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Early-stage administration of hydroxytyrosol extends lifespan and delays aging in *C. elegans*

Nannan Du[1†], Lishuo Song[1,2†], Ruigang Yang[1], Kexin Liu[3], Zubiao Niu[1], Zhanfeng Zhang[1] and Qiang Sun[1*]

Abstract

Background This study employs *Caenorhabditis elegans* (*C. elegans*) as a model organism to explore the anti-aging effects of hydroxytyrosol (HT) and its underlying mechanisms.

Results The findings reveal that HT significantly extends the lifespan of *C. elegans* while improving their functional performance (motility and pharyngeal function), and antioxidant capacity when administered on the first day of adulthood (D1). However, its efficacy diminishes when treatment begins on or after the third day of adulthood (D3). HT prolongs lifespan through a mechanism akin to that of *skn-1* modulating the oxidative stress pathway.

Conclusions This study suggests that the administration timing is an important factor for of anti-oxidation compounds to be effective in counteracting aging.

Keywords Hydroxytyrosol, *Caenorhabditis elegans*, Anti-aging, Oxidative stress

Background

According to the World Health Organization, the proportion of the global population aged 60 and above is projected to nearly double by 2050 (https://www.who.int/news-room/fact-sheets/detail/ageing-and-health). As population aging intensifies, gaining a deep understanding of the essence of aging has become an urgent priority. Aging is a complex biological process characterized by declines at the molecular, cellular, tissue, and organ levels [1]. In recent years, the rapid advancement of technologies such as genomics, epigenetics, and metabolomics has propelled in-depth research into the mechanisms of aging [2]. With the rapid advancement of these technologies, researchers have developed innovative high-throughput screening methods, such as PICLS, which identified new compounds that can extend the lifespan of human cells, offering a novel tool for the development of anti-aging drugs [3]. Additionally, current evidence indicates that aging is closely associated with factors such as telomere shortening [4], mitochondrial dysfunction [5], protein homeostasis imbalance [6], and chronic inflammation [7]. Research by Pietro et al. has highlighted the similarities in telomeres across Arabidopsis, mice, and humans, noting that they are composed of TTAGGG sequences and safeguarded by specific proteins [8, 9]. Recent studies have suggested a strong correlation between changes in chromatin structure and cellular senescence [10]. Additionally, Yang et al. showed that inflammation plays a crucial role in the senescence of mesenchymal stem cells, suggesting targeting inflammatory pathways a promising strategy for anti-aging therapies [11]. Concurrently, strategies to delay aging have garnered widespread attention.

Moreover, there is considerable scientific interest in neurodegenerative diseases that are closely associated with cell death and aging, such as Parkinson's and Alzheimer's diseases [12]. PM20D1-NADA pathway was

†Nannan Du and Lishuo Song contributed equally to this work.

*Correspondence:
Qiang Sun
sunq@bmi.ac.cn
[1] Laboratory of Advanced Biotechnology, Beijing Institute of Biotechnology, Research Unit of Cell Death Mechanism, 2021RU008, Chinese Academy of Medical Science, Beijing 100071, China
[2] Hainan Medical University, Haikou, Hainan, China
[3] School of Medicine, South China University of Technology, Guangzhou, China

shown to play a protective role in Parkinson's disease, in which PM20D1 can alleviate oxidative stress and neuroinflammation by producing the neuroprotective metabolite NADA, thereby slowing the progression of the disease [13]. Furthermore, Jiang et al. showed that, in a mouse model of Parkinson's disease, the cGAS-STING-YY1 signaling axis accelerates neurodegenerative progression through LCN2-dependent astrocyte senescence [14]. In another aging-related condition, neutralizing acyl-CoA binding protein (ACBP/DBI) has been shown to significantly induce autophagy, reduce fatty liver and fibrosis, and even reverse liver damage during disease progression [15].

As for aging interventions, its aim is to slow the aging process and reduce the incidence of age-related diseases by modulating biological mechanisms associated with aging [16]. Significant progress has been made in this field, focusing primarily on the following areas: Firstly, activating longevity-related pathways, such as AMPK and mTOR signaling pathways, through caloric restriction (CR) or caloric restriction-mimicking drugs (such as rapamycin and metformin) [17]; secondly, targeting the clearance of senescent cells (senolysis) by using senolytic agents to selectively eliminate senescent cells and mitigate the damage caused by their secreted pro-inflammatory factors to tissues [18]; thirdly, employing epigenetic reprogramming technology to restore cellular youth by regulating gene expression [19]; and fourthly, utilizing antioxidants or mitochondria-targeted drugs to enhance mitochondrial function and reduce oxidative stress damage to cells [20, 21]. Additionally, stem cell therapy and gene editing technology offer new possibilities for aging interventions [22].

This study focuses on one of the aging intervention strategies—anti-aging drugs—and reveals that hydroxytyrosol (HT) can extend the lifespan of *Caenorhabditis elegans (C. elegans)*. HT is a polyphenolic compound naturally found in olive oil, olive leaves, and olive fruits, possessing strong antioxidant, anti-inflammatory, and anti-aging properties [23, 24]. As one of the most important bioactive components in olive oil, HT significantly contributes to the health benefits of the Mediterranean diet [25]. Its biological effects are extensive, including antioxidant, anti-inflammatory, cardiovascular protection, anti-aging, and neuroprotection [26]. These characteristics make HT a promising candidate for applications in functional foods, pharmaceutical development, and cosmetics [27]. In this study, the lifespan-extending effect of HT in *C. elegans* further confirms its anti-aging potential, supporting for its application and promotion in the field of anti-aging.

Methods

Strains and culture of *C. elegans*

This study utilized wild-type *C. elegans* (N2) along with one mutant strains *skn-1(zj15)* as model organisms. All strains were maintained at 20 °C on standard nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 as the food source. Experiments were conducted using synchronized worms at early adulthood.

HT treatment

HT was dissolved in sterile water to prepare a stock solution at the desired concentration. The stock solution was aliquoted and stored at − 80 °C until use. Before each experiment, the stock was diluted 100-fold with sterile water to prepare the working solution. To prepare the NGM plates, 200 µL of *Escherichia coli* OP50 suspension was pipetted onto the center of each plate and incubated at 20 °C for 48 h to form a uniform bacterial lawn. The plates were then exposed to ultraviolet light to thoroughly kill the bacteria. Subsequently, 200 µL of the HT working solution was evenly distributed across the bacterial lawn using a pipette, ensuring complete coverage. The plates were left at 20 °C overnight to allow for uniform absorption of the HT. For the control group, sterile water was used in place of the HT solution, following the same procedure.

Staged drug administration

Drug intervention was initiated at specific time points corresponding to the adult stages of *C. elegans*: day 1 (adult stage), day 3, day 5, day 7, day 9, day 11, day 13, and day 15. Each group consisted of at least 200 synchronized worms, with one group serving as the control (treated exclusively with sterile water). At the designated time points, 0.25 mg/mL HT working solution was evenly applied to the bacterial lawn on NGM plates using a pipette, ensuring complete coverage. The plates were left overnight to ensure uniform distribution of the compound. During non-treatment periods, both the control and treatment groups were treated with sterile water instead of HT to eliminate any potential procedural effects on the results.

Lifespan measurement

Synchronized worms at early adulthood were transferred to NGM plates containing either the treatment or control solutions, with at least 200 worms per group. Worm survival was monitored daily until all individuals had died. The time of death for each worm was recorded to calculate the median and maximum lifespan. In this study, survival curves for the first 20 days were presented. To prevent interference from bacterial overgrowth, worms

were transferred to fresh HT-treated plates every 2 days during the experiment.

Motility assessment

Worm motility was assessed using a stereomicroscope through a swimming assay. Individual worms were transferred to a 96-well plate containing 1 mL of M9 buffer, and the number of body thrashes within 30 s was recorded. Each experimental group included at least 10 worms to ensure the reliability of the data.

Pharyngeal pumping frequency measurement

Pharyngeal pumping was observed under a stereomicroscope, and the number of pumps within 15 s was recorded. At least 10 worms were observed per group, and the average pharyngeal pumping frequency was calculated.

Heat stress assay

Treatment was administered to wild-type C. elegans starting on the first and third days of adulthood. During the initial 3 days of adulthood, the worms were transferred daily to fresh NGM plates. On the third day, these plates were incubated at 37 °C. The number of surviving, deceased, and missing worms on each plate was recorded at 2, 4, and 8 h post-incubation.

Statistical analysis

All experimental data are presented as mean ± standard deviation (Mean ± SD). Statistical analyses were performed using GraphPad Prism software. Group comparisons were conducted using a two-tailed paired t-test, with a P-value of less than 0.05 considered statistically significant. For lifespan data, Kaplan–Meier survival analysis was used to compare differences between treatment groups, and the Log-rank test was applied to assess statistical significance.

Quality control

Control groups were included in all experiments to ensure consistency in experimental conditions. Worm health was regularly monitored, and any abnormal individuals were excluded. All experiments were repeated at least twice to ensure the reliability and reproducibility of the results.

Results

Early administration of HT extends lifespan of *C. elegans*

In our initial screening of anti-aging compounds (including Resveratrol, Fisetin, Quercetin, Hydroxytyrosol, ABT263 and Aspirin, Fig. 1a–f), we unexpectedly found that HT significantly extends both the median and maximum lifespan of *C. elegans* (Fig. 1d). To delve deeper into how the timing of drug administration influences lifespan extension, we systematically examined the effects of

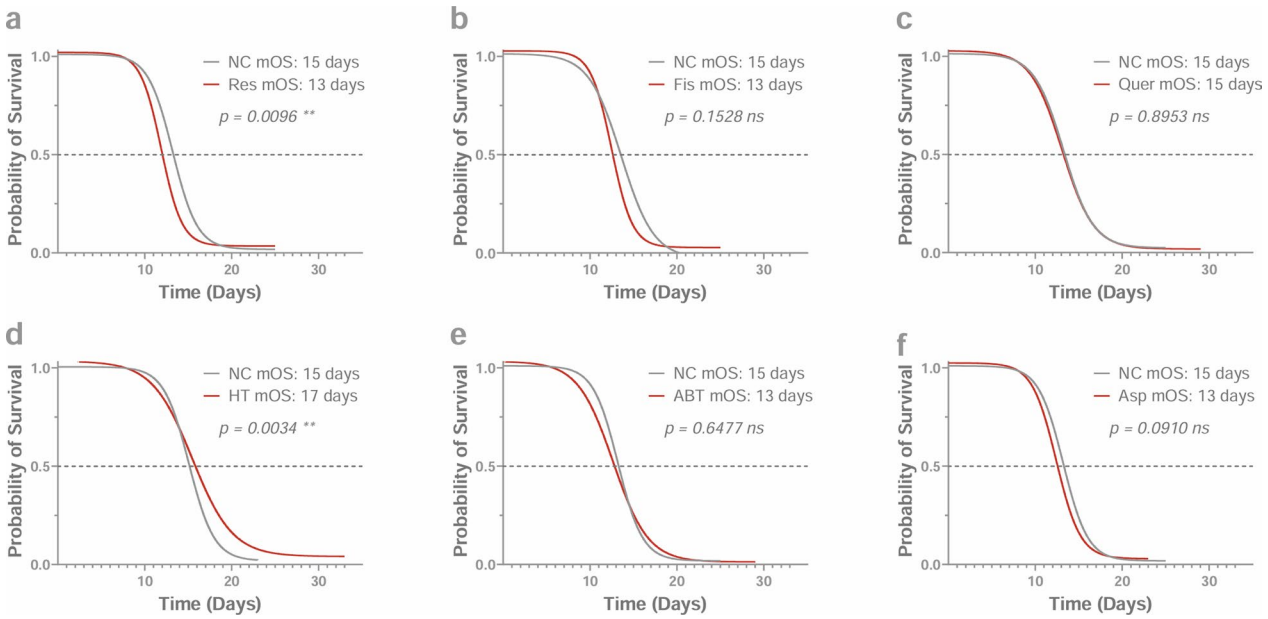

Fig. 1 Administration of HT extends lifespan of *C. elegans*. **a**–**f** Lifespan analyses of WT animals in the presence of 50 uM Resveratrol (**a**), 200 uM Fisetin (**b**), 100 uM Quercetin (**c**), 0.25 mg/mL Hydroxytyrosol (**d**), 10 uM ABT263 (**e**), 500 uM Aspirin (**f**). Compounds were administrated every 2 days from the first day of adulthood to the end of the experiment; n ≥ 100 worms/each group. ns, No significant difference; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

HT at various adult stages. The results revealed a pronounced time-dependence in the lifespan-extending effects of HT. Specifically, administration on the first day of adulthood (D1) yielded the most substantial lifespan extension (Fig. 2b), while administration on D3 resulted in a relatively weaker effect (Fig. 2c), contrary to our initial hypothesis. Notably, when HT was administrated at D5 or beyond, no lifespan-extending effects could be observed anymore (Fig. 2d–i).

Early administration of HT enhances functional performance of *C. elegans*

To comprehensively evaluate the effects of HT on the physiological functions of *C. elegans*, we conducted a series of functional assays. On the one hand, we measured the number of thrashes in a swimming assay to assess motility. At the D5 time point, compared to the control group, administration on D3 and D7 did not significantly enhance the motility of *C. elegans* (Fig. 3a). Although administration on D1 showed a trend of increased motility, it did not reach statistical significance. However, administration on D5 significantly enhanced motility (Fig. 3a), suggesting a time-dependent effect of HT on motility. At the D7 time point, compared to the control group, administration on D1, D3, and D7 did not significantly affect motility (Fig. 3b), while administration on D5 still showed a trend of increased motility (Fig. 3b). This result further supports the hypothesis that HT may significantly enhance motility within a short period (48 h). At the D9 time point, compared to the control group, only administration on D1 significantly affected motility (Fig. 3c).

On the other hand, we assessed pharyngeal pumping function by quantifying the number of pumps. At the D5 time point, compared to the control group, administration on D1, D3, and D5 significantly enhanced pharyngeal pumping function (Fig. 3d). Interestingly, administration on D7 (prior to D5 administration) also

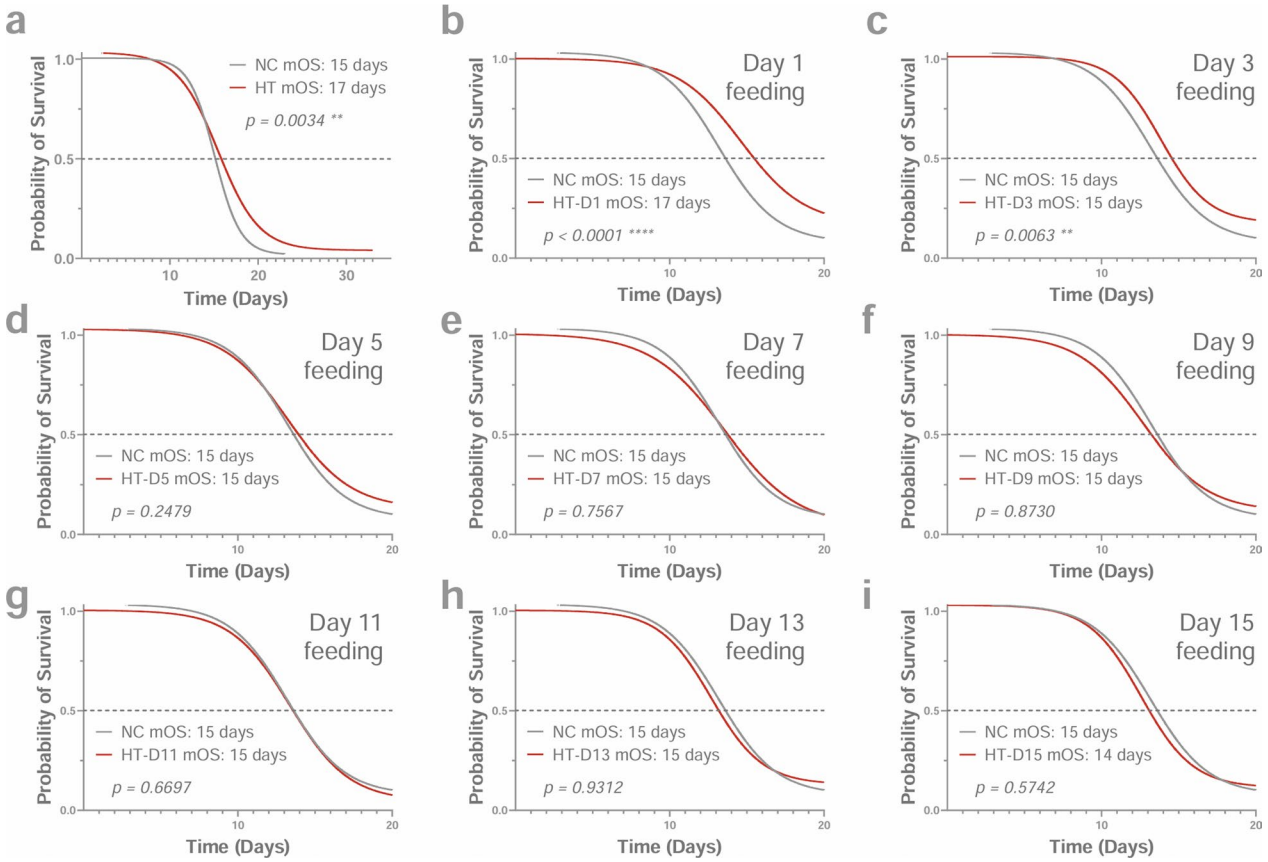

Fig. 2 Early administration of HT extends lifespan of *C. elegans*. **a** Survival analyses of wild-type *C. elegans* treated with 0.25 mg/mL HT with vehicle (NC) as control, mOS was extended by 2 days; Compounds were administrated every 2 days from the first day of adulthood to the end of the experiment. $p = 0.0034$**; n ≥ 100 worms/each group. **b**–**i** Lifespan analyses of wild-type *C. elegans* treated with 0.25 mg/mL HT starting at different time points. Significant mOS extension was detected only when HT administration started at early stages of adult (D1 and D3). HT administration started at the indicated developmental stages of adulthood, every 2 days to the end of the experiment; n ≥ 100 worms/each group. ns, No significant difference; *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

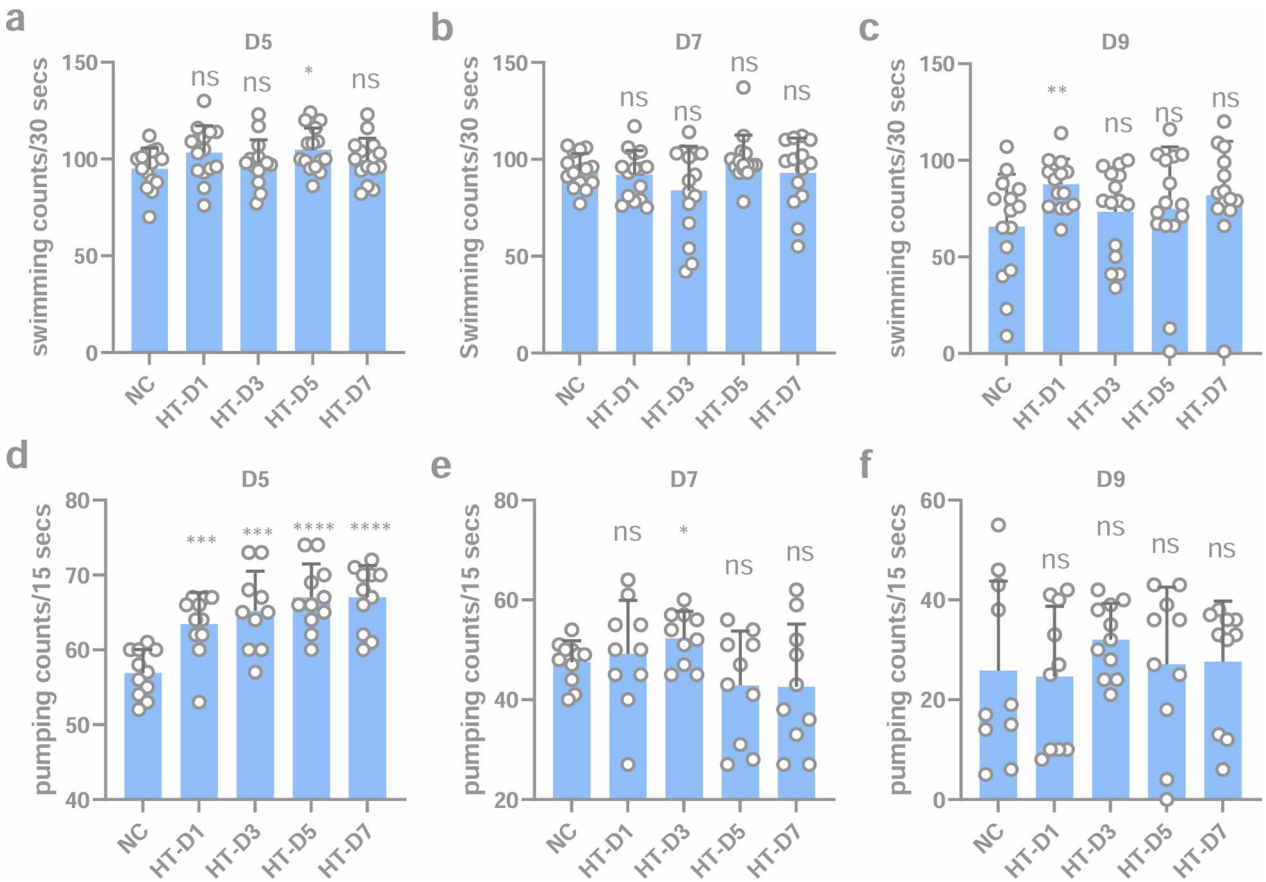

Fig. 3 Early administration of HT enhances functional performance of *C. elegans*. **a**–**c** Motility analysis of wild-type *C. elegans* in the presence of 0.25 mg/mL HT starting at D1, D3, D5, D7, respectively. Measurements were performed for 30 s on days 5 (**a**), 7 (**b**) and 9 (**c**), n ≥ 10 worms/each group. **d**–**f** Pharyngeal pumping frequency of wild-type *C. elegans* in the presence of 0.25 mg/mL HT starting at D1, D3, D5, D7, respectively. Measurements were performed for 15 s on days 5 (**d**), 7 (**e**) and 9 (**f**); n ≥ 10 worms/each group. ns, No significant difference; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

showed a significant enhancement in pharyngeal pumping (Fig. 3d), which may be related to fluctuations in the physiological state of *C. elegans*. At the D7 time point, compared to the control group, only administration on D3 significantly enhanced pharyngeal pumping (Fig. 3e). At the D9 time point, compared to the control group, administration on D1, D5, and D7 did not enhance pharyngeal pumping (Fig. 3f), although administration on D3 showed a trend of enhancement, it did not reach statistical significance (Fig. 3f).

HT promotes survival over oxidative stress akin to *skn-1*

Recognizing HT as a potent antioxidant, this study seeks to explore its impact on the antioxidant capacity and lifespan of *C. elegans*. We evaluated the antioxidant capacity of *C. elegans* using a heat shock assay and systematically recorded survival rates at three time points (2 h, 4 h, and 8 h) (Fig. 4a, b). The results indicated that, at the 8-h time point, compared to the control group, the survival rate of *C. elegans* with HT administered on D1 was significantly higher, while the survival rate of those with HT administration on D3 was only slightly higher than the control group. This finding is in agreement with the result that administration on D1 and D3 can improve the physiological functions of *C. elegans*. Notably, compared to administration on D1, the survival rate of *C. elegans* administered on D3 significantly decreased, consistent with the conclusion that HT has the most pronounced lifespan-extending effect when administered on D1.

Next, we examined whether the lifespan-extending effects of HT are closely linked to the oxidative stress pathway. To test this idea, we carried out experiments using *C. elegans* line (*skn-1(zj15)*), a gain of function mutant resistant to oxidative stress. The results revealed that the *skn-1* mutant displayed a significantly extended lifespan, which, however, could not be further extended by HT treatment (Fig. 4c, d). This finding suggests that

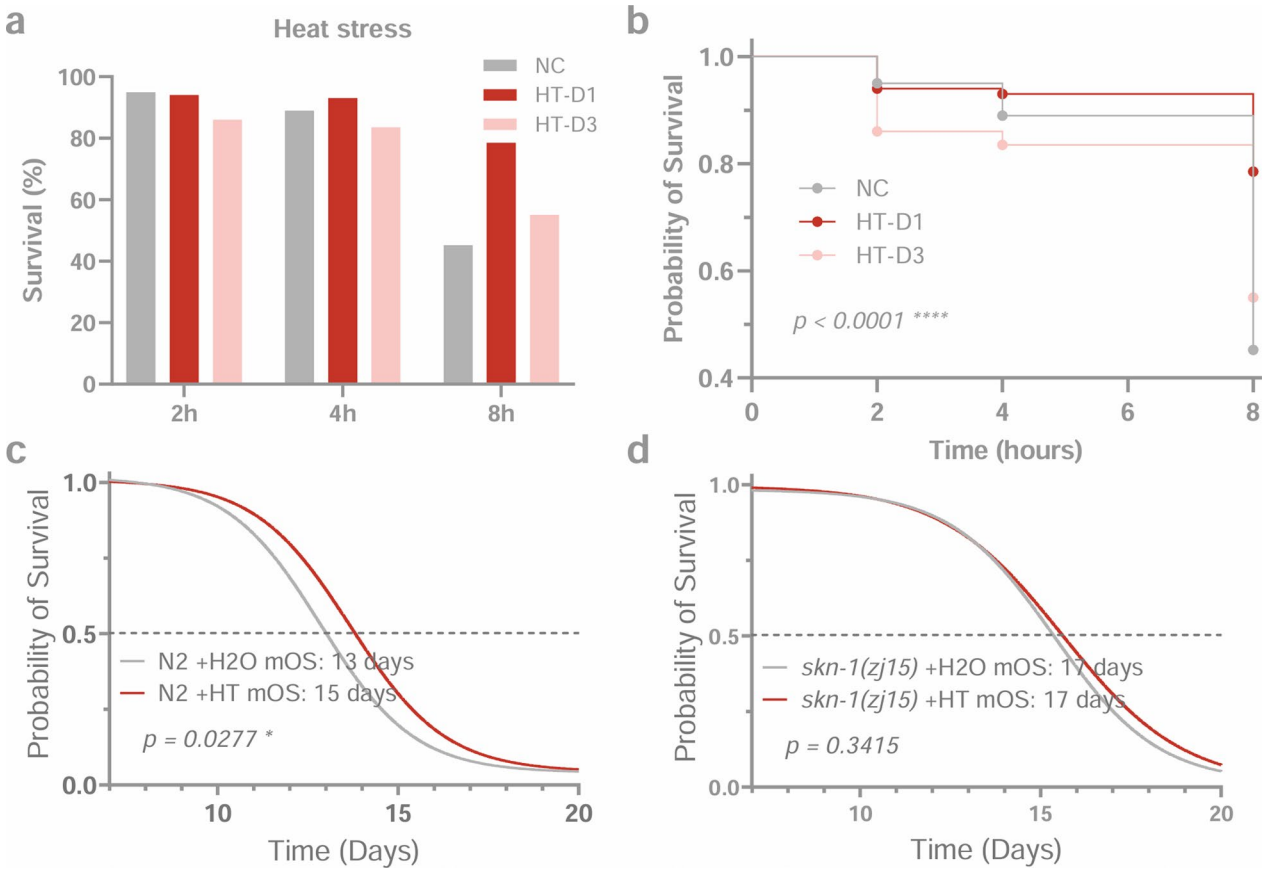

Fig. 4 HT promotes survival over oxidative stress akin to skn-1. **a** Survival rate of wild-type *C. elegans* with HT treatment starting at D1 and D3, followed by incubation at 37 °C for 2 h, 4 h and 8 h, as indicated. n ≥ 100 worms/each group. **b** Lifespan analyses of wild-type *C. elegans* with HT treatment starting at D1 and D3, followed by incubation at 37 °C for 2 h, 4 h and 8 h, as indicated; n ≥ 100 worms/each group. **c** Lifespan analyses of N2 *C. elegans* (control for *skn-1*) with HT treatment starting at D1; n ≥ 100 worms/each group. **d** Lifespan analyses of *skn-1(zj15) C. elegans* with HT treatment starting at D1; n ≥ 100 worms/each group. ns, no significant difference; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

HT may operate through mechanisms akin to those of *skn-1* within the oxidative stress pathway.

Discussion

This study reveals a tight association between the timing of HT administration and lifespan extension in *C. elegans*, demonstrating that earlier administration results in a more pronounced extension of lifespan. This innovative finding holds significant theoretical importance in the field of anti-aging research and provides new insights and directions for future studies and applications.

From a molecular perspective, early administration allows HT to rapidly intervene in the physiological and metabolic processes of *C. elegans*. As a potent antioxidant, HT efficiently and precisely eliminates reactive oxygen species (ROS) generated during the early active metabolic stages of *C. elegans* [28]. If not promptly removed, ROS, being highly oxidative, can cause severe damage to cellular structures and biomolecules such as DNA, proteins, and lipids [29–31]. The antioxidant action of HT effectively prevents such damage, maintaining normal cellular function and metabolic homeostasis [32], thus laying a solid foundation for the long-term survival of *C. elegans*.

Comparative analyses of different administration timings reveal that the lifespan extension effect in the late administration group is significantly less pronounced than in the early administration group. By the late stage, substantial irreparable damages have accumulated within the worms [33]. For instance, mitochondrial function, which is crucial for energy production, is often severely impaired in late-stage worms, leading to disrupted energy metabolism [34]. Moreover, recent research indicates that quinolinic acid accelerates microglial cell senescence by inhibiting mitophagy, which in turn shortens the lifespan of *C. elegans* [35]. This finding underscores a critical role mitochondrial health and function play in determining an organism's lifespan. Although HT can reduce ROS

through its antioxidant action at this stage, the structural and functional damages within the mitochondria are too severe to be fully restored by antioxidant measures alone, in turn, has an impact on the lifespan of *C. elegans* [34, 36, 37].

Notably, this study also found that HT extends the lifespan of *C. elegans* through mechanisms similar to those of the transcription factor *skn-1* by modulating the oxidative stress pathway. *skn-1* plays a critical role in regulating oxidative stress responses [38, 39]. Early administration of HT may mimic the activation process of *skn-1*, upregulating the expression of a series of antioxidant enzyme genes, such as superoxide dismutase (SOD) and catalase (CAT), thereby enhancing the antioxidant capacity of *C. elegans* and effectively extending their lifespan [40, 41]. In experiments with mutant *C. elegans* (*skn-1(zj15)*), HT treatment did not result in significant changes in lifespan, further confirming the similar mechanistic roles of HT and *skn-1* in the oxidative stress pathway.

Furthermore, early administration may activate longevity-related signaling pathways in *C. elegans*, such as the insulin/IGF-1 signaling pathway (IIS) [42], at an earlier stage. The IIS pathway plays a central role in regulating the lifespan of *C. elegans* [43, 44]. HT, acting early on *C. elegans*, may modulate the expression of key genes in the IIS pathway, such as daf-2 and daf-16, promoting the nuclear translocation of daf-16 and regulating the expression of a series of downstream genes [45]. This enhances the antioxidant capacity, stress resistance, and cellular repair abilities of *C. elegans*, leading to a significant extension of lifespan [40]. It's conceivable that the regulation of the oxidative stress pathway by HT may synergize with the IIS pathway to promote lifespan extension, warranting further investigation. Moreover, a link between oxidative stress and AMPK has been established. Hu et al. found that the loss of AMPK activity during oocyte aging results in organelle dysfunction and increased oxidative stress, underscoring a pivotal role of AMPK in preserving oocyte function and delaying the aging process [46]. This finding suggests that multiple signaling pathways in the body are interconnected with oxidative stress, and the extension of lifespan in *C. elegans* may be a result of the collective impacts of these pathways on oxidative stress.

Conclusion

HT significantly extends lifespan when administrated on the early stages of adulthood in *C. elegans,* which works out in a way in line with the oxidative stress pathway akin to those of *skn-1*, thereby delaying aging and enhancing functional performance. Our findings highlight the importance of timing for anti-aging intervening, setting a theoretical basis for developing natural polyphenol-based anti-aging and antioxidant therapies.

Abbreviations

C. elegans	*Caenorhabditis elegans*
HT	Hydroxytyrosol
D1	The first day of adulthood
D3	The third day of adulthood
CR	Caloric restriction
NGM	Nematode growth medium
ROS	Reactive oxygen species
SOD	Superoxide dismutase
CAT	Catalase
IIS	Insulin/IGF-1 signaling pathway

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Author contributions

Project supervision and concept: QS; data collection and analysis: ND and LS with help from RY, KL, ZN and ZZ; manuscript: ND and QS; All authors reviewed and approved the manuscript.

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Availability of data and materials

Data supporting findings reported in this study are available in the supplementary materials.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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