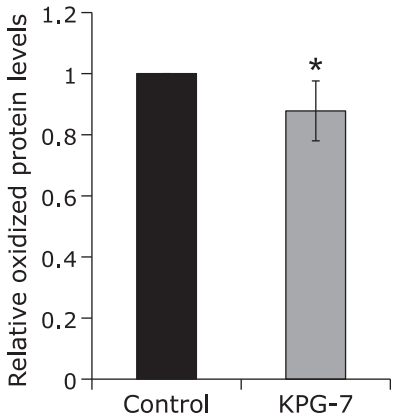

Fig. 2. Accumulation of oxidized proteins in *C. elegans* treated with KPG-7. The JK816 (*fem-3* mutant) strain was raised at 25°C on NGM plates supplemented with or without 10% KPG-7 for 9 days from L1 larvae. The worms were collected and cell-free extracts were prepared. Carbonylated proteins in the extracts were measured by Oxyblot™. Measurements were made three times independently. Error bars represent SD. The mark * means statistically different ($p<0.05$) by the Student's *t* test.

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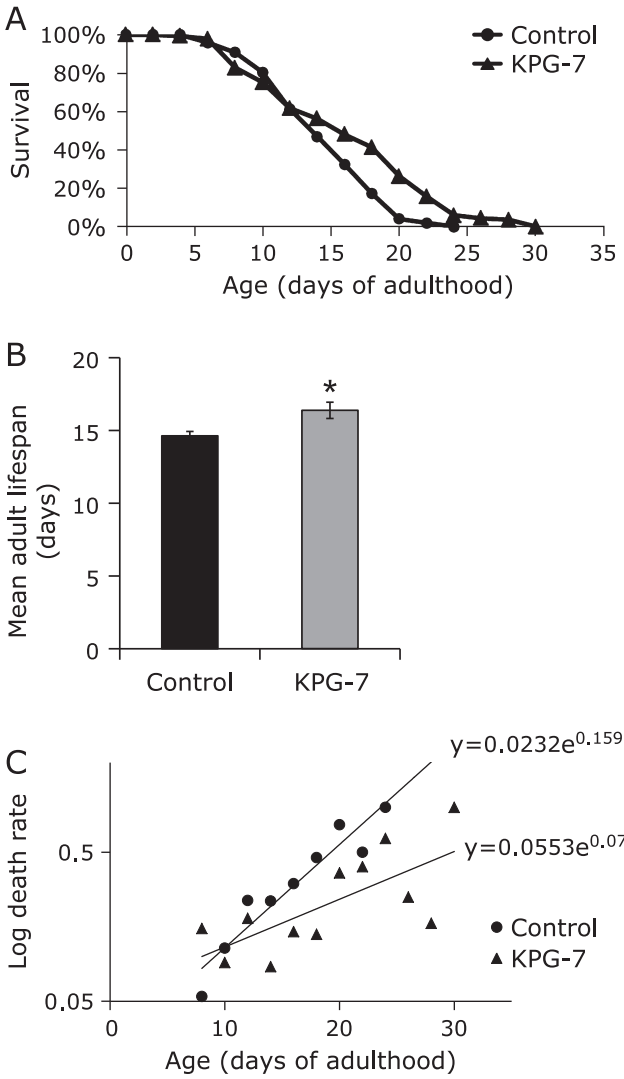

Fig. 3. Lifespan of wild-type N2 worm with or without KPG-7. Lifespan was measured four times independently. L4 larvae were transferred to NGM plates containing 10 mM 5-fluoro-2'-deoxyuridine in the absence or presence of 10% KPG-7. Dead worms were counted every 2 days. (A) Survival curves on NGM plates containing KPG-7 (▲) (n = 133) or distilled water (●) (n = 194). (B) Mean adult lifespan. The mean adult lifespan of untrated control worms was 14.63 ± 0.30 days, and that of KPG-7-treeted worms, 16.38 ± 0.56 days. Error bars represent SE. The mark * means statistically different (p<0.05) by the Student's t test. (C) Gompeltz curves on lifespan with (▲) or without (●) KPG-7. The slope was 0.16 for control worms and 0.07 for KPG-7-treated worms.

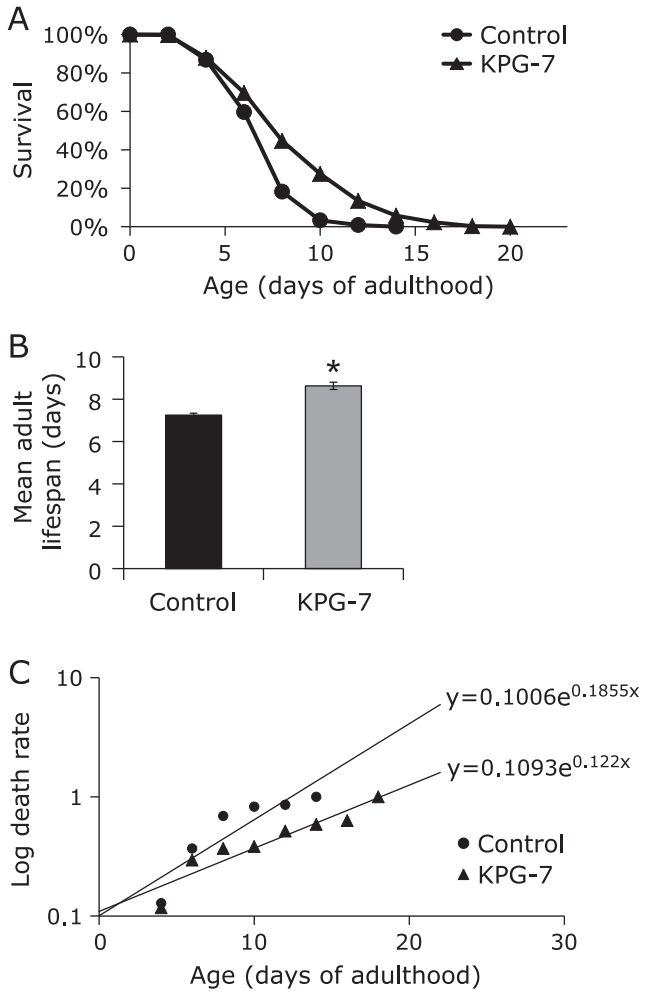

Fig. 4. Lifespan of *C. elegans* JK816 (*fem-3* mutant) strain with or without KPG-7. Lifespan was measured three times independently. L4 larvae of *C. elegans* JK816 strain were transferred to NGM plates and cultured at 25°C in the absence or presence of 10% KPG-7. Dead worms were counted every 2 days. (A) Survival on NGM plates containing KPG-7 (▲) (n = 349) or distilled water (●) (n = 456). (B) Mean adult lifespan. The mean adult lifespan of untreated control worms was 7.38 ± 0.09 days, and that of KPG-7-treated worms, 9.03 ± 0.19 days. Error bars represent SE. The mark * means statistically different (p<0.05) by the Student t test. (C) Gompeltz curves on lifespan with (▲) or without (●) KPG-7. The slope was 0.19 for control worms and 0.12 for KPG-7-treated worms.

nized L1 larvae were scattered onto agar plates supplemented with 10% (v/v) KPG-7 for 9 days, and collected. Extracts were prepared from the worms and oxidized proteins (carboxylated proteins) were determined using Oxyblot™. The results are shown in Fig. 2. It was evident that the amount of oxidized protein was significantly lower in KPG-7-treated worms than untreated worms. The level of oxidized protein decreased to about 88% in the worms treated with KPG-7 (Fig. 2).

Extension of lifespan of *C. elegans* by KPG-7 treatment. KPG-7 includes various phenolic compounds with antioxidant properties, such as thyme, rosemary and curcumin.[31–33] Recent studies have shown that certain phenolic compounds increase the lifespan of *C. elegans*, *D. melanogaster* and mice.[40,41] So, we examined whether the supplementation with KPG-7 influences the longevity of *C. elegans*. Adult N2 worms were grown at 20°C on NGM plates with 10% (v/v) KPG-7. 5-fluoro-2'-deoxyuridine was added at 10 mM to the plates to suppress progeny production when the worms reached the adult stage.[36] When the adult worms were cultured on NGM plates supplemented with 10% KPG-7, lifespan was significantly extended, as shown in Figs. 3A and B. The mean adult lifespan of untreated worms and KPG-7-treated worms was 14.63 ± 0.30 days and 16.38 ± 0.56 days, respectively (Fig. 3B). The adult lifespan increased approximately 12% compared with that of worms cultured in NGM medium without KPG-7. Gompertz mortality curves were created from the data in Figs. 3A and B. The logarithm of age-specific mortality rates was plotted against age (Fig. 3C).[42] The slope for control and KPG-7-treated worms was calculated as 0.16 and 0.07, respectively. The slope was significantly reduced in the worms treated with KPG-7, indicating that KPG-7 delayed the aging of *C. elegans*. Furthermore, the lifespan of *fem-3* (*q20*) mutants at 25°C was measured to

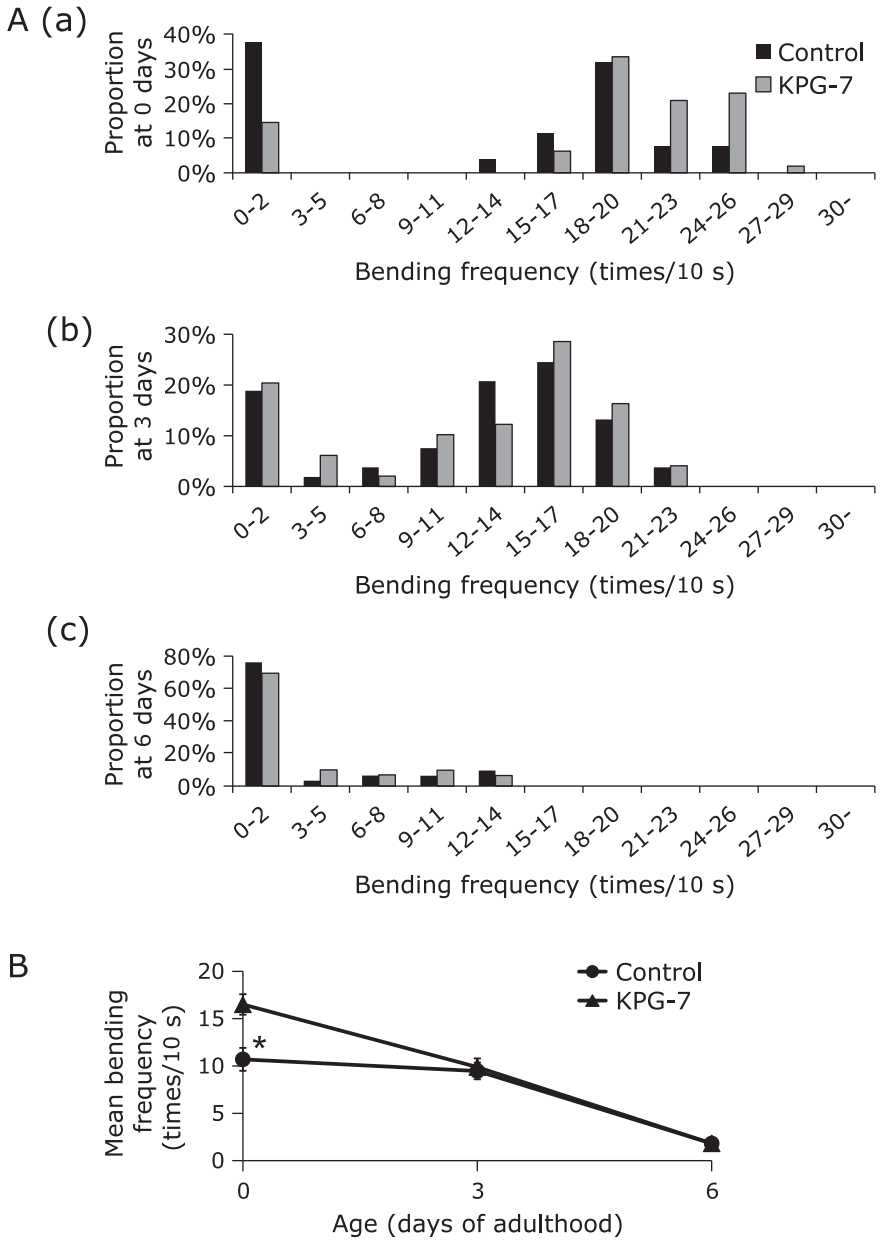

Fig. 5. Influence of KPG-7 on locomotion of *C. elegans*. L1 larvae of *C. elegans* JK816 (*fem-3* mutant) strain were cultured at 25°C on NGM plates containing 10% KPG-7. The worms were collected and used to determine bending frequency in 10 s in M9 buffer. Measurements were carried out three times independently. (A) Histograms of bending frequency. The proportion of worms was plotted against locomotive frequency. (a) 3-days-old worms, (b) 6-days-old worms, (c) 9-days-old worms. (B) Mean bending frequency at each time point. Error bars represent SD. The mark * means statistically different ($p<0.05$) by the Student's *t* test.

exclude the possibility that it was increased by 5-fluoro-2'-deoxyuridine.[36] The *fem-3* mutant becomes sterile at 25°C.[39] The mean adult lifespan of untreated control worms was 7.38 ± 0.09 days and that of KPG-7-treated worms was 9.03 ± 0.19 days (Figs. 4A and B). The lifespan of the *fem-3* mutant at 25°C increased about 22% in the worms treated with KPG-7 compared with that in untreated worms. The slope of Gompertz mortality curves was 0.19 without KPG-7 and 0.12 with 10% KPG-7 (Fig. 4C). Thus, using the *fem-3* mutant, it became evident that the treatment with KPG-7 extended the lifespan of *C. elegans*.

Bending frequency and ATP levels in KPG-7-treated worms. Locomotion, an essential feature of animals, slows with aging.[43] So, we examined whether KPG-7 influences locomotive capacity in *C. elegans*. The frequency of curvature movements was measured for 300 frames (10 s) in M9 buffer. The results are shown in Fig. 5. In all cases, the motion was highest immediately after the worms became adults (3 days of age), and then gradually decreased with age. KPG-7 markedly enhanced the motion of 3-days-old worms (Fig. 5A). Locomotive capacity was at the same level as in the untreated control in 6- and 9-days-old worms (Fig. 5A and B). To know the relation between the capacity for motion and ATP level, the amount of cellular ATP was determined as the intensity of ATP fluorescence per mg protein. The total amount of ATP was highest immediately after the worms became adults (3 days of age) and decreased about 20% in 6- and 9-days-old worms. On the other hand, when the worms were

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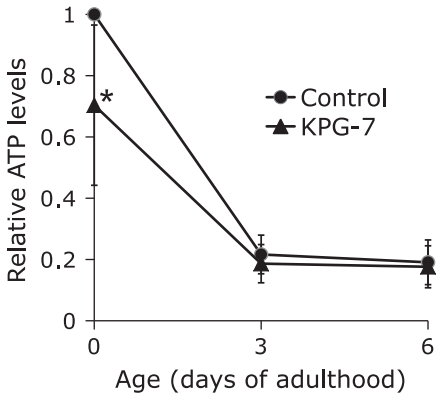

Fig. 6. The amount of cellular ATP in *C. elegans* cultured with KPG-7. L1 larvae of *C. elegans* JK816 (*fem-3* mutant) strain were transferred to NGM plates containing 10% KPG-7 and cultured at 25°C. The worms were collected and an extract was prepared. The amount of ATP was determined based on luciferase luminescence. Measurements were made three times independently. Error bars represent SD. The mark * means statistically different ($p<0.05$) by the Student's *t* test.

treated with KPG-7, the ATP content in 3-days-old worms was significantly lower than that in the control worms without KPG-7, which was at the same level as in the control 6- and 9-days-old worms (Fig. 6).

Suppression of juglone-induced expression of the *oxr1* gene in *C. elegans* by KPG-7 treatment. OXR1 is an oxidation resistant protein. Yeast *oxr1* mutant is sensitive to H_2O_2.[44] Expression of human OXR1 complements the frequency of spontaneous mutations in base excision repair-deficient *E. coli*.[44] The protein is widely conserved in eukaryotes.[44,45] We recently identified a homolog of human OXR1 in *C. elegans* (F52.E1.13, *oxr1*). The yeast and human OXR1 proteins are induced to express by H_2O_2 and heat stress.[44–46] In this study, we examined whether the expression of the *oxr1* gene in transgenic *C. elegans* is induced by oxidative stress and whether the induction is suppressed by KPG-7. Under normal conditions without juglone, a superoxide-generating agent,[47] the *oxr1*::GFP gene was markedly expressed in the head and intestine (Fig. 7A). On the other hand, the expression of *oxr1*::GFP was significantly suppressed in KPG-7-treated worms (Fig. 7B). Fig. 7C shows that juglone enhanced the expression of the *oxr1* gene, especially in the tail, in untreated control worms. It was also evident that KPG-7 efficiently suppressed the expression of the *oxr1* gene in juglone-treated *C. elegans* (Fig. 7D). These results indicate that KPG-7 protects against oxidative stress in *C. elegans*.

Discussion

Many health supplements are believed to have anti-aging effects. However, the biochemical mechanisms involved are still poorly understood. Several herbs, a popular supplement, have been shown to have antioxidant activity.[48–55] Despite the widespread use of herbal medicines to treat diseases and control aging, few studies have focused on the biological activity of mixtures of herbal medicines because of the complexity involved. It is possible that overtake single antioxidants might change to pro-oxidant by cause the production of ROS.[56] As well-known, ascorbate (vitamin C) in animals act as a cofactor for several enzymes.[57] On the other hand, ascorbate could reduce the iron Fe (III)-EDTA to Fe^{2+} and then through the Fenton reaction finally generated $^{\bullet}OH$ in cells.[58,59] Also ascorbate/Cu^{2+} could inactivate many enzymes.[57] There are several researches have reported that green tea is another potential antioxidant and pro-oxidant material.[57,60] Polyphenols rapidly oxidize in commonly-used cell culture media, generating H_2O_2 and quinine that can injure cells.[61] So, pro-oxidant effects of supplements are a problem in the food industry.[62] The possible advantages of taking a mixture of herbal medicines are to reduce the dosage of each medicine and to achieve a greater (synergistic) effect.[63] For example, single use β-carotene, α-tocopherol (vitamin E) or ascorbic acid could act as pro-oxidant but mixture of the three compounds exhibited greater protective effects than any individual compound.[64] Antioxidant mixture containing vitamin C, ferulic acid, and phloretin could against ultraviolet-induced photodamage in human skin.[65] AOB is a grain food mixture that showed grateful effects in antioxidants.[51] Kampo medicines are traditional in Japan, they are

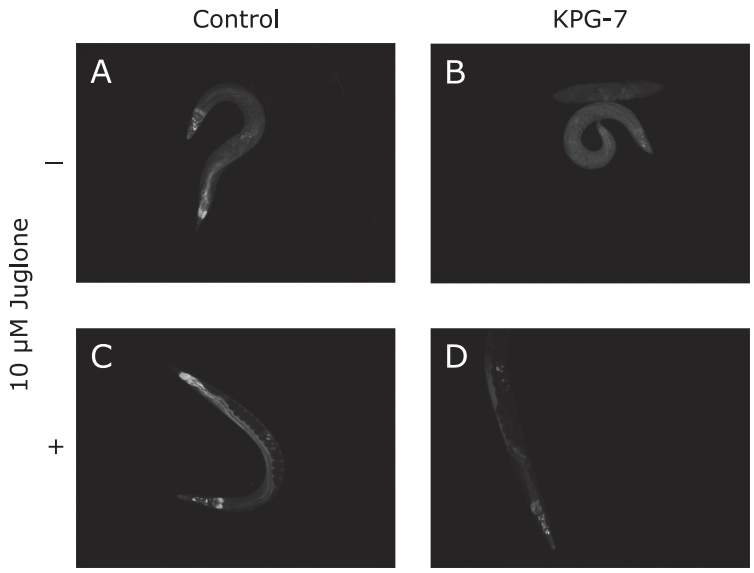

Fig. 7. The expression of the *oxr1* gene in *C. elegans* cultured with KPG-7 determined by a GFP reporter strain. L1 larvae of BC10281 strain were transferred and cultured at 20°C for 3 days on NGM plates containing 0% (A and C) or 10% (B and D) of KPG-7. These worms were collected and incubated in M9 buffer containing 0 μM (A and B) or 10 μM (C and D) of juglone for 6 h at 20°C. The expression of *oxr1* was assayed by determining the intensity of GFP fluorescence.

extracts of multiple herbs and other compounds showed powerful effects on many dysgenesis treatment. KPG-7 is a mixture of several herbs, some of which are known to have antioxidant effects.[66]A supercritical fluid from rosemary extract has been shown to enhance antioxidant activity in aged rats.[31] Curcumin, a yellow coloring agent present in the spice turmeric which belongs to the ginger (*Zingiberaceae*) family, exhibits anti-inflammatory, anti-carcinogenic and antioxidant activities.[32] Recently, Liao *et al.*[32] also reported that curcumin extends lifespan in *C. elegans*. Dietary supplementation with thyme (*Thymus vulgaris*) essential oil throughout the lifetime of rats has effects on antioxidant status in liver, kidney and heart tissues.[33] In this study, we showed that KPG-7 has an effective antioxidant effects.

C. elegans is a good animal efficient model for studying the mechanisms of aging and lifespan.[34] In this study, we established a system to evaluate the activities of mixtures of herbal medicines using *C. elegans*. We treated *C. elegans* with KPG-7 by culture plates. We found that KPG-7 extended the lifespan of the worms and delayed aging (Figs. 3 and 4).

A relation between the increased longevity and the antioxidant activity of KPG-7 was obvious from the following. Proteins are often modified by a large number of reactions involving ROS.[67] Oxidatively damaged proteins accumulate with age in many species.[68] Levels of carboxylated proteins increase with age in many species including *C. elegans*.[69] In this study, we also observed that the amount of carbonylated proteins was suppressed in the worms cultured in the presence of KPG-7 (Fig. 2).

Previous studies have suggested that antioxidants have anti-aging effects.[70] For example, superoxide-scavenging activities are important for survival, because the deletion of either cytoplasmic or mitochondrial superoxide dismutase (*sod*) genes in yeast, flies and mice results in a decreased lifespan.[71–74] Furthermore, overexpression of the SOD1 protein increases longevity in the fruit fly *Drosophila melanogaster*.[75] It is possible that decreasing cellular ROS levels through antioxidant defenses results in anti-aging effects. Therefore, in this study, we determined the influence of KPG-7 on aging in *C. elegans*. As shown in Fig. 3 and 4, KPG-7 extended the lifespan of *C. elegans*. Moreover, by analyzing Gompertz curves, it was found that the increase in lifespan well correlated to the slowing of aging.

Eleutherococcus senticosus has the capacity to increase cellular ATP levels.[76] So, we hypothesized that the increase in locomotive activity caused by KPG-7 is due to higher ATP levels. However, the worms treated with KPG-7 showed low levels of cellular ATP at 3 days of age (Fig. 6). The results suggest that the increased locomotion by KPG-7-treated worms is due to the more efficient utilization of ATP. It is worth noting that ATP levels are very important to the activity of organisms . The present results indicate that worms become more active without an increase in levels of ATP and harmful byproducts, ROS. Previous studies concerning anti-aging factors have focused on protecting cells from ROS by suppressing ATP consumption.[77] However, it may be important to utilize ATP more efficiently. Previous studies showed that dietary restriction reduces ATP levels, but increases locomotion, depending on AMP-activated protein kinase.[78] To analyze the precise mechanisms responsible for the slowing of aging by KPG-7, changes in the turnover of ATP are under investigation.

The oxidation resistance 1 gene (OXR1) is a member of a conserved family of genes found in eukaryotes but not in prokaryotes.[44,45] The human and yeast genes are induced by oxidative stress such as H_2O_2 and heat.[45,46] Yeast *oxr1* mutant is sensitive to H_2O_2.[44] *Oxr1* deletion mutant mice showed cerebellar neurodegeneration.[79] Loss of *oxr1* in mouse cerebellum significantly increased the amount of 8-oxoG in DNA. *oxr1* is therefore an important factor to protect cells from oxidative stress. In *C. elegans*, as in yeast and human cells, the expression of *oxr1* was induced under oxidative stress caused by juglone (Fig. 7). Juglone enhances cellular oxidative stress by generating $O_2^{\bullet-}$ in cells.[47] In this study, the expression of the *oxr1* gene was induced by jugulone treatment in *C. elegans*. The *oxr1* gene was expressed at lower levels when the worms were treated with KPG-7 (Fig. 7). These results also indicate that KPG-7 has antioxidant properties *in vivo*. We are currently investigating the structure and biological functions of *C. elegans* OXR1 protein.

Acknowledgments

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Conflict of Interest

No potential conflicts of interest were disclosed.

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