**Ginkgo biloba** Extract EGb 761 and Wisconsin Ginseng Delay Sarcopenia in *Caenorhabditis elegans*

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Sarcopenia refers to the progressive loss of skeletal muscle mass and strength in senescence (1), a condition that accompanies the natural aging process. In humans, skeletal muscles undergo a gradual 3%–8% decline in muscle mass per decade after the age of 30. The pace and extent of sarcopenia proceeds faster in populations older than 60 years (6%–15%), resulting in a loss of approximately 40% of skeletal muscle fibers in persons older than 80 years (2–4). Despite the well-accepted fact that muscle fiber atrophy and muscle fiber loss, as well as a decrease in the cross-sectional area of the remaining muscle fibers, are strongly correlated with sarcopenia (2,5–7), the underlying mechanisms involved in its progression still remain unresolved.

A significant reduction in muscle function parallels the muscle mass decline involved in sarcopenia, leading to impaired physiological function and physical performance in older adults. Although sarcopenia is not directly lethal to affected individuals, it can lead to the development of a number of clinical consequences, which include decreased mobility, increased risks of falling and fractures, reduced energy intake, and impaired thermoregulatory and respiratory function and insulin resistance (8–12). The severity of sarcopenia could prevent older individuals from living independently and have an adverse impact on the quality of life of the geriatric population. Due to the increasing elderly population, sarcopenia has been recognized as one of the marked problems in the developed world because of its health care consequences and its socioeconomic implications (13,14). Age-related muscle deterioration not only occurs in the skeletal muscle of human and other mammalian species, but also in invertebrates. It has been demonstrated that sarcopenia accompanies the age-dependent functional decline in the nematode *Caenorhabditis elegans*, similar to those longer-lived vertebrates (15,16).

*Caenorhabditis elegans* is a well-studied and widely used model organism for many genetic and biomedical investigations. In the past decade, this species has become a valuable genetic system for the study of aging (17) and for identifying targets for possible pharmacological modulation of life spans (18). Herndon and colleagues (15) explored the major cellular changes of a number of important cell types in aging worms. They revealed that the nervous system in senescent nematodes stays largely intact, although the muscles experience a gradual and progressive deterioration, i.e., sarcopenia. As in humans, sarcopenia compromises mobility in the worms and serves as a marker for increased mortality (15). Consistent with this view, dissociated muscle integrity was shown to correlate with age-related functional deficits (19) and could be reduced by treatment of the worms with muscarinic agonist (20) or natural antioxidants (21).

Taken together, findings obtained from human and worm studies strongly suggest that sarcopenia represents an important biomarker of aging and can serve as an independent predictor for morbidity and mortality in population-based studies (22). Sarcopenia is a complex process involving many molecular, cellular, and functional alterations. However, the underlying mechanisms responsible for these deleterious changes remain unclear. The similarity of worm muscle degeneration to sarcopenia in humans raises the possibility of using *C. elegans* to explore candidate factors contributing to muscle aging and could therefore facilitate...
the elucidation of sarcopenia mechanisms in humans. Based on the intimate link between aging and sarcopenia, it is hypothesized that drug interventions aimed at slowing senescence and extending life expectancy might render some protection against age-dependent muscle deterioration.

Increasing experimental evidence supports the life-span extension and delaying of aging by pharmacological agents in C. elegans (23–26), including EGb 761 reported from our laboratory (27). EGb 761 is a standardized extract from the leaves of Ginkgo biloba. In addition to its many clinical uses for dementia (28), EGb 761 has been shown to enhance cognition, stress resistance, and longevity in mammals (29–33). American ginseng is another commonly used herbal medicine in the United States. It is derived from the root of the ginseng plant and is known to improve physical performance and health maintenance and to have immunostimulating, anticancer, and neuroprotective activities (34,35). American ginseng cultivated in Wisconsin (WG) and Illinois exhibits a different ginsenoside profile (36,37).

The original goal of this study was to examine the potential of EGb 761 and WG for delaying age-associated tissue degeneration such as sarcopenia in the C. elegans model and to test the hypothesis that age-associated sarcopenia can be modulated by certain life-span-extending drugs. Using a combination of fluorescent and transmission electron microscope (TEM) techniques as well as behavioral assays, we demonstrate that both EGb 761 and WG delay age-dependent muscle cell degeneration. The onset of their effects on age-related decline of muscle deterioration and locomotory behaviors is different in aging worms. The insights from using EGb761 and WG extract on muscle deterioration in the nematodes may provide a basis for developing pharmacological interventions for human sarcopenia.

METHODS

Herbal Extract and Reagents

Schwabe Pharmaceuticals (Karlsruhe, Germany) provided the standardized Ginkgo biloba leaf extract EGb 761. WG extract from Panax quinquefolius was a gift from Dr. I. Khan at the University of Mississippi, National Center for Natural Product Research. The stock solutions of both herbal extracts were dissolved in 100% ethanol (EtOH) and further diluted into the worm food to get final concentrations of (100 μg/mL). The final concentration of EtOH did not exceed 0.01% in the food, which is used as a vehicle control.

Strains of C. elegans

Wild type C. elegans (N2, Bristol) was obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis). Transgenic mutant strain PD4251, which carries an integrated myo-3::gfp transgene in body wall muscle cells, was a gift from Dr. Monica Driscoll at the State University of New Jersey. Assessments of life span, motility, and pharyngeal pumping were performed on solid nematode growth medium (NGM), with a spot of 100 μL of Escherichia coli OP50 for food (38). Reproductive adults were transferred to fresh NGM plates and allowed to lay eggs for 2–4 hours, producing age-synchronized groups. All worms were cultivated at 20°C in a temperature-controlled incubator. EGb 761 or WG was added directly to the OP50 food source to feed the worms.

Fluorescent Microscopy and Quantitation of GFP Reporter Gene Expression

Age-synchronized PD4251 transgenic nematodes (about 24 in each treatment group) were transferred to a new plate every other day. At different ages, the worms were picked and placed on a glass slide. Live worms were embedded within 2% agarose pads, and a GFP reporter gene expression was examined directly under a fluorescent microscope. Epifluorescent images were acquired at the same exposure parameter using the 40X objective of a microscope (BX 60; Olympus, Tokyo, Japan) equipped with a digital camera (Micropublisher 5.0; QIMAGING, Burnaby BC, Canada). For quantifying a population of GFP reporter animals, each 40X image was analyzed using Image-ProPlus 4.5.1 software (Media Cybernetics, Silver Spring, MD).

Electron Microscopy of Muscle Ultrastructure of the Worms

Electromicroscopy (EM) was used to examine age-related muscle cell degeneration. Age-synchronized worms fed with different agents were maintained at 20°C. The day of synchronized egg hatching was defined as day 1. The synchronized worms were transferred to fresh plates (30 nematodes per plate) at day 3 after hatching. When the worms started laying eggs, they were transferred daily for 4–5 consecutive days until the cessation of egg laying to avoid overlapping generations. After that, the worms were transferred every other day. Worms were scored as dead if they did not display pharyngeal pumping and respond to touch stimulus.

A, B, C Motion Scoring

Locomotive behaviors were scored in three categories as described by Herndon (15). “A” motion is defined as spontaneous movement and significant coordinated sinuoidal locomotion in response to prodding. “B” motion is
defined as little spontaneous locomotion and discontinuous or sluggish (non sinusoidal) movement in response to prodding, and “C” motion is defined as immobility with only head and/or tail movement or twitch in response to touch. The adult synchronized worms were scored every other day for classes A, B, and C as described above until all animals had died.

Body Bend and Pharyngeal Pumping Assay
The worms at days 5, 8, 11, and 14 were selected for measurement of the locomotory rate by scoring the number of body bends in 20 seconds and pharyngeal pumping within 1 minute. Each 25 control or treated adult (3 day old) worms were transferred to fresh plates and examined at day 5, day 8, day 11, and day 14. The animals were maintained by transferring every day (during reproductive period) or every other day until subjected to the experiment. On the experiment day, 10 selected, age-matched animals were examined for pharyngeal pumping. The same worms were then picked and washed twice with S basal buffer and transferred to 10 cm Petri plates seeded with bacteria lawns. The number of body bends was recorded in 20-second intervals after the animals had been on the plate for 10 minutes. To keep consistency, each animal was gently tapped at the back with a platinum loop. The stimulus was sufficient to induce their response but did not cause any damage to the tapped region. In all cases, plates were coded so that the experimenter was blind to control or treated animals.

Statistical Analysis
Statistical comparison between treatments was done with an unpaired Student t test using Origin 6.0 software (MicroCal Software, Inc., Northampton, MA). Standard errors of the mean were used in the figures. Differences with *p < .05 were defined as statistically significant.

RESULTS

Age-Associated Deterioration of Body Wall Muscle Cells Is Alleviated by EGb 761 and Ginseng Treatment
One recently developed noninvasive method for assessing muscle structure in living C. elegans is an aging-related change in the nuclear distribution of a muscle-specific GFP (15). To determine if life-span-extending chemicals such as EGb 761 would improve age-associated tissue deterioration, we first used GFP-tagged myo-3 gene transgenic worms to examine the muscle degeneration in living animals by using a fluorescent microscope. The transgenic GFP reporter worm PD4251 expresses a GFP-tagged MYO-3 protein that is localized in body wall muscle nuclei. The age-associated muscle cellular deterioration can be reflected by a progressive decline in the number of GFP-labeled nuclei, which can be detected and counted under the fluorescent microscope (15). As represented in Figure 1A and B, there is a linear decline of the fluorescence signal as the worms age (day 4, 7, 10, 15; Figure A left panels Ctrl, and Figure B open bars). In the worms fed with EGb 761, the rate of decrease of the number of fluorescent nuclei was slower to some extent compared with untreated ones (Figure 1A). Quantitative analysis of GFP-labeled nuclei in PD4251 worms revealed (Figure 1B) that both EGb 761 and WG extracts were able to delay the progression of muscle degeneration in aged worms. Apparently, the effect of EGb 761 on the worm muscle cells started in midlife and exerted a significant effect on sarcopenia at day 10, compared with age-matched control worms (Ctrl 51.4 ± 0.8, vs EGb 55.7 ± 1.9; total worms 24, p = .004) or worms fed with ginseng (Ctrl 51.4 ± 0.8 vs WG 52.0 ± 2.0 [p = .22]).

Figure 1. Age-associated deterioration of Caenorhabditis elegans body wall muscle shown as green fluorescent protein (GFP) fluorescence declines. A, Representative images of the anterior body of transgenic C. elegans (PD4251) expressing GFP in the nuclei of body wall muscles at ages indicated. Age-synchronized worms were treated with vehicles (Ctrl), with EGb 761 (EGb; 100 μg/mL), or with Wisconsin Ginseng (WG; 100 μg/mL) for 4 days starting at day 1 of age. Fluorescent images were captured in live worms under a 4× microscope objective. B, Quantification of the number of GFP-labeled nuclei in the transgenic worms treated with vehicle (white bars) or EGb 761 (black bars), or WG (hatched bars). Results were obtained from two independent experimental trials with 12 worms in each condition. The decline of GFP signals in control worms over time was faster than that of drug-treated animals. Error bars indicate the standard errors. EGb-treated day 10 animals retain significantly more GFP-labeled nuclei of muscle cells than do other animals (*p < .05). The delay of the muscle cell deterioration in worms fed WG is significantly different than that of the control animals at day 15 (*p < .05).
However, the effect of WG on muscle cell deterioration appeared relatively late, and the significant effect was obvious at day 15 (Ctrl 37.6 ± 2.3 vs WG 46.0 ± 1.9 [p = .001] vs EGb 39.3 ± 1.5 [p = .4]).

Aside from the fluorescence assay, we used TEM to analyze ultrastructural changes of body wall muscle cells under different treatments, as a visual support of the data presented in Figure 1. Age-related decline in sarcomere integrity was demonstrated dramatically as viewed by TEM (15). The muscle sarcomere integrity of a young worm is shown in Figure 2A. White arrows indicate sarcomeres bordered with dense bodies (black arrows). Compared with the young worm, a representative TEM section of a 11-day-old worm exhibits disintegrating sarcomeres (white arrow), revealed as disorganized and with reduced dense bodies (Figure 2B). In age-matched worms fed with EGb 761, the muscle integrity viewed by TEM did not exhibit remarkable differences in comparison with the vehicle-fed, 11-day-old controls (Figure 2C). However, the worms (11 day) fed with WG (Figure 2D) displayed an ultrastructural change similar to the young, 3-day-old animals (Figure 2A). Results were obtained from representative sections (at least 8) taken from a total of 3 worms in each treatment.

**Age-Associated Declines of Locomotive Behavior Are Reduced by EGb 761 and WG Extracts**

The body movement of young adult worms exhibits a spontaneous, well-coordinated, sinusoidal pattern. The motor activity is reduced with increasing age; the aging animals move slowly and ultimately stop moving. Herndon and colleagues (15) established a behavioral phenotype analysis to characterize locomotion in aging worms. In brief, they sorted worms into three classes. The “A” locomotory class stands for highly mobile worms, the “B” locomotory class represents worms with significantly reduced mobility, and members of the “C” class are immobile individuals. It was found that all animals begin adulthood in the A class, then proceed into the B and finally the C class prior to death (15). The A, B, C locomotion scoring allowed us to look closely at effects of drugs on the development of sarcopenia at the behavioral level. Because the development of locomotory defects in aging worms always follows the order A→B→C, we categorized worms via counting the number of worms falling into corresponding classes and reclassified them every other day until all animals had died.

Both EGb 761 and WG extracts (100 μg/mL each, fed from day 1 of age until the worms were collected) altered...
the ratio of the number of worms in a given category (A, B, or C) to the total number of worms present at the start of the experiment from midlife to the late stage of life (Figure 3A). Among those behavioral phenotypes, the behavior in A motion is particularly interesting, for it represents highly mobile worms that still retain good muscle function or only suffer mild locomotory deficits without evidence of significant muscle deterioration. We reasoned that if the treatment with EGb 761 could attenuate sarcopenia resulting in delaying the onset of age-related muscle deterioration, the fraction of A class animals would remain higher in drug-treated nematodes in than in untreated animals. As expected, the statistical analysis showed that there was a significant increase in the fraction of A class animals treated with EGb 761 or WG on the given days (Figure 3B). Interestingly, EGb 761 significantly delayed the onset of locomotory impairment and retained more A motion animals in midlife (day 10, p = .016; day 12, p = .007; day 14, p = .003, total control worms: n = 118, total worms fed with EGb 761: n = 114). The significant effect of WG on the development of sarcopenia is at days 12, 14, and 16 (day 12, p = .006; day 14, p = .004; day 16, p = .047; total control worms: n = 118; total worms fed with WG: n = 118). The pattern of EGb 761 and WG affecting sarcopenia in the worms over age...
correlates with that observed in the structural assays by quantitative GFP fluorescence (Figure 1B). Furthermore, no dose response of EGb 761 and WG in A motion was observed among concentrations tested (10–100 µg/mL) suggesting that concentrations < 1 µg/mL might have been effective (Figure 3, C and D).

Declines of Locomotory Rate and Pharyngeal Pumping in Aged Animals Are Reduced by Treatment of EGb 761 or WG

The age-associated declines in locomotory behavior and pharyngeal pumping are positively correlated with each other and with aging (39). We note that the onset of sarcopenia appears at different times among individual animals and that the progressive decline of muscle function proceeds at variable rates in aging nematodes. For this reason, a defined category of animals (A motion) was chosen to assess the locomotory rate and pharyngeal pumping (10 worms were selected of 25 animals, if death occurred from the corresponding plate) on the given day. The locomotory rate was measured by examining the number of body bends in 20 seconds at different time points. Figure 4A shows that only ginseng significantly reduced the age-related decline of the number of body bends at day 14 (n = 50, p = .007). Surprisingly, there is no significant improvement of locomotory rate by EGb 761 treatment on any given day (n = 50, p = .175).

Progressive muscle deterioration not only occurs in body wall muscles, but also in pharyngeal muscles in C. elegans (15,19). The pharynx is a neuromuscular organ that displays rhythmic contractions. The pumping rate was measured by counting the number of contractions in 20 seconds. Figure 4B shows that both EGb and WG could increase the pharyngeal pumping in the midlife of worms. The significant increase of pumping by EGb 761 treatment was observed at day 11 (n = 40, p = .028), but WG had a significant effect on pumping on a later day (day 14, n = 40, p = .028).

The survival assay, or life-span assay, was conducted next in the same population of worms under defined drug treatments (Figure 4C). Consistent with our previous report, EGb 761 significantly extended the mean life span in the nematode. Treatment of wild-type worms (N2) with EGb 761 extended their mean life span by 9% (p = .018). However, we failed to observe a significant increase of the worm’s mean life span by WG treatment (p = .086).
life span by WG ($p = .086$). At the point of 50% survival rate, both EGb 761 and WG show similarity in extending the life span from 15 days to 17 days (Figure 4C).

**DISCUSSION**

The present study describes the efficacy of natural extract EGb 761 and WG to modulate the process of muscle aging, or sarcopenia, and the associated decline in locomotive behaviors. Interestingly, the reduced decline of muscle cell integrity (Figures 1 and 2) by the two extracts displayed onsets at different ages, which seems to correlate with their effects on age-dependent decline in motor behavior (Figures 3 and 4). These data support our hypothesis that age-associated sarcopenia can be modulated by certain life-span-extending drugs.

Both EGb 761 and WG have long been known to have anti-aging properties (40). Despite their different chemical components and applications, they share some common features including anti-oxidative, anti-inflammatory, and anti-apoptotic properties (41,42). Their common features may explain a general anti-aging mechanism. It is well known that oxidative stress plays an important role in aging (43). Studies have suggested that oxidatively damaged macromolecules are increased overall in skeletal muscle tissue during ageing (44), which has been correlated with a reduction in anti-oxidative capacity (45). Skeletal muscle is a postmitotic tissue that lacks high repair capacity. Lower levels of anti-oxidative defense in aging satellite cells, which are usually recruited to replace damaged fibers, may negatively affect the potential regeneration of muscle fibers (46,47). Aiken and colleagues (48) reported that mitochondrial DNA deletions subsequent to oxidative damage result in dysfunction of the electron transport system and an abnormal energy supply in the affected segment of skeletal muscle, which will cause muscle fiber breakage and loss as observed with aging. Taken together, the oxidative insult accumulated in the skeletal muscle with the aging process may be an important factor in sarcopenia. A positive correlation of anti-oxidative stress and longevity in *C. elegans* has been well established (49). The interventions aimed at preventing oxidative damage and environmental stresses has been shown to extend life span in worms (24,25,50–52). Nevertheless, chemicals other than anti-oxidants also extend life span in *C. elegans* (23,53), suggesting that multiple mechanisms exist.

Although EGb 761, but not WG, extends mean life span by the survival assay, the actual survival curves overlap at the mean life span (Figure 4). Thus both EGb 761 and WG modestly increased mean life span. It should be noted that EGb 761 has an effect at midlife onward, and WG later in life (Figure 1), and their life-span curves fit this idea. What might differ between WG and EGb 761 that accounts for the different onset of their effects? Ginsenoside has been known to be a typical “adaptive” chemical, i.e., it is able to work bidirectionally, depending on the immediate environment. For example, in patients with high blood pressure, it reduces it, whereas the same chemical increases it in patients with low blood pressure. This property of ginsenosides may explain why WG had a late onset in its effect on aging and sarcopenia.

The specific protective mechanisms of EGb 761 and WG against age-related cell deterioration are unknown, partially due to the complexity of their makeup. However, studies of active constituents of the extracts implicate some molecular mechanisms of action. Among active ingredients of Panax ginseng, ginsenosides Rg3 and Rh2 are major inhibitors of Ca$^{2+}$ channels and show some Ca$^{2+}$ channel selectivity (54). Rg3 is also a novel Na$^+$ channel inhibitor capable of acting on the resting and open states of Na$^+$ channels via interactions with the S4 voltage-sensor segment of domain II (55). Furthermore, some ginsenosides as well as ginsenoside metabolites can influence 5-HT3A receptor channel activity in *Xenopus* oocytes (56) and modulate excitatory (N-methyl-D-aspartic acid [NMDA]) and inhibitory (gamma-aminobutyric acid [GABA]) neurotransmitter receptors (57). In contrast, the protective mechanism of EGb 761 can be attributed to a range of biochemical and pharmacological effects (28), including ginkgolide B as an antagonist of platelet-activating factor (PAF) receptor (33), enhancement of neuronal plasticity (58), anti-inflammatory effects (59), and anti-apoptotic effects in neuronal cells (60,61). Accumulating evidence suggests that many of the actions of EGb 761 are so-called “polyvalent” actions, that is, the therapeutic activity of EGb 761 is the net effect of interactions among various biological activities of the individual substances in the extract. Presumably, this is one of the advantages of using chemicals obtained from natural products for the prevention and treatment of infirmity, as well as the maintenance of health (62). Several lines of experimental evidence indicate that the development of sarcopenia is not attributed to one or two contributors in its pathogenesis, rather, it is most likely the result of interplaying actions of multiple intrinsic and extrinsic factors (63,64). It has been proposed that a number of factors might play important roles in the onset and progression of sarcopenia with advancing age. These factors include deficient satellite cell recruitment (65,66), a variety of hormonal changes (67,68), oxidative stress, mitochondrial DNA (mtDNA) damage (69–71), apoptosis (69,72), inadequate nutrition (10,73), and reduced physical activity (74,75). Nevertheless, oxidative stress is one of the most important factors. The evidence is that the stressed *C. elegans* models also exhibit accelerated sarcopenia. Experimental evidence indicates that age-related behavior declines were delayed in *C. elegans* mutants in the daf-2/insulin-like pathway, which governs longevity and has reduced oxidative stress (19). Similar results were observed in other stress models such as in *Drosophila*, mammals, and humans (76–78).

The relationship between specific tissue maintenance and life span has a significant impact on the treatment on life span versus muscle integrity over time. Because sarcopenia could predict life span, that is, a positive correlation between sarcopenia and life span, pharmacology of life span would have to start early enough to overcome age-associated muscle integrity decline. However, it is not realistic to use drugs beginning at an early age for the long term to enhance life span. Lifestyle change is certainly a better approach, except for genetic disorders of aging, or premature aging, for which long-term use of pharmacological therapy would be necessary.
ACKNOWLEDGMENTS
This study was supported by IPSEN (Paris, France) and partially by National Institutes of Health (NIH) grant R01AT001928-04 (NIH National Center for Complimentary and Alternative Medicine; YL).

We thank Dr. Catherine Wolkow from the National Institute on Aging for insightful discussion and Marishka Brown for reading and commenting on the manuscript.

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Received January 31, 2007
Accepted August 16, 2007
Decision Editor: Huber R. Warner, PhD