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# Long-Term Dietary Supplementation with a Yang-Invigorating Chinese Herbal Formula Increases Lifespan and Mitigates Age-Associated Declines in Mitochondrial Antioxidant Status and Functional Ability of Various Tissues in Male and Female C57BL/6J Mice

Kam Ming Ko, Po Yee Chiu, Hoi Yan Leung, Ada Hoi Ling Siu, Na Chen, Eriol Pou Kwan Leong, and Michel K.T. Poon

## Abstract

To investigate whether Vigconic 28 (VI-28), a Yang-invigorating Chinese herbal formula, could affect survival of aging animals, male and female C57BL/6J mice were given a VI-28–supplemented diet (0.05 and 0.5%, wt/wt) starting at 36 weeks of age, until death. VI-28 dietary supplementation at 0.05% significantly increased median lifespans of both male and female mice as compared to controls. Survival enhancement was associated with protection against age-associated impairments in mitochondrial antioxidant status and functional ability in various tissues. In conclusion, VI-28 could retard the aging process in mice, probably by mitigating age-associated declines in mitochondrial antioxidant status and functional ability in tissues.

## Introduction

Mitochondria are primarily responsible for adenosine triphosphate (ATP) generation in most eukaryotic cells. In addition, these organelles participate in intermediary metabolism, calcium signaling, and apoptosis.[1] Reactive oxygen species (ROS) arising from mitochondrial respiration accelerate accumulation of mitochondrial DNA damage, leading to a progressive decline in respiratory function over time.[2,3] Although any causal relationship between mitochondrial dysfunction and aging remains to be established, it is likely that maintenance of mitochondrial antioxidant status and functional ability can enhance survival of aging individuals. Vigconic 28 (VI-28) is a Yang-invigorating Chinese herbal formula used for the promotion of general wellness in Chinese medicine. An earlier study in our laboratory demonstrated that long-term treatment with VI-28 enhanced both mitochondrial antioxidant status and functional ability in various tissues of male and female rats.[4] A recent study showed that long-term VI-28 treatment affords protection against oxidative injury in various rat tissues.[5] It is unclear, however, whether long-termVI-28 treatment affects survival. In the present study, we examined the effects of VI-28 dietary supplementation on lifespan in male and female C57BL/6J mice. The effects of supplementation on age-associated impairments in mitochondrial antioxidant status and functional abilities of various tissues were also investigated.

## Materials and Methods

### *Chemicals*

Reduced glutathione (GSH), oxidized GSH, GSH reductase, xanthine oxidase, xanthine, cytochrome *c*, α-tocopherol (α-TOC), ATP, and adenosine diphosphate (ADP) were purchased from Sigma Chemical Co. (St. Louis, MO). Luciferase and 2′,7′-dichlorofluorescein diacetate (DCFDA) were obtained from Fluka (Basel, Switzerland) and Perkin Elmer (Boston, MA), respectively. All other chemicals were of analytical grade. Solvents used for high-performance liquid chromatography (HPLC) were of HPLC grade.

### *Animal care and treatment*

Male and female C57BL/6J mice were bred in the Animal Care Facility at the Hong Kong University of Science and Technology (HKUST), Hong Kong. All animals, born within a 2- week interval, were housed in an air-conditioned room with humidity control, under a 12-h dark/light cycle at about 22°C, and allowed food and water *ad libitum*. Experimental

Department of Biochemistry, the Hong Kong University of Science & Technology, Clear Water Bay, Hong Kong SAR, China.

protocols were approved by the Research Practice Committee of HKUST. Animals were randomly divided into longevity and sacrifice groups. In each of the two groups, animals were assigned to control or VI-28–supplemented subgroups. In the supplemented subgroups, starting from 36 weeks of age, animals were fed a diet containing VI-28 at a concentration of 0.05% (D1) or 0.5% (wt/wt) (D2) until death or sacrifice. During the first 9 months of VI-28 supplementation, the average daily intake of 3–5 g of VI-28–supplemented diet (0.05%, wt/wt, VI-28) would yield a daily VI-28 dose of 50–80 mg/kg. Male and female mice in the longevity group ($n = 41$–60 in each subgroup) were monitored separately for survival changes during aging, whereas mice in the sacrifice group ($n = 30$ in each subgroup) were killed at 72, 96, and 120 weeks of age, and tissue mitochondrial parameters were measured.

### *Herbal preparation*

VI-28, a multicomponent herbal formula composed of Radix Ginseng, Cornu Cervi, Cordyceps, Radix Salviae, Semen Allii, Fructus Cnidii, Fructus Evodiae, and Rhizoma Kaempferiae, was prepared as described.[6] A commercial sample was manufactured and supplied by Vigconic (International) Ltd., Hong Kong, with standardized contents of total saponins (1.2%, wt/wt), total flavonoids (0.5%, wt/wt), and total lignans (1.0%, wt/wt).[4]

### *Preparation of mitochondrial fractions*

Tissue homogenates were prepared from various tissues (brain, heart, liver, and kidney) as previously described.[4,5] Mitochondrial pellets were isolated from tissue homogenates by differential centrifugation. Tissue homogenates were centrifuged at $600\times g$ at 4°C for 20 min. The resulting supernatants were then centrifuged at $9200\times g$ at 4°C for 30 min to obtain the mitochondrial pellets. Mitochondrial pellets were resuspended in 1 mL of the homogenizing buffer appropriate for the particular tissue and constituted mitochondrial fractions.

### *Biochemical analyses*

**Measurements of antioxidant levels and enzyme activities.** Aliquots (200 μL) of mitochondrial fractions were used to measure mitochondrial GSH and α-TOC levels by an enzymatic method[7] and HPLC analysis,[8] respectively, using authentic GSH and α-TOC as standards. Mitochondrial manganese superoxide dismutase (Mn-SOD) activity was assessed by monitoring cytochrome *c* oxidation caused by superoxide radicals generated in the xanthine oxidase–xanthine reaction.[9]

**Measurement of mitochondrial ATP generation capacity.** Mitochondrial ATP generation capacity (ATP-GC) was measured using bioluminescence method, as described.[4]

### *Protein level*

The protein concentration of mitochondrial fractions was measured using a Bio-Rad (Hercules, CA) protein assay kit.

### *Statistical analysis*

Data on survival rates were analyzed by the log rank test, and median lifespan values were estimated. Mitochondrial and behavioral parameters were analyzed by one-way analysis of variance (ANOVA). Post-hoc multiple comparisons were performed using the least significant difference (LSD) test. $p$ values $< 0.05$ were regarded as statistically significant.

## Results

### *Survival of control and VI-28-supplemented male and female mice*

VI-28 dietary supplementation (0.05 or 0.5%, both wt/wt, starting at 36 weeks of age) significantly extended the lifespan of male mice, with the median lifespans being 127 or 126 weeks ($p = 0.0356$ and $p = 0.0083$), respectively, compared to the control value (111 weeks) (Fig. 1 A). The largest difference in survival rate decline between control and VI-28–supplemented male mice was observed from 100 to 120 weeks of age. VI-28 dietary supplementation at 0.05% (wt/wt) significantly extended the lifespan of female mice, with the median lifespan being 123 weeks ($p = 0.0035$), compared to the control value (113 weeks) (Fig. 1A). However, female mice fed a diet supplemented with VI-28 at 0.5% (wt/wt) showed only a marginal increase in median lifespan (119 weeks, $p = 0.3161$). The largest difference in survival rate decline between control and VI-28-supplemented female mice was seen from 80 to 100 weeks of age.

### *Age-associated changes in mitochondrial antioxidant status and functional abilities of various tissues in control and VI-28-supplemented mice*

Aging caused progressive decreases in the levels and activities of mitochondrial antioxidant components (GSH, α-TOC, and Mn-SOD) in all tested tissues of male and female mice, up to the age of 120 weeks, with the overall mean extents of depletion (encompassing three antioxidant components in four tissues) being larger in male mice (15–60% versus 11–43%) and the rate of depletion being higher between 72 and 96 weeks or 96 and 120 weeks of age (data not shown). Aging also caused decreases in mitochondrial ATP-GC of all tested tissues in male and female mice, with the mean degrees of impairment (in four tissues) being larger in female than male mice (7–35% versus 7–46%) (data not shown). A higher rate of decrease in mitochondrial ATP-GC was observed between 72 and 96 weeks or 96 and 120 weeks of age.

VI-28 dietary supplementation dose-dependently counteracted the age-associated depletion in mitochondrial antioxidant components of various tissues in male and female mice, with the mean degrees of protection diminishing with increasing age from 72 to120 weeks (male, 39% to 7% and 65% to 10%; female, 34% to 8% and 59% to 18%, in mice fed VI-28 at 0.05% and 0.5% [both wt/wt], respectively) (data not shown). The age-associated decrease in mitochondrial ATP-GC in various tissues of male and female mice was also inhibited by VI-28 supplementation, with protection diminishing with age and the effect being more prominent in male mice (male, 31% to13% and 60% to 22%; female, 22% to 7% and 40% to 13%) (data not shown). Figure 1B shows the changes in mitochondrial antioxidant and functional parameters at 72 weeks of age in control and VI-28–supplemented male and female mice.

## Discussion

VI-28 dietary supplementation, commencing at 36 weeks of age and at a low concentration of 0.05%, significantly

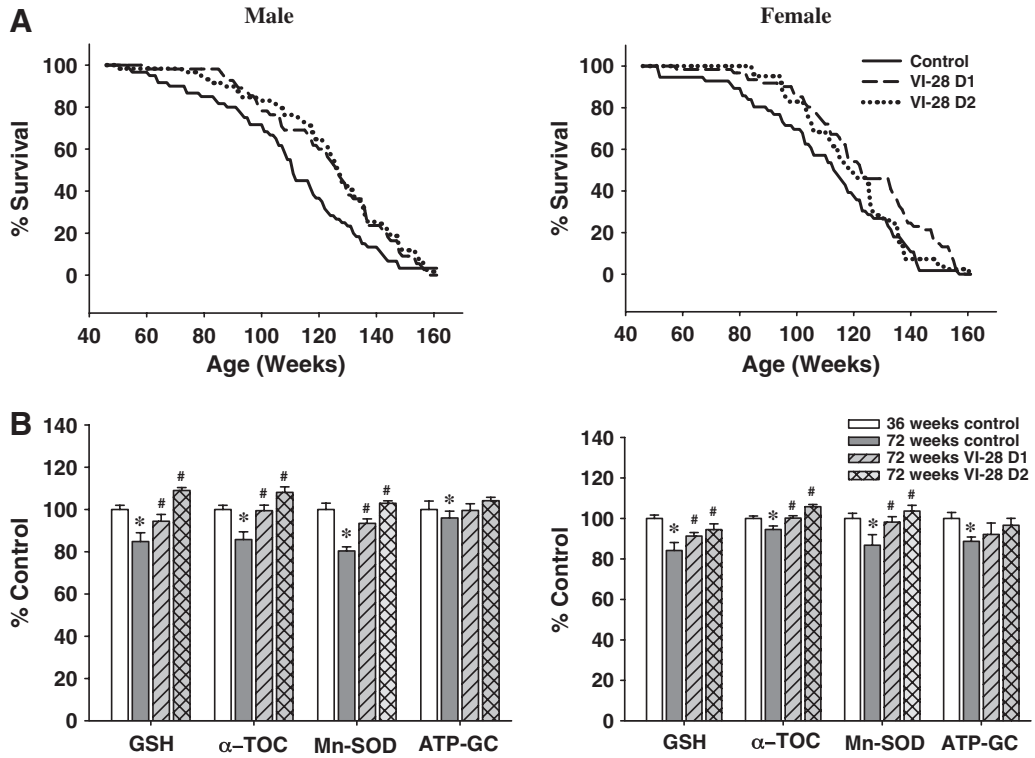


**FIG. 1.** Survival curves (**A**) and changes in mitochondrial antioxidant status and functional ability in brain tissue (**B**) of control and VI-28–supplemented male and female mice. Commencing at 36 weeks of age, male and female mice in the longevity and sacrifice groups were fed a diet supplemented with VI-28 [0.05% (D1) or 0.5% (D2), (both wt/wt)] until death or sacrifice at 72 weeks of age. Control animals were given a normal diet. The number of animals in the longevity groups was: male, control = 54, VI-28 D1 = 61, VI-28 D2 = 50; female, control = 56, VI-28 D1 = 58, VI-28 D2 = 41. Animal mortality was checked on a weekly basis. In the sacrifice groups, animals were sacrificed at 72 weeks of age, and reduced glutathione (GSH) and α-tocopherol (α-TOC) levels, manganese-superoxide dismutase (Mn-SOD) activity, as well as adenosine triphosphate generation capacity (ATP-GC), were measured in brain mitochondria. Data are expressed as a percentage of 36-week control value (mean ± standard error of the mean [SEM], with $n = 5$). (*) Significantly different from the 36 weeks control; (#) significantly different from the 72 weeks control.

increased the median lifespans of both male and female C57BL/6J mice. The mean and maximum survival times of control male or female mice (110.5 and 167 weeks or 110.4 and 157 weeks, respectively) are in the accepted range of well-maintained C57BL/6J mouse colonies, and similar results were seen in careful longevity studies.[10–12] Survival enhancement by VI-28 supplementation was unlikely to be caused by reduction in food (or calorie) intake, as evidenced by moderate changes in body weights of supplemented animals, compared to the controls, throughout the study period (data not shown).

Aging in rodents is associated with progressive impairment in mitochondrial antioxidant status and a decline in mitochondrial respiratory function.[13,14] In the present study, C57BL/6J mice showed age-dependent decreases in mitochondrial GSH and α-TOC levels, and Mn-SOD activity, in various tissues, indicative of impairment in mitochondrial antioxidant capacity. Aging was also accompanied by reduced mitochondrial ATP generation capacity in various tissues of mice. Dietary VI-28 supplementation starting at 36 weeks of age mitigated declines in mitochondrial antioxidant capacity and functional ability in various tissues of aging mice. VI-28 supplementation was effective in reducing the numbers of early deaths occurring from 100 to 120 weeks or 80 to 100 weeks of age in male and female mice, respectively, with the aging rates of control and VI-28–supplemented animals being similar thereafter. Apparently, up-regulation of mitochondrial antioxidant capacity and functional ability earlier in life may be beneficial for later survival enhancement. In this regard, long-term VI-28 treatment has been shown to afford generalized tissue protection against oxidant injury in rats aged 8–10 weeks,[5] which may be related to a decrease in the mortality resulting from age-related pathological changes in various organs of older mice.

In conclusion, long-term VI-28 dietary supplementation mitigated age-dependent declines in mitochondrial antioxidant capacity and functional ability in C57BL/6J mice. Retardation of mitochondrial decay at younger ages was associated with later survival enhancement. Thus, VI-28 may be a valuable hormetic agent in humans, sustaining mitochondrial structural and functional integrity during aging, and offering the prospect of longer survival.

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Address correspondence:
*Kam Ming Ko*
*Department of Biochemistry*
*Hong Kong University of Science & Technology*
*Clear Water Bay*
*Hong Kong SAR*
*China*

*E-mail:* bcrko@ust.hk