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Novel approach to aging well and longevity

~balancing mitochondrial activation and antioxidant effects~

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1. Introduction

The global population aging is accelerating at an unprecedented rate. Aging has profound effects on economies, healthcare systems, and demographic structures, making the promotion of healthy aging a major global challenge [1]. By 2030, one in six individuals worldwide is projected to be aged ≥ 60 years. The population of those aged ≥ 60 years is expected to grow from 1 billion in 2020 to 1.4 billion by 2030, and double to 2.1 billion by 2050. Furthermore, the number of individuals aged ≥ 80 years is anticipated to triple between 2020 and 2050, reaching 426 million [2].

Aging is not solely a biological process; it is also intricately linked to social and behavioural factors. The observable indicators typically associated with aging include changes in hair colour and patterning, skin characteristics, body morphology, movement, clothing style, hairstyle, and makeup preferences. Such visible manifestations of aging may indirectly affect health outcomes and overall well-being. As aging is an inevitable process, the following question arises: Is it possible to delay the progression of aging?

Addressing this issue requires a comprehensive understanding of the mechanisms underlying aging. However, aging remains an exceptionally complex biological phenomenon and is one of the greatest unsolved challenges in the natural sciences. The nematode *Caenorhabditis elegans* (*C. elegans*) possesses homologues of approximately two-thirds of all human disease-associated genes. Owing Due to its well-characterised physiological aging features and experimental advantages, *C. elegans* has been widely recognised in recent decades as a valuable model organism for studying aging, age-related diseases, mechanisms of longevity, and drug screening [3]. Unlike cell-based models, *C. elegans* enables the investigation of aging at the entire organism level, facilitating the study of systemic interactions among diverse tissues, including the nervous, muscular, and digestive systems.

Mitochondria are essential cellular organelles that play a pivotal role in the aging process. They produce adenosine triphosphate (ATP) through oxidative phosphorylation, while concurrently generating reactive oxygen species (ROS) as by products. Interestingly, a mild reduction in mitochondrial activity has been shown to extend the lifespan of *C. elegans* [4]. Conversely, mitochondrial dysfunction is associated with several various age-related diseases [5]. This paradox has fuelled significant debate in the field of aging research.

This study proposes a novel approach to suppress aging by simultaneously enhancing mitochondrial activity and reducing ROS levels through the application of 5-hydroxy-4-phenylbutenolide (5H4PB), a compound found in aromatic vinegar [6].

2. Materials and Methods

2-1. Materials

5H4PB was obtained from a batch purified samples in our laboratory. In this study, 5H4PB was dissolved in dimethyl sulfoxide (DMSO) and used.

2-2. *C. elegans* culture conditions

Caenorhabditis elegans N2 Bristol wild-type and mutant strains, including DH26 (*rrf-3(b26)*) and QV225 (skn-1*(zj15)*), were obtained from the *Caenorhabditis* Genetics Center (St. Paul, MN, USA). Worms were routinely maintained on NGM agar using standard techniques and seeded with OP50, an internationally established feed [7]. Eggs were extracted from egg-bearing worms in a sodium hypochlorite sodium hydroxide solution and transferred onto NGM plates seeded with OP50. Synchronised L1 worms were grown at a restricted temperature (20 °C) to obtain sterile L4/young adult worms.

2-3. *C. elegans* life span and killing assays

For life span and killing assay experiments, the DH26 (*rrf-3(b26)*) mutants were used because they are unable to produce progeny at 25 °C without alteration in the *C. elegans* phenotype [8]. Synchronised L1 larvae were fed OP50 and reared into young adults. The resultant synchronised hermaphrodites were transferred to NGM plates containing 0, 100, and 500 μM of 5H4PB (15 worms per 35 mm plate). 5H4PB was dissolved in DMSO to a final concentration of 0.1%. After the plates were incubated, the number of live or dead worms was scored every 2 days. Worms were inferred as “dead” when they failed to respond to a gentle touch with a worm picker. Worms that crawled off the plate and underwent non-natural death, such as internal hatching or adherence to the plate wall, were regarded as lost and excluded from the analysis. More than 65 worms from each group were used for the longevity assay. The experimental procedures were repeated at least three times to ensure the robustness and reliability of the results.

2-4. Locomotion assays

5-day-old young adult N2 Bristol wild-type worms were placed on NGM plates seeded with different 5H4PB concentration treatments until they developed into 10-day-old adult worms at 20°C. The locomotor performance of the worms was assessed by measuring the number of body bends. The bending rate was measured by placing live animals on a plate containing S-basal buffer, allowing them to recover for 2 min and counting the number of bends for 1 min. Each strain was tested at least three times.

2-5. RNA sequencing and data analysis

2-5-1. Sample Preparation

Approximately 2000 worms were used for RNA sequencing. Five-day-old young adult DH26 (*rrf-3(b26)*) mutant worms were transferred to NGM plates with or without 500 μM 5H4PB until they developed into 6-day-old adult worms. Six-day-old worms were collected and washed three times with S-basal buffer.

2-5-2. mRNA sequencing

Total RNA was extracted with TRIzol Reagent (Eugene, OR, USA) according to the manufacturer's protocol and stored at −20 °C. RNA quality was evaluated using a Nano Photometer spectrophotometer (IMPLEN, Munich, Germany), and RNA integrity was characterised using a Bioanalyzer 2100 (Agilent, Santa Clara, CA). All the RNAs were confirmed to be intact. Libraries for mRNA sequencing were created using the NEBNext Ultra II RNA Library Prep Kit for Illumina and NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, MA, USA) according to the manufacturer's instructions. The concentration and size distribution of the libraries were measured using an Agilent DNA 7500 kit (Agilent) on a bioanalyzer. All samples were subjected to analyses using NGS equipment. The libraries were pooled, and the concentrations were adjusted to 10 nM. The pooled libraries were denatured and neutralized by with further dilution. Subsequently, the libraries were subjected to an NGS run on a NovaSeq X Plus System (Illumina, San Diego, USA) with a 10 B Reagent Kit (300 cycles) (Illumina). The sequencing was performed using paired-end reads of 150 bases. After the sequencing run, FASTQ files were exported, and basic information of the NGS run data was checked for quality control using the CLC Genomics Workbench software (version 24.0; CLC, Qiagen, Venlo, The Netherlands). The results showed that 99.83% of all reads had a PHRED score > 20, confirming successful data acquisition in the NGS run.

2-5-3. Bioinformatics analysis

Bioinformatics analysis was performed using the CLC Genomics Workbench software, which proposed statistical analysis, obtaining quantification values, and creating heatmaps, followed by mapping to the reference sequence of *C. elegans* (WBcel235) with general transfer format (GTF) file. Second, the data were analysed using Microsoft Excel (Office 2019, Microsoft) to extract the target genes. A Venn diagram was created using Venny software (version 2.1.0; https://bioinfogp.cnb.csic.es/tools/venny/), as a web tool. Gene enrichment and gene ontology analyses were performed using Metascape [9] as a web tool by applying the gene lists filtered by each threshold.

2-6. Thermorecovery assay

To assess thermal shock stress resistance, 5-day-old young adult N2 Bristol wild-type worms were placed on NGM plates seeded with different concentrations of 5H4PB until they developed to 10-day-old adult worms. Ten-day-old worms were transferred to new treatment free NGM plates and incubated at 35 °C for 6 h. Thereafter, the worms were fed at 20 °C for an additional 24 h and the number of worms which are dead and alive were counted. Each strain was tested at least three times.

2-7. Stress responses for Paraquat

One hundred L1 N2 Bristol wild-type worms were placed on NGM plates containing paraquat 0.4 mM and different concentrations of 5H4PB for 4 days. The percentage of worms that reached adulthood was expressed as the survival rate. Each strain was tested at least three times.

2-8. Lipofuscin accumulation in the living worms

Five-day-old young adult N2 Bristol wild-type worms were placed on NGM plates seeded with different concentrations of 5H4PB until they developed into 15-day-old adult worms. Randomly selected worms were washed with S-basal buffer for 30 min and placed on a 3% agar pad coated with 1 M sodium azide to anaesthetise the worms. Lipofuscin autofluorescence images were obtained detected with excitation at 340–-380 nm and emission at 425 nm using a LEICA imaging system (DM4 B; Leica Microsystems Wetzlar, Germany). Lipofuscin-positive areas were quantified by densitometry using the ImageJ software. Each strain was tested at least three times.

2-9. Fluorescent straining of H2O2 in the living worms

Intracellular H_2O_2 levels were assessed in *C. elegans* using the fluorescent probe BES-H_2O_2-Ac (Fujifilm Wako Pure Chemical Corp., Tokyo, Japan). The probe was diluted to 200 μM in S-basal buffer. Synchronized 5-day-old N2 Bristol wild-type worms were treated with varying concentrations of 5H4PB on NGM plates for 15 days. Fifteen worms per condition were incubated in 450 μL of staining solution for 1 h, washed with S-basal buffer (30 min), and anaesthetised with 1 M sodium azide on 3% agar pads. Fluorescence was detected using a LEICA DM4 B microscope (excitation: 460–500 nm; emission: 512–542 nm). Each strain was tested at least three times.

2-10. Metabolites extraction and metabonomic profile of *C. elegans*

Worm samples were prepared as described in Section 2-5-1.

Metabolomics profiling was performed as described previously [10]. The extraction process for low-molecular-weight metabolites involved mixing samples with 250 μL of a methanol-water-chloroform solvent mixture (2.5:1:1, v/v/v) that included 10 μL of an aqueous solution of sinapinic acid (0.5 mg/mL in distilled water; Sigma-Aldrich, Tokyo, Japan) as an internal standard. After 30 min of shaking at 1,200 rpm and 37 ºC, the mixture was centrifuged for 3 min at 4 ºC at 22,000 × g. Subsequently, 200 μL of distilled water was added to 225 μL of the supernatant and placed into a new tube. After centrifuging the mixture once more for 3 min at 4 ºC at 22,000 × g, 250 μL of the supernatant was transferred into a fresh tube and lyophilised using a freeze-dryer. The lyophilised samples were combined with 20 μL of methoxyamine hydrochloride (20 mg/mL in pyridine; Sigma-Aldrich) and shaken at 1,200 rpm for 90 min at 30 ºC to perform oximation. The mixture was then centrifuged at 22,000 × g for 5 min at 4 ºC after 10 μL of N-methyl-N-trimethylsilyl-trifluoroacetamide (GL Science, Tokyo, Japan) was added for derivatisation. The mixture was incubated at 1,200 rpm for 30 min at 37 ºC. The supernatant was analysed using gas chromatography/mass spectrometry (GC/MS). Utilising a GCMS-QP2010 Ultra (Shimadzu, Kyoto, Japan) with a fused silica capillary column (CP-SIL 8 CB low bleed/MS; 30m × 0.25 mm inner diameter, 0.25 μm film thickness; Agilent), GC/MS analysis was performed. The column helium gas flow rate was 39.0 cm/s, and the front inlet temperature was 230 ºC. The column was maintained at 80 ºC for 2 min isothermally, after which temperature was increased to 330 ºC at 15 ºC/min and maintained for 6 min isothermally. The transfer line and ion-source temperatures were 250 ºC and 200 ºC, respectively. After maintaining the column at 80 ºC for 2 min, the temperature was increased to 330 ºC at a rate of 15 ºC/min and maintained for 6 min. The ion-source and transfer line temperatures were 200 ºC and 250 ºC, respectively. Twenty scans per second were acquired, spanning a mass range of 85–500 m/z. The MS-DIAL software [11] was used for peak detection, alignment, and identification. The quantification process involved computing the peak height of each ion, normalising it to the peak height of sinapinic acid as an internal standard, and adjusting the sample weight.

3. Results

3-1. Effect of 5H4PB on the Lifespan and the locomotive capability of *C. elegans*

First, the effects of 5H4PB (0, 100, and 500 µM) on the lifespan of *C. elegans* were examined. As shown in Figure 1a, the mean lifespans of the worms treated with 500 µM of 5H4PB were significantly increased compared with that of the control group. The mean and maximum lifespans of worms treated with 500 µM of 5H4PB were extended 11.4% and 8.0%, respectively. This suggests that 5H4PB may help slow down *C. elegans* aging.

Locomotive capability was assessed as an indicator of muscle function. As shown in Figure 1b, the proportion of worms displaying vigorous locomotion was significantly higher in 5H4PB worms. Locomotion rate is also considered to be highly correlated with longevity. Therefore, this result suggests that 5H4PB may help increase the quality of life by extending the locomotor health span.

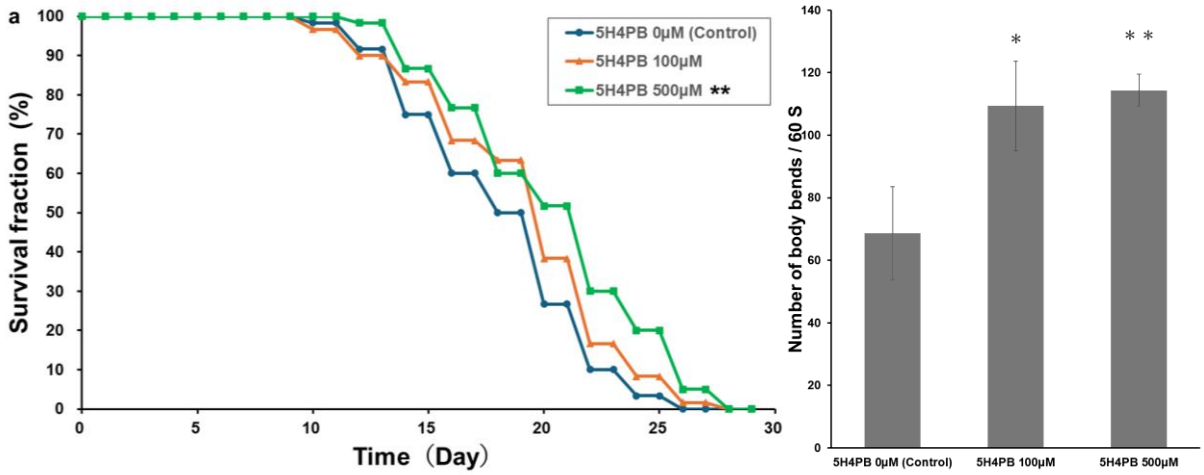

Figure 1. a Effects of 5H4PB on the lifespan of *C. elegans*. **b** Effect of 5H4PB on the locomotor capability. Asterisks indicate significant differences compared to the control treatment at *p ≤ 0.05 and **p ≤ 0.01 according to a two-tailed Student's t-test; mean ± SD, n=90(a) n=15(b)

3-2. Effects of 5H4PB treatment on *C. elegans* transcriptome Differential gene identification

To investigate the effect of 5H4PB treatment on *C. elegans*, RNA-sequencing data were analysed to identify the differentially expressed genes (DEGs) between the treatment groups. Using p-value < 0.05 and fold change |log2FC| ≥ 1.5 as cut-off criteria.

Enrichment of the GO pathway was analysed based on the DEGs of 5H4PB-treated worms, using threshold-corrected p-values of < 0.05. In this study, 240 GO terms were significantly enriched based on DEGs. The results showed that the molecular functions primarily included glucuronosyltransferase, UDP-glycosyltransferase, hexosyltransferase, iron ion binding, glycosyltransferase, and glutathione transferase activities. The expression of genes encoding oxidoreductase activity and heme binding, was also induced. Biological processes mainly included modified amino acid, glutathione, amide, sulphur compound, and diol metabolism. The cellular components primarily included membrane rafts and microdomain (Figure 2a-c).

The KEGG-based pathway analysis using DEG data revealed that the nematode exposed to FG1 was oxidative phosphorylation, drug metabolism, metabolism of xenobiotics by cytochrome P450, retinol metabolism, and longevity regulating pathway (Figure 2d).

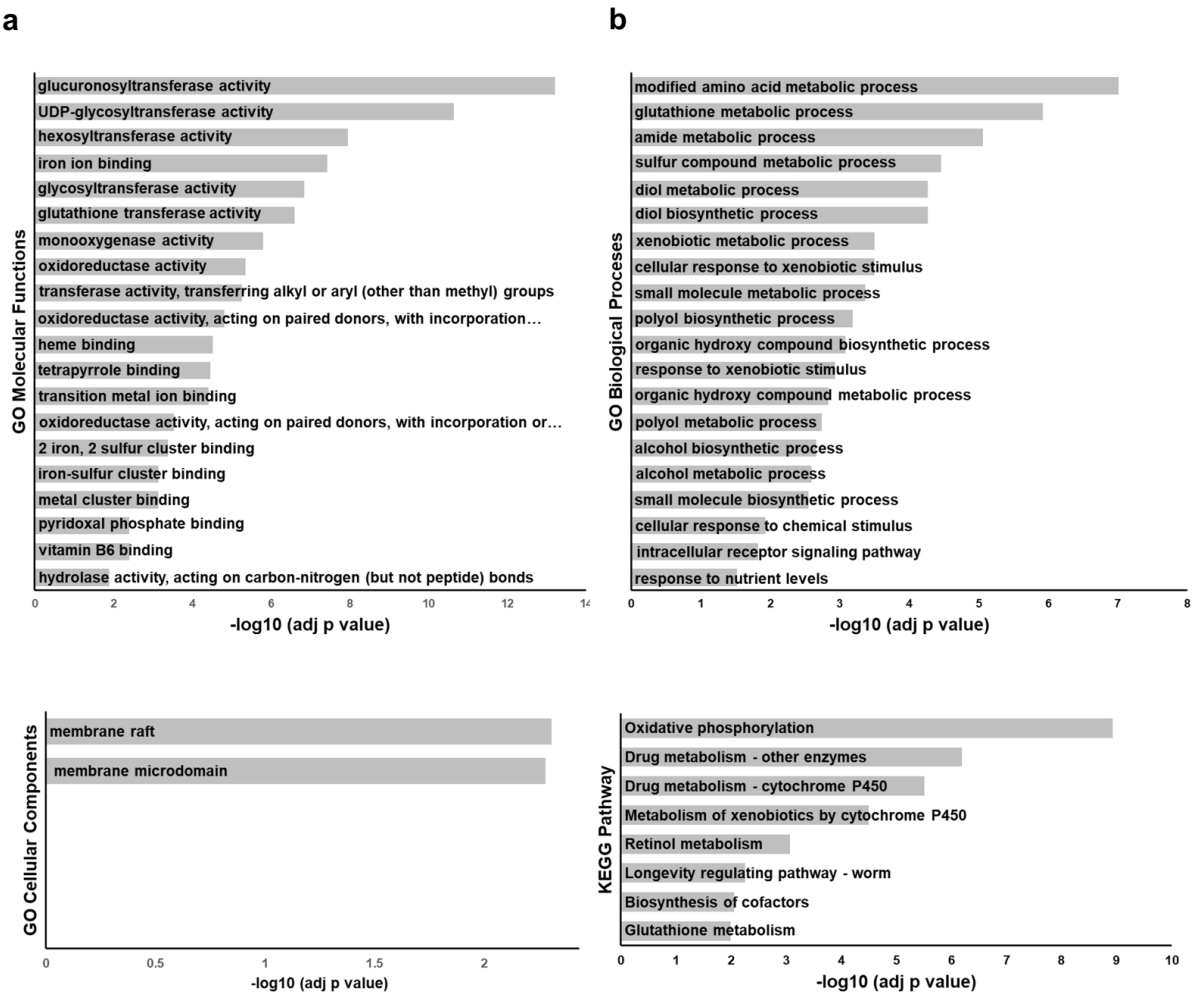

Figure 2. Gene ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis plots of the differentially expressed genes (DEGs) across treatments.

Based on these observations, we classified the gene expression changes in the groups of genes into two types. Type I changes are closely linked to the detoxification genes. Xenobiotic detoxification occurs in three phases: phase I (cytochrome p450 enzymes (CYPs) chemically modify endotoxins), phase II (UDP-glucuronosyl transferases and glutathione S-transferases (GSTs) increase their solubility), and finally, phase III (modified endotoxins are released into the extracellular space by ATP-binding cassette transporters). Type II changes are closely linked to the mitochondria. Mitochondria supply energy to organisms through oxidative phosphorylation, in which iron ions modulate the entire mitochondrial energy metabolism process.

3-3. Effect of 5H4PB on the Redox Active Signalling Pathway

RNA sequencing revealed that 5H4PB treatment modulated the expression of genes associated with UDP-glycosyltransferase, glycosyltransferase, and glutathione transferase activities, suggesting the involvement of a redox-active signalling pathway. In *C. elegans*, this pathway is regulated by SKN-1, an orthologue of human Nrf2 [12]. To assess the role of SKN-1, lifespan assays were conducted using the SKN-1 deficient strain QV225 (skn-1(zj15)). The lifespan extension observed in the N2 wild-type worms treated with 5H4PB was abolished in the SKN-1 deficient strain (Figure 3). This indicates that the prolonged effect of 5H4PB requires functional SKN-1 activity.

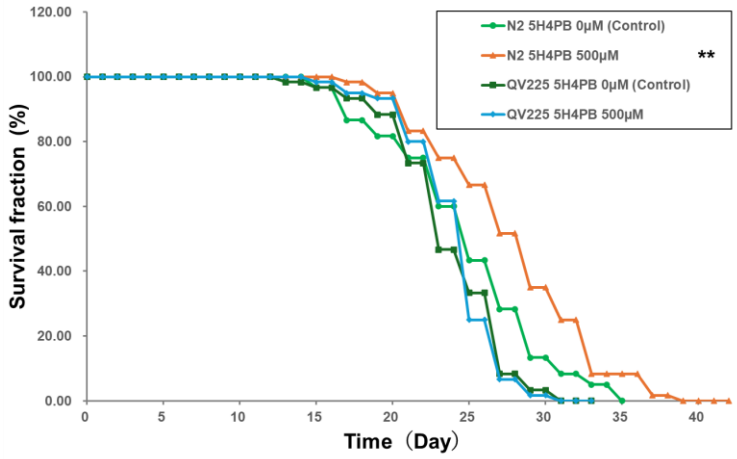

Figure 3. Effects of 5H4PB on the lifespan of *C. elegans* QV225. Asterisks indicate significant differences compared to each control treatment at *p ≤ 0.05 and **p ≤ 0.01 according to a two-tailed Student's t-test, n=90

3-4. Effects of 5H4PB on *in vivo* antioxidant capacity in *C. elegans*

Lipofuscin accumulation and muscle function are established markers of aging in *C. elegans* [13]. Lipofuscin, a lipid peroxidation byproduct, is assessed via autofluorescence and progressively increases slowly during adulthood in the reproductive phase and more rapidly after reproduction. Thus, its accumulation reflects physiological, rather than chronological, aging. In this study, autofluorescence intensity was significantly reduced in worms treated with 100 μM and 500 μM 5H4PB compared to 0 μM controls (Figure 4a).

Associations between ROS levels and the age-related phenotype [14]. When the worms treated with the respective drugs were stained with BES-H_2O_2-Ac as a fluorescent probe for detecting H_2O_2, the intestinal tract and its neighbouring tissues were significantly stained in the control worms compared to the 5H4PB-treated worms (Figure 4b).

Therefore, these results suggest that 5H4PB may enhance *in vivo* antioxidant capacity.

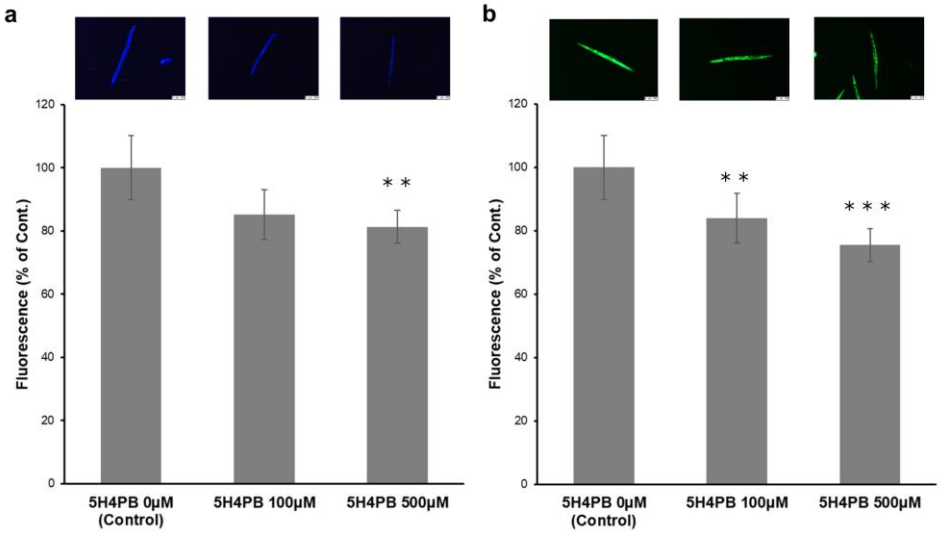

Figure 4. a Effects of 5H4PB on lipofuscin accumulation **b** Effects of 5H4PB on H_2O_2 accumulation. Asterisks indicate significant differences compared to the control at *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 according to a two-tailed Student's t-test; mean ± SD, n=15

3-5. Stress responses for exogenous stimulus

Enhanced stress resistance extends the lifespan of *C. elegans* [15]. Accordingly, we conducted stress resistance assays using various stressors. Heat stress, which induces mitochondrial damage via ROS accumulation, was mitigated by 5H4PB, with the highest survival rate observed at 500 μM, indicating its protective effect. Paraquat, a free radical generator that induces oxidative stress, disrupts cellular structures, and impairs nematode lifespan and respiration. Paraquat resistance was assessed by measuring the developmental success of paraquat. The proportion of normally developed individuals was higher in *C. elegans* treated with 5H4PB than in the untreated controls, suggesting enhanced oxidative stress resistance.

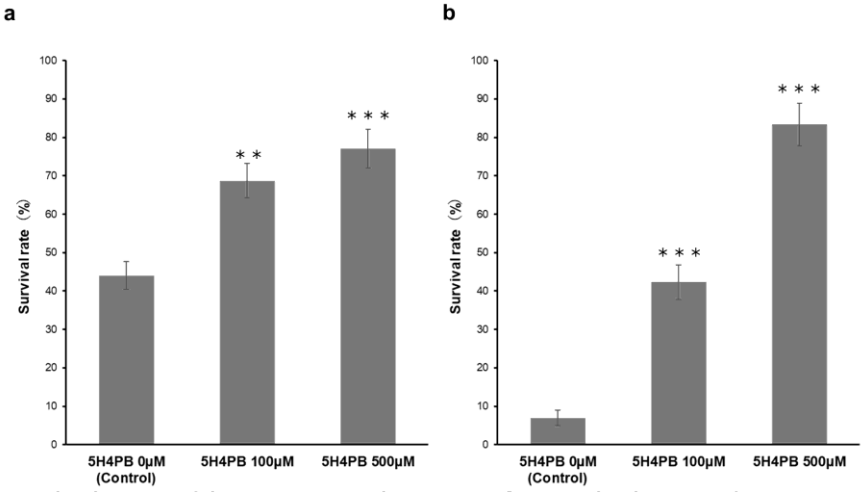

Figure 5. a Survival rate of heat-stressed worms. **b** Survival rate of H_2O_2-stressed worms. Asterisks indicate significant differences compared to the control at *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 according to a two-tailed Student's t-test; mean ± SD, N=3.

3-6. Effects of 5H4PB treatment on *C. elegans* metabolic profiles

To further investigate the biological effects of 5H4PB on metabolism, we performed a global metabolomics analysis. We quantified 33 known metabolites extracted from the control and 5H4PB-treated worms. Metabolic pathway analysis of significantly upregulated metabolites ($p < 0.05$) identified the TCA cycle intermediates representing a higher flux and activation of mitochondria as the enriched pathway (Fig. 6). These findings suggest an enhanced mitochondrial flux and activation. In addition, the glutathione system and the related thiol network were identified. These findings suggest an enhanced antioxidant defense system.

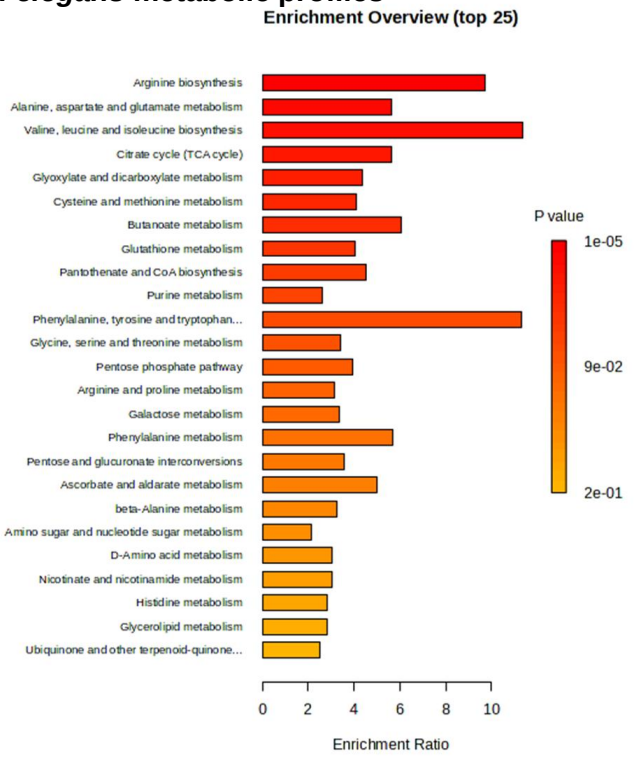

Figure 6. Metabolite set enrichment analysis reveals.

4. Discussion

4-1. 5H4PB exert anti-aging effects by increasing the antioxidant activity *in vivo*

This study demonstrates that 5-hydroxy-4-phenylbutenolide (5H4PB), isolated from aromatic vinegar, extends the lifespan of *C. elegans* and preserves its locomotor activity. 5H4PB treatment activated SKN-1 downstream genes, whereas lifespan extension was abolished in the SKN-1 mutants, highlighting the critical role of SKN-1 in regulating detoxification, metabolism, and immune responses. Consistent with previous findings linking SKN-1 activation to antioxidant gene expression, the 5H4PB treatment significantly upregulated detoxification enzymes such as UDP-glycosyltransferases and glutathione S-transferases. Importantly, 5H4PB lacks direct radical-scavenging activity, as demonstrated through experiments measuring the consumption of fluorescein in the presence of AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride, a peroxyl radical generator) [16]. These findings clearly indicate that 5H4PB antioxidant effects are primarily mediated through the enhancement of endogenous antioxidant systems rather than through direct ROS neutralisation.

The free radical theory posits that accumulated ROS disrupt redox homeostasis and promote oxidative stress and aging [17]. Stress responsive pathways in *C. elegans* are crucial for lifespan regulation, and enhanced stress resistance is associated with longevity. In this study, 5H4PB treatment suppressed the accumulation of ROS and lipofuscin *in vivo* and improved heat shock and oxidative stress tolerance. Furthermore, we previously reported that pretreatment with 5H4PB exerts a marked cytoprotective effect against H_2O_2-induced cell death in mouse cultured fibroblasts and primary cultured hepatocytes [16].

These results suggest that 5H4PB may exert anti-aging effects by enhancing *in vivo* antioxidant capacity.

4-2. Role of Mitochondria in Lifespan Determination

Another notable finding was the potential enhancement of mitochondrial function following 5H4PB intake. The mitochondria are critical for cellular metabolism, apoptosis, and ROS production. The energy requirements of cells are met by mitochondrial respiration, an ATP-generating process driven by a series of protein complexes collectively known as the electron transport chain (ETC), which is located on the inner membrane of the mitochondria. However, ROS, such as superoxide, singlet oxygen, and peroxides, are inevitably produced during respiration and disrupt macromolecular and cellular structures. The oxidative damage caused by mitochondrial ROS production has been established as the molecular basis of multiple pathophysiological conditions, including aging and cancer. In *C. elegans*, inhibition of the ETC reduces ATP production and extends lifespan [4]; however, mitochondrial dysfunction is also implicated in aging and disease [5], suggesting contradictory roles for mitochondrial activity in lifespan regulation.

In this study, 5H4PB administration extended the lifespan of the worms, implying that maintaining ATP levels alongside protection against ROS is crucial. Thus, aging may be modulated by balancing mitochondrial function and antioxidant capacity.

4-3. Anti-Aging and Protective Cosmetic Applications

The skin is a high-turnover organ and its constant renewal depends on the rapid proliferation of progenitor cells. With increasing evidence supporting the close association between mitochondria and skin health, their therapeutic targeting in the skin, either via an ATP production boost or free radical scavenging has gained attention from clinicians and aestheticians. Collectively, these results support the notion that 5H4PB, through its role in

maintaining the equilibrium between mitochondrial function and antioxidant defences, has significant potential as an anti-aging agent for the skin.

5. Conclusion

Our study revealed that 5H4PB enhanced mitochondrial function while simultaneously attenuating ROS levels through indirect antioxidant pathways. These dual actions synergistically promote both lifespan and health, highlighting a potential therapeutic strategy for aging that harmonises mitochondrial activation with the regulation of oxidative stress.

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