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Original Contribution

Significant longevity-extending effects of EGCG on *Caenorhabditis elegans* under stress

Longze Zhang [a,b], Guoliang Jie [c], Junjing Zhang [a], Baolu Zhao [a,b,d,e,f,*]

[a] State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, People's Republic of China
[b] Graduate School of the Chinese Academy of Sciences, Beijing 100029, People's Republic of China
[c] Zhejiang University, Hangzhou, Zhejiang Province 310058, People's Republic of China
[d] Division of Nitric Oxide and Inflammatory Medicine, E-Institutes of the Shanghai Universities, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, People's Republic of China
[e] State Key Laboratory of Mental Health, Institute of Psychology, Chinese Academy of Sciences, Beijing 100101, People's Republic of China
[f] E-Institute of Shanghai Municipal Education Commission, Shanghai 200003, People's Republic of China

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ABSTRACT

Epigallocatechin gallate (EGCG), a main active ingredient of green tea, is believed to be beneficial in association with anticarcinogenesis, antiobesity, and blood pressure reduction. Here we report that EGCG extended *Caenorhabditis elegans* longevity under stress. Under heat stress (35°C), EGCG improved the mean longevity by 13.1% at 0.1 μg/ml, 8.0% at 1.0 μg/ml, and 11.8% at 10.0 μg/ml. Under oxidative stress, EGCG could improve the mean longevity of *C. elegans* by 172.9% at 0.1 μg/ml, 177.7% at 1.0 μg/ml, and 88.5% at 10.0 μg/ml. However, EGCG could not extend the life span of *C. elegans* under normal culture conditions. Further studies demonstrated that the significant longevity-extending effects of EGCG on *C. elegans* could be attributed to its *in vitro* and *in vivo* free radical-scavenging effects and its up-regulating effects on stress-resistance-related proteins, including superoxide dismutase-3 (SOD-3) and heat shock protein-16.2 (HSP-16.2), in transgenic *C. elegans* with SOD-3::green fluorescent protein (GFP) and HSP-16.2::GFP expression. Quantitative real-time PCR results showed that the up-regulation of aging-associated genes such as *daf-16, sod-3,* and *skn-1* could also contribute to the stress resistance attributed to EGCG. As the death rate of a population is closely related to the mortality caused by external stress, it could be concluded that the survival-enhancing effects of EGCG on *C. elegans* under stress are very important for antiaging research.

EGCG, the most abundant catechin in green tea, is credited with the majority of health benefits associated with green tea consumption and beneficial effects in studies of many diseases, such as Parkinson disease [1–4] and Alzheimer disease [5–7]. EGCG has also been found beneficial in obesity [8–10] and diabetes [9,11,12].

The beneficial properties of green tea polyphenols, especially EGCG, provoked great interest in the antiaging potential of green tea. However, only a few studies have reported on the beneficial properties of EGCG in the aging process of a model organism. Bae *et al.* suggested in a recent study that EGCG might be a potential agent for the prevention and treatment of skin photoaging [13]. Srividhya *et al.* in 2008 demonstrated that EGCG could augment the activities of enzymatic antioxidants such as SOD and improve on the nonenzymatic antioxidants such as glutathione in aged rat brain [14]. In an earlier study, Brown *et al.* reported the beneficial effects of EGCG on life span and age-dependent behavioral declines in *Caenorhabditis elegans,* suggesting that EGCG could attenuate the rate of decline in pharyngeal pumping behavior and other behaviors in *C. elegans* [15]. In these studies, EGCG was found to be a potential antiaging agent. Brown *et al.* provided the only evidence for the effects of EGCG on the life span of wild-type *C. elegans* N2 under normal culture conditions.

In this study, we investigated the antiaging effects of EGCG in *C. elegans.* Importantly, we found that, although EGCG could not extend the worm's life span under normal culture conditions, EGCG could significantly improve the longevity of *C. elegans* under stress conditions. Our current study suggests that EGCG might provide strong protection against stress and extend the longevity of *C. elegans* by scavenging reactive oxygen species (ROS) and up-regulating the expression of stress-resistance-associated genes such as *sod-3, daf-16, skn-1,* and *hsp-16.2.* We believe these findings will provide insight into the antiaging research on EGCG and green tea.

Abbreviations: ROS, reactive oxygen species; GFP, green fluorescent protein; EGCG, (−)-epigallocatechin gallate; SOD, superoxide dismutase; FUDR, 5-fluoro-2′-deoxyuridine; ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate); H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; juglone, 5-hydroxy-1,4-naphthoquinone; HSP, heat shock protein; IC_{50}, concentration of antioxidant when 50% of the reaction was inhibited.

* Corresponding author. State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, People's Republic of China. Fax: +86 10 64871293.
E-mail address: zhaobl@sun5.ibp.ac.cn (B. Zhao).

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Experimental procedures

Reagents

EGCG, 98% (Sigma, St. Louis, MO, USA), was stored in water solution at −20°C. FUDR (5-fluoro-2′-deoxyuridine), 98%, was bought from Sigma. ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)] (Sigma) and pyrogallol (Sigma) were used as free radical providers. H_2DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate) (Sigma) was used as a fluorescent probe. Juglone (5-hydroxy-1,4-naphthoquinone), a reactive oxygen species-generating compound, was used to induce oxidative stress in worms.

Worm strains and maintenance

Standard nematode growth medium (NGM) [42] was used for *C. elegans* growth and maintenance at 20°C. Unless stated otherwise, plates were seeded with live *Escherichia coli* OP50 bacteria [42]. Bristol N2 (Caenorhabditis Genetics Center; CGC) was used as the wild-type strain. The transgenic strain CF1553 (muIs84) containing the SOD-3::GFP-linked reporter, used to visualize SOD-3 expression, was from the CGC. The CL2070 (dvIs70) strain, containing the HSP-16.2::GFP-linked reporter used to visualize HSP-16.2 expression, was a generous gift from Y. Luo of the University of Maryland (College Park, MD, USA).

Life span assays and stress resistance

Life span assays were performed at 20 and 25°C. Synchronized hermaphroditic animals were transferred to treatment plates when the young adults began to lay eggs of the indicated genotypes. The worms were then transferred to fresh treatment plates every 2 days for the first 10 days of the assays. Treatment plates were prepared using standard NGM with the reproductive suppressant FUDR (Sigma; 100 mg/L) and EGCG of various concentrations. EGCG at 0.1, 1.0, or 10.0 μg/ml was diluted into live *E. coli* OP50 suspension and added to the surface of the dry NGM plates to the indicated final concentrations [16,17].

Heat-shock assays were performed at 35°C using 2-day-old adults. The worms were treated on plates containing EGCG (0, 0.1, 1.0, or 10.0 μg/ml) for 2 days and then transferred to an incubator set to 35°C. The number of dead worms was recorded every hour [17,18].

The expression of HSP-16.2::GFP in CL2070 worms was investigated by fluorescence microscopy. The worms were treated for 2 days with or without 0.1 μg/ml EGCG, followed by heat shock (treatment at 25 or 30°C for half an hour and then 35°C for an hour) and recovery for 24 h [19,20].

Juglone sensitivity was assessed at 20°C using 2-day-old adults. Worms were incubated on treatment plates with EGCG (0, 0.1, 1.0, or 10.0 μg/ml) for 2 days and then transferred to plates with 500 μM juglone. The number of dead worms was counted and recorded every hour.

For all life span assays, every experiment was repeated three times and conducted in a double-blind manner [20].

ABTS assay

ABTS assay was carried out based on the method in Ref. [21]. ABTS (38.4 mg) and $K_2S_2O_8$ (6.6 mg) were dissolved in 5 ml water. The solution was kept at room temperature for 12–16 h to form $ABTS^+$ solution by oxidizing ABTS with potassium persulfate. The $ABTS^+$ solution was diluted 1:100 in absolute ethanol to prepare the working solution. After 1.8 ml working solution was mixed with 0.2 ml EGCG solution, the absorbance at 734 nm was measured for 2 min (Beckman UV–Vis spectrophotometer, Model DU640B). The final concentrations of EGCG used were 1.0, 2.0, 3.0, 4.0, and 5.0 μg/ml.

Pyrogallol self-oxidation assay

The *in vitro* superoxide anion-scavenging effects of EGCG were measured by monitoring the chemiluminescence in the pyrogallol–luminol system. All reagents were equilibrated in a water bath at a constant temperature (25°C) and then added to a glass luminescence tube (1×5 cm) in a water bath in the following order: 50 μl 1 mmol/L pyrogallol and 950 μl 0.1 mmol/L luminol (in sodium carbonate buffer, pH 10.2). The final concentrations of EGCG were 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, and 20.0 μg/ml. Light emission was observed after a 15-s delay at 25°C [22].

Measurement of intracellular ROS in C. elegans

Intracellular ROS in *C. elegans* were measured with H_2DCF-DA as the molecular probe. For ROS detection under normal culture conditions, the worms, which had just reached adulthood, were treated with or without EGCG (0.1 μg/ml) for 2 days. For the ROS test under oxidative stress, worms that had just reached adulthood were treated with 300 μM juglone for 1 h and then treated with or without EGCG (0.1 μg/ml) for 2 days. At the end of the specified treatment time, the *C. elegans* were collected into 100 μl phosphate-buffered saline (PBS) with 1% Tween 20 in Eppendorf tubes. The worms were then sonicated (Branson Sonifier 250; VWR Scientific, Suwanee, GA, USA) and pipetted into the wells of a Costar 96-well microtiter plate (black, clear, flat-bottom wells) containing H_2DCF-DA (final concentration 50 μM in PBS). Samples were read every 20 min for 2 h in a Thermo Labsystems Fluoroskan Ascent microplate reader at 37°C with excitation 485 nm and emission 530 nm [23,24].

Fluorescence quantification and visualization

Overall GFP fluorescence of GFP-expressing populations was assayed using a Thermo Labsystems Fluoroskan Ascent microplate reader. Adult worms were treated with or without 0.1 μg/ml EGCG for 2 days. Twenty control or treated adult animals of the indicated age were transferred in 100 μl of PBS to a well of a Costar 96-well microtiter plate (black, clear, flat-bottom wells), and total GFP fluorescence was measured using 485 nm excitation and 530 nm emission filters [25]. Quadruple populations were used for each determination.

For fluorescence microscopy, the worms were mounted with a drop of heavy mineral oil placed on a coverslip covered with 2% agarose. The GFP pictures of transgenic worms were taken on an Olympus FluoView FV500 microscope [20,25].

Quantitative real-time PCR

Adult worms were treated with or without 0.1 μg/ml EGCG for 2 days. Total RNA was extracted from adult worms with TRIzol reagent (Invitrogen), and cDNA was produced by oligo(dT) priming. The RT-PCR primers were as follows: *daf-2* (NM_065249), 5′-GGCCGATGGACGTTATTTTG-3′ and 5′-TTCCACAGTGAAGAAGCCTGG-3′; *daf-16* (NM_001026247), 5′-TTTCCGTCCCCGAACTCAA-3′ and 5′-ATTCGCCAACCCATGATGG-3′; *sod-3* (NM_078363), 5′-AGCATCATGCCACCTACGTGA-3′ and 5′-CACCACCATTGAATTTCAGCG-3′; *skn-1* (NM_171347), 5′-AGTGTCGGCGTTCCAGATTTC-3′ and 5′-GTCGACGAATCTTGCGAATCA-3′ *ama-1*, 5′-CTGACCCAAAGAACACGGTGA-3′ and 5′-TCCAATTCGATCCGAAGAAGC-3′.

ama-1 was taken as the internal control. mRNA expression was assessed by quantitative real-time PCR on a Bio-Rad IQ5 Multicolor real-time PCR detection system using SYBR green as the detection method. The gene expression data were analyzed using the comparative $2^{-\Delta\Delta Ct}$ method [26], taking *ama-1* mRNA as the normalizer.

Statistical tools

The data of the life span assays and stress-resistance assays were processed using the Kaplan–Meir survival analysis of SPSS 13.0.

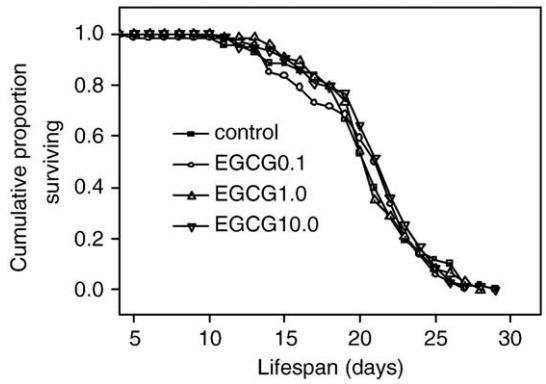

Fig. 1. Effect of EGCG on the life span of wild-type *C. elegans* N2 under normal culture conditions at 20°C. Each survival curve was drawn based on three individual experiments. Compared with the control (N=255), at concentrations of 0.1 (N=210), 1.0 (N=208), and 10.0 μg/ml (N=194), EGCG did not affect the mean life span of wild-type *C. elegans* N2. The data were processed using the Kaplan–Meier survival analysis of SPSS 13.0.

Accidentally lost worms were calculated as censored. The p values were calculated by Kaplan–Meier log-rank pairwise comparison between the control and the EGCG-treated groups. Other data were analyzed with Origin 7.0. Standard error bars of the averages are used in the figures.

Results

EGCG cannot significantly extend the life span of wild-type C. elegans N2 under normal culture conditions

Because there is only one report that EGCG could not extend the life span of *C. elegans* under normal culture conditions [15], we believe it is necessary to confirm the life-span-extending effects of EGCG under normal conditions. This study confirmed that EGCG did not significantly affect the worm life span at 20°C, which is the optimal culture temperature for *C. elegans*. Compared with the control, the EGCG-treated groups at 0.1, 1.0, and 10.0 μg/ml showed no significant increase in the mean life span of *C. elegans* (Fig. 1, Supplemental Fig. 1, and Table 1).

We also investigated whether EGCG could extend the mean life span of *C. elegans* at 25°C, at which the nematodes have a shorter life span compared to 20°C. Again, we found that EGCG demonstrated no significant life-span-extending effects in *C. elegans* (data not shown). We conclude that under normal culture conditions, EGCG could not significantly extend the mean life span of *C. elegans*.

EGCG improves the stress resistance of C. elegans under stress conditions

To detect any longevity-extending effects of EGCG under environmental stress, assays were carried out under thermal stress and oxidative stress. In the thermo-tolerance assay, the worms, shortly after reaching adulthood, were pretreated with EGCG for 48 h before being exposed to heat shock at 35°C. Our results showed that EGCG pretreatment enhanced the worms' resistance to heat stress, thus generating an increased survival rate under the heat shock. The data showed that the mean survival rate was significantly increased by 13.1% at 0.1 μg/ml, 8.0% at 1.0 μg/ml, and 11.8% at 10.0 μg/ml (Fig. 2A, Supplemental Fig. 2A, and Table 2). In the EGCG-treated groups, the maximum longevity at 35°C was raised from 17 to 19 h (Fig. 2A and Supplemental Fig. 2A).

To evaluate the potential effect of EGCG on wild-type *C. elegans* N2 under oxidative stress, the worms were exposed to juglone (500 μM) after 48 h of pretreatment with EGCG shortly after reaching adulthood. Juglone, a pro-oxidant that can be reduced with NAD(P)H by diaphorases, converts oxygen to superoxide anion and consequently increases intracellular oxidative stress [20,27,28]. The results showed that EGCG pretreatment improved the worms' resistance to oxidative stress induced by juglone. Under the oxidative stress, EGCG at all designated concentrations (0.1, 1.0, and 10.0 μg/ml) showed strong protective effects. When all the worms in the control group and the EGCG 10.0 μg/ml group had died, there were still 52.8 and 50.0% alive (taken as censored in the statistical process) in the EGCG 0.1 and EGCG 1.0 μg/ml groups, respectively. Statistical results showed that, compared with the control, the mean survival rate was significantly increased by 172.9% at 0.1 μg/ml, 177.7% at 1.0 μg/ml, and 88.5% at 10.0 μg/ml in the EGCG-treated groups (Fig. 2B, Supplemental Fig. 2B, and Table 3).

Apparently, EGCG could enhance the stress-resistance of *C. elegans* under heat stress and especially under oxidative stress, suggesting that EGCG might obtain this effect by scavenging free radicals.

EGCG decreases the intracellular ROS level in C. elegans under normal culture conditions and oxidative stress

To explore how EGCG could significantly enhance the stress resistance of *C. elegans* under environmental stress, the free radical-scavenging abilities of EGCG were evaluated in the subsequent experiments. First, it was investigated in chemical systems *in vitro*. ABTS was first used to detect the total antioxidant ability of EGCG. The data showed that EGCG could effectively scavenge $ABTS^{+}$, and the IC_{50} (the concentration of antioxidant at which 50% of the reaction was inhibited) of EGCG in this reaction system was 2.8 μg/ml (Fig. 3A). Second, the activity of EGCG to remove superoxide anions was

Table 1
Effects of EGCG on wild-type *C. elegans* N2 feeding on live *E. coli* OP50 at 20°C

Trial	Food	Genotype	Treatment (20°C)	Total (N)	Censored (N)	Mean			Log-rank test	
						Estimate	SE	% Extended[a]	χ^2	p
1	Live-OP50	N2	Control	68	0	20.5	0.49			
			EGCG 0.1	65	0	20.5	0.48	−0.2	0.04	0.8483
			EGCG 1.0	68	5	20.8	0.43	1.2	0.01	0.9308
			EGCG 10.0	73	1	21.0	0.44	2.3	0.16	0.6919
2	Live-OP50	N2	Control	97	23	20.0	0.32			
			EGCG 0.1	62	0	20.1	0.51	0.6	2.85	0.0913
			EGCG 1.0	83	0	20.2	0.46	1.4	3.87	0.0492
			EGCG 10.0	60	1	20.2	0.57	1.1	3.83	0.0503
3	Live-OP50	N2	Control	90	20	19.2	0.49			
			EGCG 0.1	83	2	18.4	0.46	−4.1	3.89	0.0487
			EGCG 1.0	57	5	19.6	0.61	2.3	0.81	0.3686
			EGCG 10.0	61	0	19.5	0.47	1.5	0.08	0.7760
Total	Live-OP50	N2	Control	255	43	19.9	0.25			
			EGCG 0.1	210	2	19.5	0.29	−1.8	0.08	0.7733
			EGCG 1.0	208	10	20.2	0.29	1.7	1.98	0.1590
			EGCG 10.0	194	2	20.3	0.29	1.8	1.67	0.1966

[a] Percentage is relative to the control.

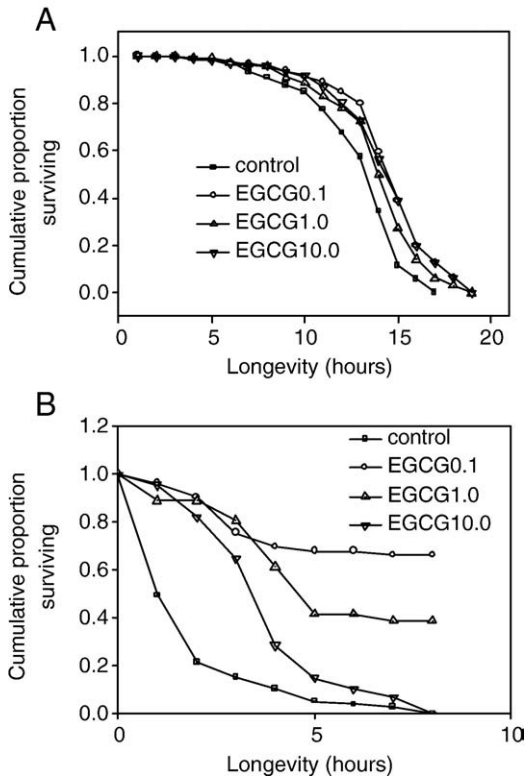

Fig. 2. Protective effect of EGCG on wild-type *C. elegans* N2 under heat stress and oxidative stress. (A) At 35°C, compared with the control (N=327), EGCG extended the worm longevity by 13.1% at 0.1 μg/ml (N=420), 8.0% at 1.0 μg/ml (N=256), and 11.8% at 10.0 μg/ml (N=329). (B) Under oxidative stress, compared with the control (N=292), EGCG extended the worm longevity by 172.9% at 0.1 μg/ml (N=212), 177.7% at 1.0 μg/ml (N=168), and 88.5% at 10.0 μg/ml (N=203). All survival curves are presented based on three individual experiments. The data were processed with the Kaplan–Meier survival analysis of SPSS 13.0.

detected in a pyrogallol self-oxidation system and the data showed that EGCG could effectively scavenge the free radicals produced by pyrogallol self-oxidation with an IC_{50} value of 4.72 μg/ml (Fig. 3B).

Importantly, EGCG also demonstrated a ROS scavenging ability in *C. elegans*. Under normal culture conditions, EGCG at the concentration 0.1 μg/ml could significantly inhibit the production of ROS *in vivo* (compared with the control, $p<0.01$) (Fig. 3C). In the next assays, the ROS levels were detected in juglone (300 μM)-treated wild-type *C. elegans* N2. The data showed that, under juglone-generated oxidative stress, EGCG at the concentration 0.1 μg/ml could effectively reduce ROS accumulation, detected in a 90-min course (compared with the control, $p<0.01$) (Fig. 3D).

Therefore, we conclude that EGCG is a versatile free radical scavenger both *in vitro* and *in vivo*.

EGCG up-regulates SOD-3::GFP expression in transgenic C. elegans CF1553

To further study the protective effects of EGCG in *C. elegans*, SOD-3::GFP reporter gene expression in transgenic CF1553 with or without EGCG treatment was investigated. Compared with the control group, the EGCG-treated group demonstrated higher SOD-3::GFP intensity in pictures taken with confocal laser scanning microscopy (Figs. 4A and 4B). With quantification by a Thermo Labsystems Fluoroskan Ascent microplate reader, the data showed that EGCG at 0.1 μg/ml could significantly up-regulate SOD-3::GFP expression by 49.0% in transgenic CF1553 (Fig. 4C). In a following study, the SOD-3::GFP expression was monitored in a 9-day course, starting at the third day of the worm adulthood, aiming to investigate the long-term effect of EGCG on the expression of *sod-3*. At every designated time point, 0.1 μg/ml EGCG-treated worms showed higher SOD-3::GFP levels than the control worms (Fig. 4D), indicating that EGCG could slow the decline of SOD-3::GFP expression during the aging process in *C. elegans*.

EGCG up-regulates the expression of heat shock protein HSP-16.2 in transgenic C. elegans CL2070

HSP-16.2 could serve as a stress-sensitive reporter to predict longevity in *C. elegans* [19,29]. Higher levels of HSP-16.2::GFP predict longer mean remaining longevity of *C. elegans* [19,29]. In this study, the effect of EGCG on the expression of HSP-16.2 was investigated to provide more clues to the protective effects of EGCG on *C. elegans* under environmental stress. CL2070 worms with the HSP-16.2::GFP reporter gene were treated with heat shock at 35°C for 1 h and allowed to recover at 20°C for 24 h. Compared with the control group, the 0.1 μg/ml EGCG-treated group showed higher HSP-16.2::GFP intensity in pictures taken with confocal laser scanning microscopy (Figs. 5A and 5B). With quantification by a Thermo Labsystems Fluoroskan

Table 2
Protective effects of EGCG on wild-type *C. elegans* N2 feeding on live *E. coli* OP50 under heat stress at 35°C

Trial	Food	Genotype	Treatment (35°C)	Total (N)	Censored (N)	Mean			Log-rank test	
						Estimate	SE	% Extended[a]	χ^2	p
1	Live-OP50	N2	Control	100	0	12.6	0.22			
			EGCG 0.1	135	0	13.4	0.22	6.0	11.68	0.0006
			EGCG 1.0	86	0	12.7	0.21	0.5	0.09	0.7683
			EGCG 10.0	102	0	13.4	0.30	6.0	13.74	0.0002
2	Live-OP50	N2	Control	74	0	11.5	0.31			
			EGCG 0.1	97	0	13.8	0.34	20.4	43.83	<0.0001
			EGCG 1.0	54	0	12.3	0.41	7.3	5.25	0.0220
			EGCG 10.0	75	0	13.8	0.33	20.3	32.59	<0.0001
3	Live-OP50	N2	Control	63	0	11.5	0.45			
			EGCG 0.1	115	0	13.5	0.26	16.6	12.80	0.0003
			EGCG 1.0	60	0	13.0	0.42	12.4	6.62	0.0101
			EGCG 10.0	104	0	13.8	0.31	19.9	23.64	<0.0001
4	Live-OP50	N2	Control	90	0	12.2	0.29			
			EGCG 0.1	73	0	14.0	0.35	15.2	34.76	<0.0001
			EGCG 1.0	56	0	14.2	0.38	16.4	34.37	<0.0001
			EGCG 10.0	48	0	12.3	0.27	0.8	1.08	0.2998
Total	Live-OP50	N2	Control	327	0	12.0	0.15			
			EGCG 0.1	420	0	13.6	0.14	13.1	93.23	<0.0001
			EGCG 1.0	256	0	13.0	0.18	8.0	28.47	<0.0001
			EGCG 10.0	329	0	13.5	0.16	11.8	68.06	<0.0001

[a] Percentage is relative to the control.

Table 3
Protective effects of EGCG on wild-type *C. elegans* N2 feeding on live *E. coli* OP50 under oxidative stress

Trial	Food	Genotype	Treatment (20°C; juglone)	Total (*N*)	Censored (*N*)	Mean			Log-rank test	
						Estimate	SE	% Extended[a]	χ^2	*p*
1	Live-OP50	N2	Control	157	0	2.1	0.13			
			EGCG 0.1	53	35	6.3	0.34	204.4	111.05	<0.0001
			EGCG 1.0	36	14	5.4	0.40	160.1	59.29	<0.0001
			EGCG 10.0	88	0	4.0	0.18	93.1	48.07	<0.0001
2	Live-OP50	N2	Control	69	0	2.3	0.18			
			EGCG 0.1	85	52	6.6	0.22	186.9	118.57	<0.0001
			EGCG 1.0	81	32	5.6	0.28	141.1	69.57	<0.0001
			EGCG 10.0	61	0	3.0	0.16	30.9	14.15	<0.0001
3	Live-OP50	N2	Control	66	0	2.2	0.23			
			EGCG 0.1	74	25	4.8	0.30	115.7	49.15	<0.0001
			EGCG 1.0	51	38	7.2	0.23	221.8	95.84	<0.0001
			EGCG 10.0	54	0	5.4	0.31	142.0	38.06	<0.0001
Total	Live-OP50	N2	Control	292	0	2.2	0.10			
			EGCG 0.1	212	112	5.9	0.17	172.9	286.88	<0.0001
			EGCG 1.0	168	84	6.0	0.19	177.7	249.11	<0.0001
			EGCG 10.0	203	0	4.1	0.14	88.5	102.52	<0.0001

[a] Percentage is relative to the control.

Ascent microplate reader, the data show that EGCG could significantly up-regulate HSP-16.2::GFP expression by 11.9% in CL2070 (Fig. 5C, $p<0.01$ compared with the control). We conclude that EGCG could up-regulate the expression of the HSP-16.2::GFP reporter gene in CL2070 under thermal stress.

EGCG regulates the mRNA expression of aging-associated genes in wild-type C. elegans N2

DAF-16/forkhead transcription factor, the downstream target of the insulin-like signaling in *C. elegans*, is indispensable for both life span regulation and stress resistance [30]. DAF-2 is the insulin/IGF-1 receptor that signals through a conservative PI3-kinase/AKT pathway and ultimately down-regulates DAF-16 [31,32]. SOD-3, one of the downstream effectors of DAF-16, is also an important regulator of life span and stress resistance in *C. elegans* [31–33]. SKN-1 is another transcription factor that can positively regulate the life span and stress resistance in *C. elegans* [34,35]. Quantitative real-time PCR experiments were performed to investigate whether EGCG could regulate the expression of the aging-associated genes *daf-2*, *daf-16*, *sod-3*, and *skn-1*. Our results showed that EGCG did not affect the expression of *daf-2*, but it up-regulated the mRNA expression of *daf-16* (not statistically significant), *sod-3* ($p<0.05$) and *skn-1* ($p<0.05$) (Fig. 6). The up-regulation of EGCG on *sod-3* expression is consistent with the result on SOD-3::GFP

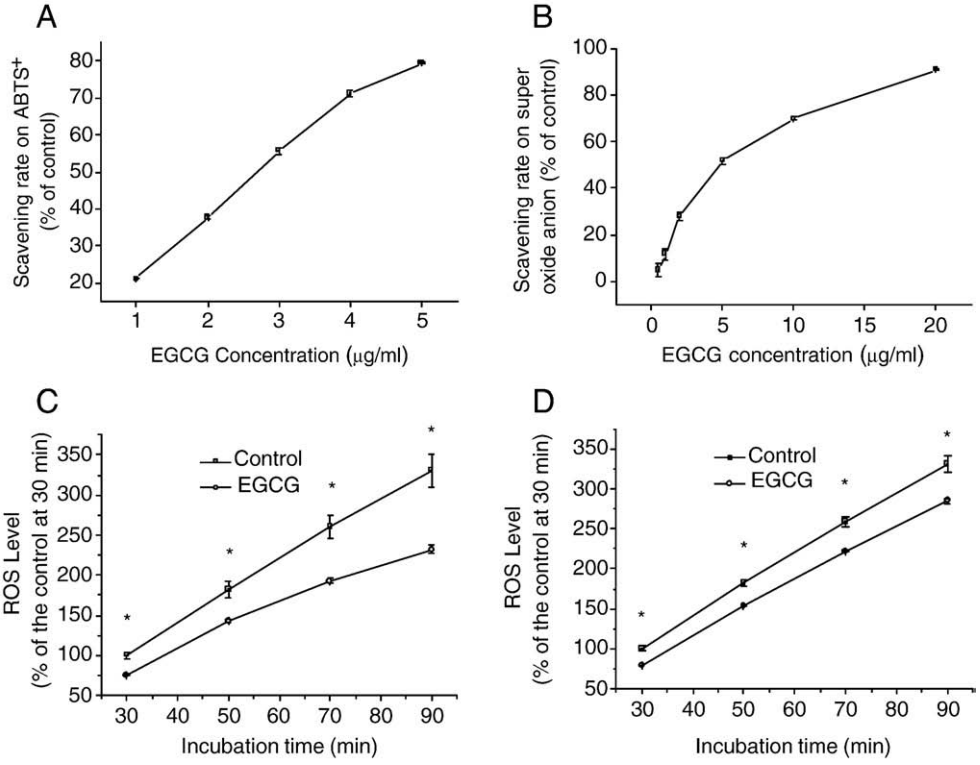

Fig. 3. Free radical-scavenging effect of EGCG *in vitro* and *in vivo*. (A) EGCG effectively scavenged $ABTS^+$ (IC_{50} 2.8 μg/ml). (B) EGCG dramatically scavenged the free radicals generated by the Fenton reaction (IC_{50} 0.07 μg/ml). (C) EGCG at the concentration 0.1 μg/ml decreased ROS accumulation in *C. elegans* under normal culture conditions, detected in a 90-min course. (D) EGCG at the concentration 0.1 μg/ml reduced ROS accumulation in *C. elegans* under juglone-generated oxidative stress, detected in a 90-min course. Error bars indicate SE; *$p<0.01$.

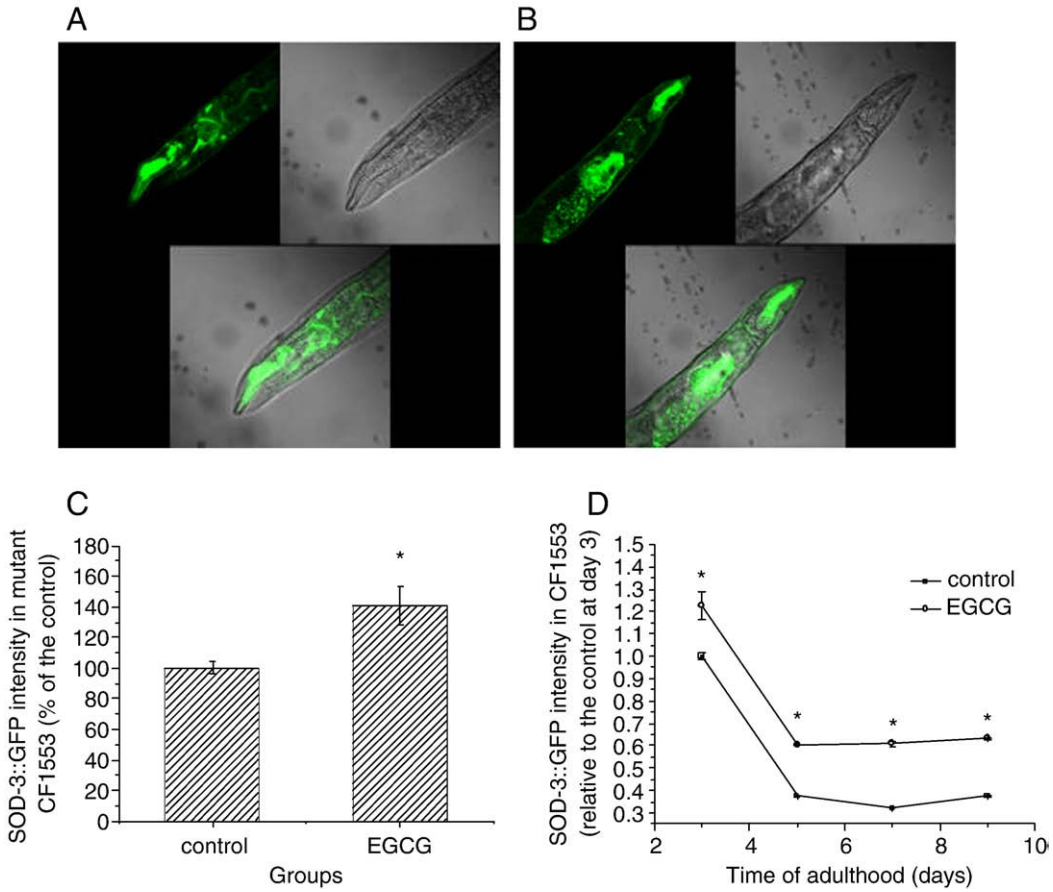

Fig. 4. EGCG up-regulates SOD-3::GFP expression in transgenic *C. elegans* CF1553. (A) Image of SOD-3::GFP expression in control worms. (B) Image of SOD-3::GFP expression in 0.1 μg/ml EGCG-treated worms. The SOD-3::GFP expression in EGCG-treated worms (B) is higher than that in control worms (A). The GFP pictures of transgenic worms were taken on an Olympus FluoView FV500 microscope. (C) Quantified GFP intensity (±SE) in CF1553 from four experiments in each group, with 20 worms in each experiment. (D) Quantified GFP intensity (±SE) in CF1553 from four experiments in each group, with 20 worms in each experiment, in a 9-day course. The quantification was performed on a Thermo Labsystems Fluoroskan Ascent microplate reader. *$p<0.01$.

expression. We conclude that EGCG could improve the stress resistance of *C. elegans* through up-regulating aging-related genes.

Discussion

In 2006, Marishka K. Brown *et al.* reported that although EGCG could not extend the life span of wild-type *C. elegans* N2 under normal culture conditions, it still demonstrated antiaging effects by diminishing free radicals; attenuating the age-related decline in pharyngeal pumping rates; enhancing the chemotaxis index in older worms, which also declines with age; and delaying paralysis in the Aβ-expressing transgenic worm strain CL4176 [15]. In another study, Y.M. Li *et al.* investigated the antiaging effects of green tea catechin extract from Longjing green tea and reported that 10 mg GTC (green tea catechin)/ml diet in *Drosophila melanogaster* could prolong its median survival time by 36% and mean life span by 16% [43]. However, EGCG was not studied separately as a pure compound in that work. These results provoked our interest to study further the antiaging effects of the GTC EGCG.

In this study, we observed that EGCG could not extend the life span of *C. elegans* under normal culture conditions, but significantly extend the survival of *C. elegans* under heat stress and oxidative stress. Because the mortality in a population is usually closely associated with causes from environmental stress, these results demonstrate the great antiaging potential of EGCG [36–38]. In 1954 D. Harman initially advanced the free radical theory of aging and hypothesized that free radical species caused deterioration of an organism [39]. On one hand, aerobic organisms develop a cellular metabolism that takes oxygen as an electron acceptor, but they continuously generate ROS, namely hydroxyl radical, superoxide anion, and hydrogen peroxide; on the other hand, they possess antioxidant defense systems that can effectively remove these ROS [39]. EGCG has been reported to be an affective antioxidant [40,41]. Does EGCG enhance the stress resistance of *C. elegans* under environmental stress to prolong its longevity by removing ROS? We got a positive answer from this study. We found that EGCG treatment significantly down-regulated ROS levels under both normal culture conditions and oxidative stress (Figs. 3C and 3D).

Because SOD-3 and HSP-16.2 are the downstream effectors of DAF-16 and can serve as stress-sensitive reporters to predict longevity in *C. elegans* [19,29], the effects of EGCG on the expression of both proteins were investigated. EGCG could up-regulate both reporters in *C. elegans,* which might explain why EGCG could significantly increase the survival of *C. elegans* under heat stress and oxidative stress. In addition, quantitative real-time PCR results suggested EGCG could significantly up-regulate the expression of the aging-associated genes *sod-3* and *skn-1,* which helped explain why EGCG could significantly extend the longevity of *C. elegans* under heat stress and oxidative stress.

In conclusion, the longevity-improving effects of EGCG in the nematode *C. elegans* under stress might be attributed to its direct ROS-scavenging activity and indirect free radical-scavenging activity through up-regulating stress-resistance-associated genes such as *sod-3, hsp-16.2,* and *skn-1.* These interesting findings highlight the

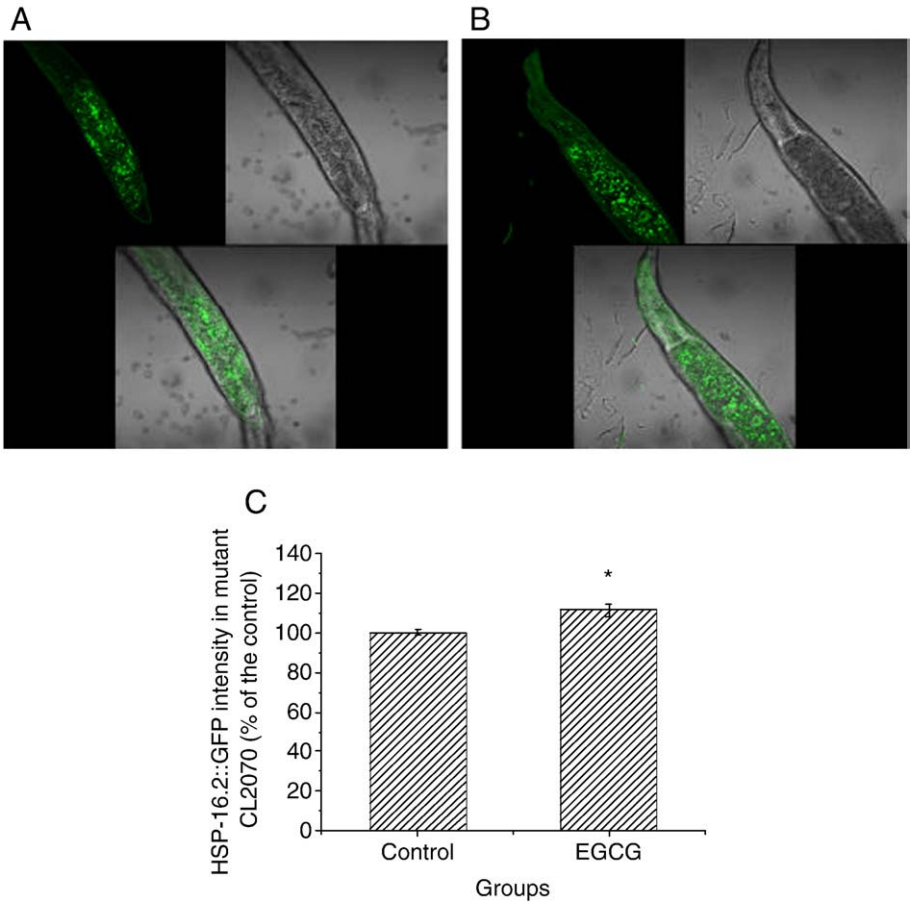

Fig. 5. Effects of EGCG on the expression of heat shock protein HSP-16.2 in CL2070. (A) Image of HSP-16.2::GFP expression in the control worms. (B) Image of HSP-16.2::GFP expression in the 0.1 μg/ml EGCG-treated group. The HSP-16.2::GFP expression in EGCG-treated worms (B) is higher than that in control worms (A). The GFP pictures of transgenic worms were taken on an Olympus FluoView FV500 microscope. (C) Quantified HSP-16.2::GFP intensity (±SE) in CL2070 from four experiments in each group, with 20 worms in each experiment, detected on a Thermo Labsystems Fluoroskan Ascent microplate reader. *$p<0.01$.

potential of EGCG to extend the human average life expectancy by providing protection against environmental stress.

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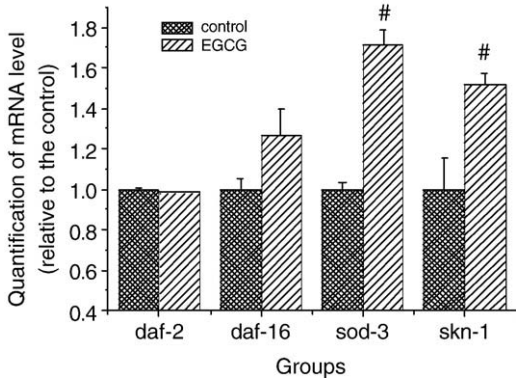

Fig. 6. Effects of EGCG on the expression of aging-associated genes in wild-type *C. elegans* N2. Quantitative real-time PCR results of the effects of EGCG on the expression of the aging-associated genes *daf-2, daf-16, sod-3,* and *skn-1* in *C. elegans.* The EGCG group was treated with 0.1 μg/ml EGCG. Error bars indicate SE; #$p<0.05$.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2008.10.041.

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