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Original Article

Senolytics Enhance the Longevity of *Caenorhabditis elegans* by Altering Betaine Metabolism

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Abstract

Aging triggers physiological changes in organisms that are tightly linked to metabolic changes. Senolytics targeting many fundamental aging processes are currently being developed. However, the host metabolic response to natural senescence and the molecular mechanism underlying the antiaging benefits of senolytics remain poorly understood. In this study, we investigated metabolic changes during natural senescence based on the *Caenorhabditis elegans* model and pinpointed potential biomarkers linked to the benefits of senolytics. These results suggest that age-dependent metabolic changes during natural aging occur in *C elegans*. Betaine was identified as a crucial metabolite in the natural aging process. We explored the metabolic effects of aging interventions by administering 3 antiaging drugs—metformin, quercetin, and minocycline—to nematodes. Notably, betaine expression significantly increased under the 3 antiaging drug treatments. Our findings demonstrated that betaine supplementation extends lifespan, primarily through pathways associated with the forkhead box transcription factor (FoxO) signaling pathway, the p38-mitogen-activated protein kinase (MAPK) signaling pathway, autophagy, the longevity regulating pathway, and the target of rapamycin (mTOR) signaling pathway. In addition, autophagy and free radicals are altered in betaine-treated nematodes. Overall, we found that betaine is a critical metabolite during natural aging and that senolytics extend the lifespan of nematodes by increasing betaine levels and promoting autophagy and antioxidant activity. This finding suggests that betaine could be a novel therapeutic target for promoting longevity.

Keywords: Autophagy, Biomarker, Metabolism, Oxidative stress

The intricate pathophysiology of aging presents a formidable challenge for mitigating age-related mortality and morbidity (1). The aging process is associated with physiological and molecular degeneration, including reduced tissue elasticity, impaired immune function, reduced infection resistance, and diminished metabolic homeostasis capacity (2). Over the past decade, research has extensively explored how host metabolism affects aging. Studies have revealed that metabolic dysbiosis may contribute to the development of aging (3). Although conclusive evidence of a causal link between metabolic dysbiosis and aging is still lacking, research indicates that therapeutic strategies such as exercise, diet control, and senolytic treatment may confer antiaging benefits through altering metabolic profiles (4,5).

The nematode *Caenorhabditis elegans* (*C elegans*), which has a short and easily monitored lifespan (3 weeks), is a powerful model for aging research. Although *C elegans* lacks traditional senescent cells, it still experiences age-related changes at the tissue and cellular levels. Drugs targeting cellular senescence in *C elegans* likely affect specific tissues by modulating conserved signaling pathways to promote healthier aging and extend lifespan (6). Senolytics, a class of drugs designed to eliminate senescent cells, hold promise for promoting antiaging. Drugs such as minocycline, metformin, rapamycin, aspirin, and quercetin, initially prescribed for treating other diseases by modulating host metabolism (7–11), have recently been reported to be effective interventions for counteracting the aging process. Minocycline, a widely used tetracycline analogue for treating acne vulgaris, was reported to have excellent anti-inflammatory effects and attenuate the aggregation of proteins (12). Metformin, a drug extensively used to treat type 2 diabetes, has been suggested to increase lifespan and delay age-related diseases (13). Additionally, quercetin affects glucose and lipid metabolism in organisms and may regulate the p38-mitogen-activated protein kinase (MAPK) pathway and the insulin-like signaling pathway to extend the lifespan

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of nematodes (14). However, the absence of a comprehensive characterization of aging through omics techniques and the limited efficacy of existing senolytic targets hinder their practical clinical application. Therefore, it is imperative to investigate how host metabolism responds to natural senescence and elucidate the relationship between metabolic alterations and the antiaging effects of senolytics.

Aging can be categorized into 2 scenarios: aging without external interference and aging with external intervention. The term "natural aging" refers to the aging process observed in organisms under normal environmental conditions, without any experimental interventions such as drugs. Therefore, first, this study aimed to identify alterations in metabolic profile during natural senescence using the *C elegans* model. Second, we investigated potential biomarkers associated with the aging process. Additionally, the study explored the mechanisms underlying the longevity-enhancing effects of senolytics, including minocycline, metformin, and quercetin, intending to identify specific metabolic targets that could be harnessed to slow the aging process.

Method

Chemical Reagents

Dimethyl sulfoxide (DMSO, AR, MACKLIN Co., Ltd., Shanghai, China), 1,1-dimethylbiguanide hydrochloride (metformin, purity ≥97.0%, Sangon Biotech Co., Ltd., Shanghai, China), quercetin (purity = 98.02%, MedChemExpress, Monmouth Junction, NJ), and minocycline (purity = 99.79%, MedChemExpress) were used.

Participants

Fasting serum samples were collected from 100 individuals (male/female, 57/43) aged 23 to 77 years. Written informed consent was obtained from all individuals.

Culture Conditions and Strains

The strains were cultured on nematode growth medium (NGM) agar plates supplemented with *Escherichia coli* (*E coli*) OP50 at 20°C as previously described (15). Adult hermaphrodite nematodes were collected from the plates into 15 mL tubes, lysed with a fresh bleaching mixture (0.5 mol/L NaOH, 2% NaClO) and washed 2 times with K media (32 mM KCl and 51 mM NaCl) (14). Nematodes at the L1 stage (12 hours after synchronization) were subsequently grown in drug-free S media. The S media contained 44 mM KH_2PO_4, 100 mM NaCl, 5.74 mM K_2HPO_4, 3 μM $MgSO_4$, 10 μM potassium citrate, 3 μM $CaCl_2$, some trace metals, and 1.29 μM cholesterol. An appropriate amount of *E coli* was added to the S media, and the nematodes were cultured until they reached day 1 (the first day of nematode adulthood, 96 hours after synchronization). The experiment required nematodes that were selected on day 1 and then cultured selectively in 6-cm Petri dishes (2 mL medium), 6-well plates (1 mL medium), or 96-well plates (200 μL medium) at a temperature of 20°C in an incubator. The medium and food were changed every 2 days. The number of nematodes required for the experiment was counted to determine the concentration of nematodes per unit volume. Due to the 3-day reproductive cycle of nematodes and to minimize metabolic disturbances caused by egg laying, during the entire growth of *C elegans*, 5-fluoro-deoxyuridine (5-FUDR) was used to control the reproduction of nematodes to reduce the impact on offspring.

Drug Exposure

The nematodes were exposed to 50 mM metformin, 100 μM quercetin, 100 μM minocycline, or single betaine (10 μM, 100 μM, 500 μM, 1 mM, 2 mM, 10 mM, or 50 mM) from young adults (day 1) until the assay endpoints were reached in 6 cm dishes at 20°C with food of live OP50, with 10 000 worms per dish. All groups were analyzed in 5 independent experiments in triplicate. During exposure, nematodes were cultured in sterile S media (44 mM KH_2PO_4, 100 mM NaCl, 5.74 mM K_2HPO_4, 3 μM $MgSO_4$, 10 μM potassium citrate, 3 μM $CaCl_2$, some trace metals, and 1.29 μM cholesterol). Test solutions were prepared by dissolving different amounts of antiaging drugs in DMSO, with a final working concentration of DMSO of no more than 1%. Additionally, 5-FUDR was introduced during the entirety of the nematode drug exposure.

Intestinal Lipofuscin Levels

After continuous exposure to the drug for 4 or 10 days, the nematodes were placed on 2% agar pads and anesthetized with levamisole to visualize intestinal autofluorescence. According to previous experiments, fluorescence images of the endogenous intestine were collected using a 525 nm bandpass filter (the central wavelength [CWL] was 525 nm, and the width at half maximum [FWHM] was 50 nm) without automatic gain control to maintain the relative intensity of fluorescence across different animals (16). Images were collected using a 525 nm bandpass filter (the CWL was 525 nm, and the FWHM was 50 nm) at a constant exposure time in order to obtain the relative intensity of the fluorescence from different nematodes. Images were taken with an Axio Imager M2 microscope (Zeiss, Oberkochen, Germany) at 100× magnification. ZEN 2 Pro software was used to measure lipofuscin levels. This assay was performed in triplicate, and 20 nematodes were necessary per treatment.

Lifespan Assay

Lifespan studies were performed at 20°C in the presence of fluorodeoxyuridine (17). Nematodes on day 1 were exposed to different antiaging drugs in 96-well plates with a liquid S-medium (200 μL) culture system. The medium was refreshed every 2 days. We recorded the number of live, missing, and dead nematodes daily under a dissecting microscope (Olympus, Tokyo, Japan) and selected the dead worms. Survival curves were generated to calculate the percentage of live nematodes in each group over time. There were 6 parallel groups, and each group had 20 nematodes.

Locomotion Behavior Assay

The movement speed of the nematodes was recorded to assess locomotion behavior. A nematode body bend is a change in direction along the y-axis of the posterior bulb of the pharynx, assuming that the nematodes move along the x-axis (18). After gently touching a nematode with platinum wire, its movement was classified as "fast" if it exhibited continuous sinusoidal motion for at least 30 seconds; otherwise, it was classified as "slow." After drug exposure in S-medium, the nematodes were transferred to a new plate without food, and then 100 μL of M9 buffer (1 mM $MgSO_4$, 5 mM KH_2PO_4, 20 mM Na_2HPO_4, and 20 mM NaCl) was added to the agar. The movement speed was recorded for 1 minute. Twenty nematodes were measured in each treatment, and assays were conducted in triplicate.

RNA Extraction and Real-Time Quantitative PCR

On day 1, a nematode group was selected and cultured in 6-well plates. Each well contained 6 000 nematodes and 1 mL of medium containing antiaging drugs. The medium was refreshed every 2 days for 10 days. Worms treated with drugs were collected with M9. Total mRNA was extracted with TRIzol reagent. Amplified cDNA was prepared from approximately 1 000 ng of RNA using HiScript II Q RT SuperMix for qPCR (Vazyme, Nanjing, China). SYBR Green real-time PCR experiments were performed using AriaMx Real-Time PCR (Agilent Technologies, Santa Clara, CA) and Taq Pro Universal SYBR qPCR Master Mix (Vazyme). Relative gene expression was normalized to tba-1 mRNA levels. The comparative ΔΔCt method was used to calculate the fold changes in gene expression. The primers used are listed in Supplementary Table 4. Each experiment was repeated 3 times, and biological replicate samples were used.

Observations of Autophagy

Nematodes on day 1 were cultured in 6-well plates. Three sets of parallel controls were established. Each well contained 4 000 nematodes and 1 mL of medium containing antiaging drugs. The incubation period was 10 days, and the medium was refreshed every 2 days. After drug exposure, the worms were stored in a fixative of 4% glutaraldehyde. After overnight fixation, the nematodes were embedded in agar. Further processing included postfixation in 2% OsO_4 and 1.5% potassium ferrocyanide and dehydration in an ascending ethanol series followed by acetone and propylene oxide. The samples were embedded in pure epoxy resin 618. Sections were stained with uranyl acetate and examined with a Tecnai Spirit electron microscope (FEI, Tecnai G2, F20 200 KV, Hillsboro, OR), which the Public Technology Service Center, Fujian Medical University provided.

Measurement of ROS

The levels of reactive oxygen species (ROS) in worms were determined by a commercial kit (Beyotime Biotechnology, Shanghai, China). 2′,7′-Dichlorofluorescein-diacetate (DCFH-DA) is effortlessly oxidized to fluorescent dichlorofluorescein (DCF) under the action of intracellular ROS; thus, the levels of ROS were quantified. Accordingly, the worms were cultured in 24-well plates and exposed to 2 mM betaine for 10 days. After treatment, the worms were incubated with 10 μM DCFH-DA for 2 hours at 20°C in the dark and washed twice with M9 buffer. Worms were placed on 2% agar pads and anesthetized with levamisole to visualize internal ROS. Using a 488 nm excitation wavelength and 525 nm emission filter, we imaged the sample with a laser scanning confocal microscope (Leica, TSC SP8, Germany) under 100× magnification. Images were analyzed by ZEN 2 Pro software. The intensity of fluorescent ROS in the intestines was measured and is expressed as relative fluorescent units. Twenty nematodes were measured in each treatment, and the assays were performed in triplicate.

Assays of Antioxidant Enzyme Activity

On day 1, nematodes were cultured in 6-well plates containing 6 000 worms per well in 1 mL of medium. The medium was renewed every 2 days. Samples were collected after betaine (2 mM) treatment for 10 days. The worms in the 2 groups (DMSO and betaine) were homogenized in cold normal saline to determine the antioxidant enzyme activity of T-SOD via commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The xanthine/xanthine oxidase method based on the production of O^{2-} anions was used to assay T-SOD activity by ELISA (BioTek Instruments, Winooski, VT, λ = 450 nm). T-SOD activity is shown as units per milligram of protein (U/mg protein).

Metabolite Extraction

Day 1 stage nematodes were selected and cultured in 6-cm petri dishes, and the number of nematodes in each dish was 10 000. The volume of medium in each dish was 2 mL, and the medium was changed every 2 days. Ten days later, the nematodes and medium were transferred to a centrifuge tube, the medium was removed by centrifugation, and the nematodes were preserved. Until analysis, frozen *C elegans* samples or serum were stored at −80°C in liquid nitrogen. Serum sample preparation was conducted as described previously (3). Nematodes (day 10) were selected for analysis, and 7 000 worms were used for NMR metabolomics. The worm samples were transferred to a precelly (containing 1.4 mm ceramic spheres, MP Biomedicals LLC, Santa Ana, CA, USA) to obtain metabolites. Then 600 μL of ice-cold methanol and ddH_2O (2:1) were added to each tube for homogenization by a Tissuelyser-24 (Shanghai Jingxin Industrial Development, China). Using a centrifuge, 12 000 rpm was applied for 30 minutes at 4°C to obtain the supernatant and then evaporated to obtain a dry metabolite pellet (Eppendorf Concentrator plus, Hamburg, Germany). Samples were redissolved in 500 μL of NMR buffer for the NMR experiments as described (19).

Data Acquisition

A Bruker Avance III HD 600-MHz NMR spectrometer equipped with a TXI probe head was used for NMR metabolic profiling and analysis. With presaturation, ^{1}H ^{1}D NMR spectra were acquired using Carr–Purcell–Meiboom–Gill (CPMG) pulse sequences (cpmgpr1d, 128 scans, 73 728 points in F1, 12019.230 Hz spectral width, 1 024 transients, recycle delay 4 s). NMR spectral data were processed as previously described (20). Bruker Topspin version 4.0.2 processed the data by multiplying the free induction decay by an exponential window, performing Fourier transformations, and calculating phase differences. MATLAB2014a and Chenomx NMR suite 8.4 were used to identify the metabolite tissue. Integrations were used to generate the orthogonal partial least squares discriminant analysis (OPLS-DA), permutation analysis, metabolite enrichment analysis, and heatmap using MetaboAnalyst 5.0. Q^2 is a quality assessment statistic that verifies the statistical significance of the identified differences.

Statistical Analysis

GraphPad Prism was used for the univariate statistical analysis (GraphPad Software, La Jolla, CA). Data are represented as the mean ± standard deviation (*SD*). R framework was performed in the analysis of RNA-seq, and a 2-tailed Student's *t* test was used to evaluate statistical significance. *p* values were calculated using a 2-tailed Student's *t* test when comparing variables pairwise. Metabolites with $p < .05$ are shown in each Supplementary Figure. *p* values of < .05 indicate statistical differences among multiple groups (1-way ANOVA) (*), < .01 (**), < .001 (***), or < .0001 (****).

Results

Age-Dependent Metabolic Changes Associated With Natural Aging in *C elegans*

Dysregulation of metabolism is a hallmark of aging. We employed ^{1}H NMR-based metabolomics to analyze the metabolic changes in nematodes throughout their natural aging process. Multivariate statistical analysis was used to assess the alterations in the metabolic profile of *C elegans* as they aged. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) of the nematodes revealed that the metabolic fingerprints of the nematodes changed with increasing age (Figure 1A). When comparing the differences in the metabolic fingerprints between nematodes of different ages, OPLS-DA revealed an increasing correlation coefficients Q^2 of 0.971 ($p = .01$) and an R^2Y of up to 0.998 ($p = .01$; Figure 1B, Supplementary Figure 1, and Supplementary Table 1) with increasing clustering. Given the stochastic nature of nematode aging and the notable onset of mortality after day 10, data points beyond this time point were excluded from the analysis. Nematodes have an approximate lifespan of 3 weeks, with numerous aging-related characteristics, such as decreased pharyngeal pumping rates, mitochondrial fission, and muscle degeneration, becoming pronounced by day 10 (21). The heatmap provided a metabolic overview of the nematodes as they aged (Figure 1C). To accurately identify metabolite alterations while minimizing potential disruptions from egg laying, we selected young adult nematodes (day 4) and older adult nematodes (day 10) as specific time points for our study. Additionally, oviposition inhibitors were administered during drug exposure to prevent egg-laying.

There was a clear distinction between young adults (day 4) and older adults (day 10) in terms of natural aging nematodes, which could be observed in the OPLS-DA plot (Supplementary Figure 2a). The correlation coefficients provided by the permutation test showed a positive Q^2 of 0.985 ($p = .02$) and an R^2Y of up to 0.985 ($p = .02$; Supplementary Figure 2b). The reduced NMR spectra analysis revealed specific changes in metabolites, including increases in leucine, isoleucine, valine, lactic acid, arginine, acetic acid, glutamic acid, dimethylamine, ethanolamine, choline, trimethylamine N-oxide, taurine, myo-inositol, uracil, xanthine, uridine diphosphate-sugars (UDP-sugars), and hypoxanthine and decreases in alanine, citric acid, methionine, trimethylamine, lysine, phosphatidylcholine (PC), betaine, glycerol, deoxycytidine triphosphate, tryptophan, indoxyl sulfate, and adenosine diphosphate (Supplementary Figure 2c and d). We conducted functional analyses to investigate age-related alterations in molecular mechanisms. RNA sequencing (RNA-seq) data were analyzed, and the top 20 enriched pathways were identified (Supplementary Figure 3). Among these pathways, several are related to longevity, such as the MAPK signaling pathway and longevity regulating pathway, which have been proposed to be related to lifespan (22,23). Moreover, we identified critical metabolic pathways, such as autophagy, the forkhead box transcription factor (FoxO) signaling pathway, and the target of rapamycin (mTOR) signaling pathway, which are closely related to physiological activities, including autophagy and oxidative stress (24) (Supplementary Figure 3a and b). Correlation analysis was conducted to identify the metabolites related to aging further. The positive and negative correlation coefficients indicate that metabolite levels increased and decreased with age. Among them, methionine ($R^2 = 0.7533$, $p < .0001$) and betaine ($R^2 = 0.6135$, $p < .0001$) showed the most significant correlations with age (Figure 1D, Supplementary Figure 3d). Methionine restriction is known to enhance longevity in several model organisms of aging (25). The synthesis of methionine requires a methyl donor. As a donor of osmolytes and methyl groups, betaine is cytoprotective and beneficial to human health (26). Our hypothesis suggested that changes in methionine levels could be attributed to the influence of betaine, a compound known to affect an organism's self-repair mechanisms. Our observations revealed a notable decrease in betaine levels as age increased, and a similar pattern was observed in human samples (Figure 1E and F). The betaine concentration in the older adults was much lower than in the young adults (Figure 1F). In previous studies, betaine has been implicated in the aging process. Mohammadi investigated the impact of betaine on the expression of aging markers in ovarian and testicular cells under hyperglycemic conditions (27). Yang demonstrated that betaine attenuated cognitive impairment by suppressing microglial pyroptosis (28). Building on these results, we hypothesized that betaine metabolism exerts a pivotal influence on the aging trajectory of nematodes, given its pronounced decline with age in *C elegans*.

Effects of Senolytics on Nematode Lifespan Extension

We hypothesized that if betaine metabolism deficiency causes aging, drugs that delay aging and have senolytic effects may influence betaine metabolism. Nematodes were divided into 4 groups and cultured with DMSO, minocycline, metformin, or quercetin to characterize the effects of these compounds. The accumulation of lipofuscin, a well-known aging biomarker, was measured to assess the impact of antiaging drugs on delaying the aging process. Compared with those in the DMSO control group (day 10), the relative fluorescence intensities in nematodes exposed to minocycline, metformin, and quercetin (day 10) decreased 0.08-fold ($p < .0001$), 0.31-fold ($p < .0001$), and 0.27-fold ($p < .0001$), respectively (Figure 2A and B). The results suggested that the antiaging drugs slowed the deposition of metabolic waste products and lipid oxidation in nematodes, with minocycline showing the greatest effectiveness. We further measured the survival of the worms in the 4 groups. Under antiaging drug conditions, the lifespan of nematodes is extended to different extents. Compared with those of the control group, the lifespan of the minocycline-fed nematodes increased by 140.5% ($p < .0001$), whereas metformin and quercetin slightly improved the lifespan by 12% ($p < .001$) and 9% ($p < .003$), respectively (Figure 2C, Supplementary Table 2). The results showed that antiaging compounds, including minocycline, metformin, and quercetin, could extend organisms' lifespan. A distinct feature of aging nematodes is decreased mobility and muscle function. Therefore, we assessed alterations in the locomotor capacity of worms after treatment with antiaging drugs. The frequencies of body bending were measured to evaluate the locomotion behavior of the nematodes. Our results showed that the frequency of body bending significantly increased by 20% in the metformin- and quercetin-treated nematodes compared to that in the control nematodes but slowed in the minocycline-fed nematodes (Figure 2D). Moreover, we identified the signaling pathways strongly related to the above drugs utilizing RNA-seq analyses and gene ontology

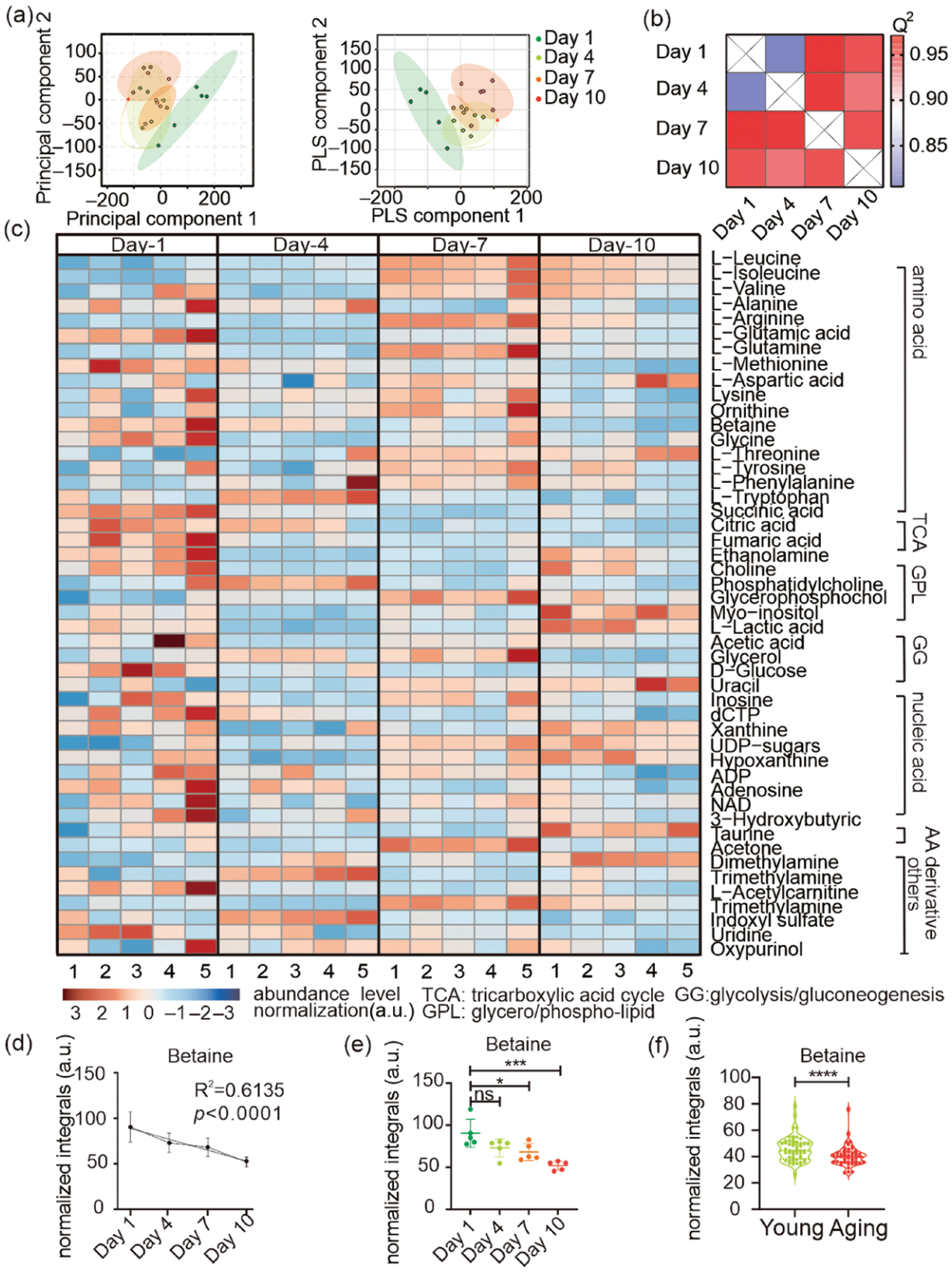

Figure 1. NMR metabolomics analysis of natural aging *Caenorhabditis elegans* samples. (A) PCA and PLS-DA plots of naturally aged *C elegans* samples. The direction of the arrows indicates trends in the metabolism of nematodes from day 1 to day 10. (B) Heatmap showing OPLS-DA-derived Q^2 values for pairwise comparisons of natural aging nematode samples of different ages. (C) Heatmap showing the relative metabolite levels in naturally aged *C elegans* samples by NMR analysis. Each group consisted of 1–5 samples. Each column displays one sample, whereas rows represent relative metabolites. Red and blue represent increases and decreases, respectively. Different classes of metabolites are classified according to their chemical composition and biomolecular nature. (D) Linear variation in betaine concentration in worms under natural aging conditions. (E) Betaine concentration in nematodes under natural aging conditions. (F) The betaine concentration in human serum samples (60 years is the age limit). *p < .05, **p < .01, ***p < .001, or ****p < .001 indicate significant differences from the control group, and ns indicates no significant difference.

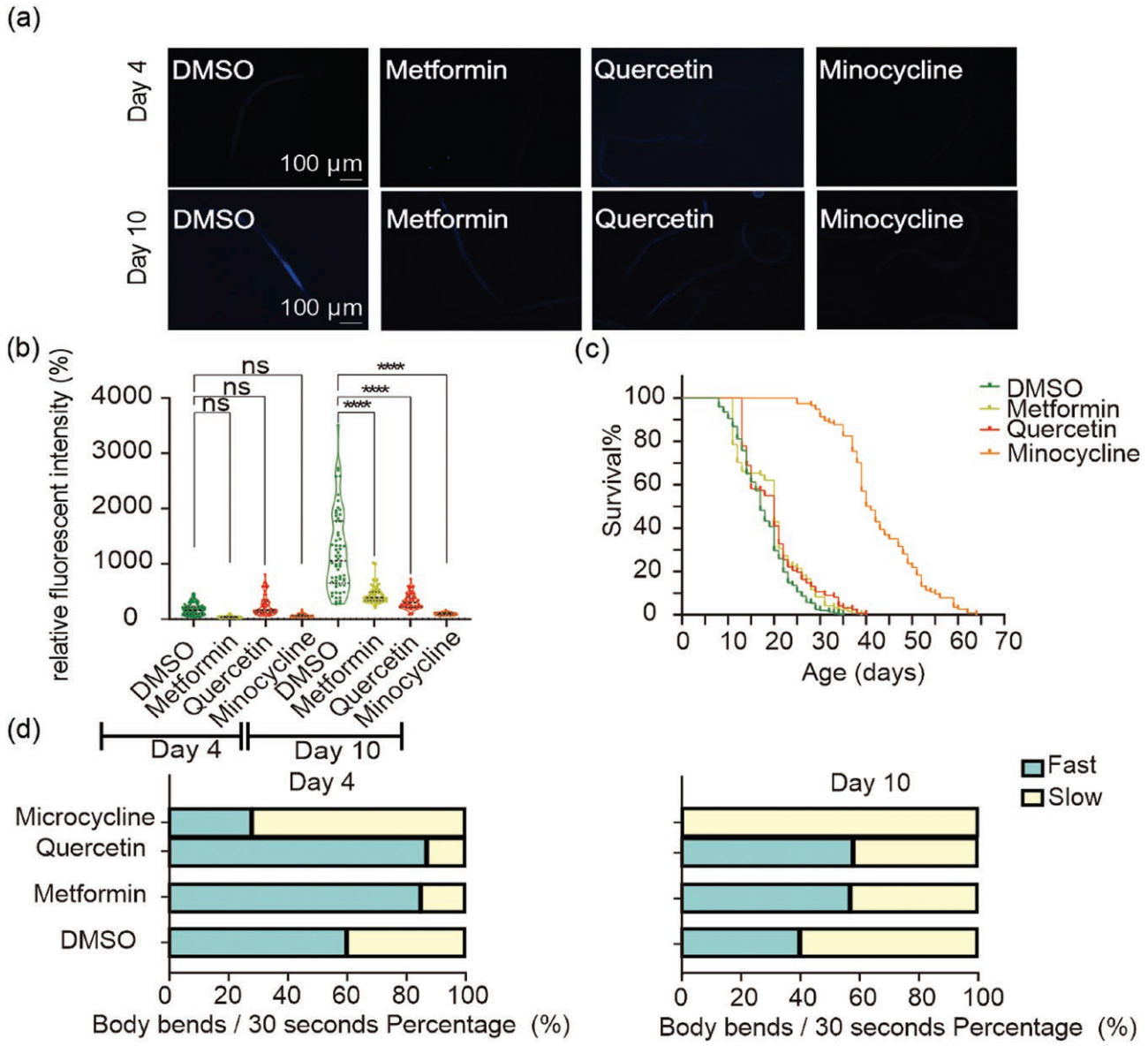

Figure 2. Antiaging compounds have been shown to extend lifespan. (A) Intestinal fluorescence accumulation in young adult (day 4) and older adult (day 10) nematodes treated with DMSO, metformin, minocycline, or quercetin. (B) Quantitation of the intestinal autofluorescence intensity in young adult (day 4) and older adult (day 10) nematodes treated with DMSO, metformin, minocycline, or quercetin. (C) The lifespan of nematodes after treatment with antiaging drugs. The median lifespans in this experiment were as follows: DMSO, 35 days; metformin, 39 days ($p = .003$); quercetin, 40 days ($p = .001$); and minocycline, 64 days ($p < .001$). (D) Effects on body bends of young adult (day 4) and older adult (day 10) nematodes exposed to antiaging compounds.

(GO) enrichment (Supplementary Figure 4a–c). In summary, we confirmed the life-prolonging effects of these 3 antiaging drugs on *C elegans*.

Changes in Metabolites Linked to Therapeutic Benefits

We further focused on the regulatory effect of antiaging drugs on nematode metabolism to uncover the mechanisms underlying lifespan extension. The PCA plot (Figure 3A) shows that the metabolite variation in older nematodes treated with antiaging drugs closely aligns with that observed in young nematode samples, likely due to the regulatory effects of the antiaging drugs. The results indicated that antiaging drugs could intervene in the metabolic profile of aged nematodes and shift their metabolome toward a younger state. OPLS-DA revealed an evident separation between the metabolomes of samples from the older control (day 10) and senolytic groups, indicating that the metabolic profiles of the nematodes were significantly different from those of the older controls (Supplementary Figure 5a–c). Differentially abundant metabolites between the older control (day 10) and senolytic groups were identified. The impact of senolytics on these metabolites is illustrated in Supplementary Figure 5d–i. Our results showed that 16 differentially abundant metabolites were shared across 3 comparisons: minocycline versus older control, metformin versus older control, and quercetin versus older control (Supplementary Figure 5j–l), including citric acid, succinic acid, glycerophosphocholine, betaine, L-alanine, trimethylamine, phosphorylcholine, L-glutamine, ethanolamine, and L-tryptophan. Among them, betaine, also identified as

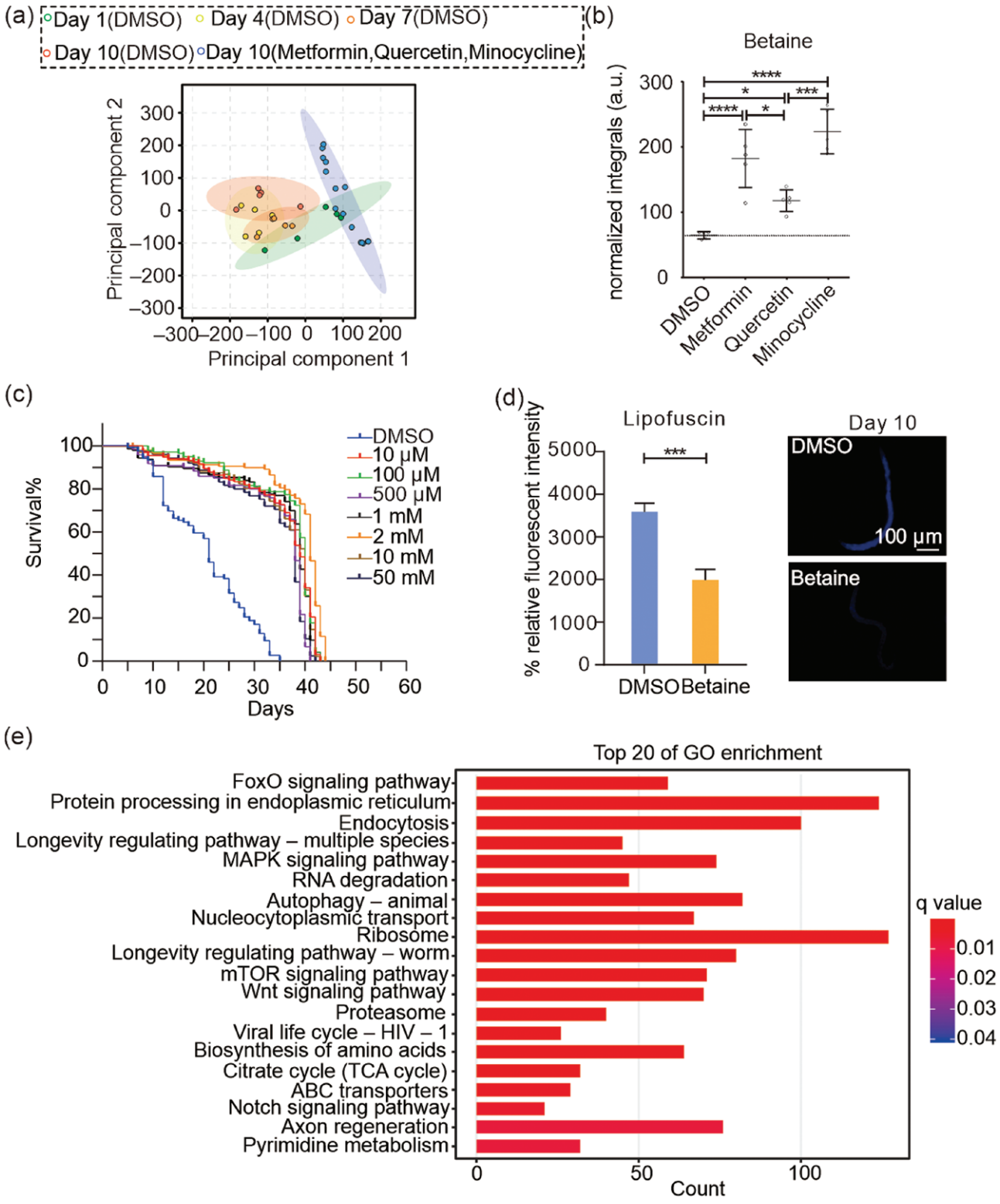

Figure 3. Enhancement of lifespan by betaine in nematodes. (A) PCA plot of nematode samples treated with natural aging and antiaging compounds cultured for 10 days. (B) Statistical analysis of betaine in nematode samples cultured with antiaging drugs. (C) The lifespan of nematodes after treatment with betaine at different concentrations. (D) A representative image of the intestinal cells of nematodes exposed to betaine shows autofluorescent lipofuscin accumulation. Scale bars are 100 μm. (E) The top 20 signaling pathways of nematodes treated with betaine according to GO analysis. Count indicates the number of genes.

an aging-related metabolite in naturally aging nematodes in our study, stood out prominently. The levels of betaine across the 4 groups were compared, as depicted in Figure 3B. Betaine was significantly increased after senolytic administration ($p < .05$), and the highest concentration was observed in minocycline-fed nematodes ($p < .0001$). Notably, the levels of metabolites such as phosphorylcholine, ethanolamine, and glycine, which are involved in the betaine metabolic pathway,

markedly increased after senolytic administration (Supplementary Figure 6). These results indicated that the 3 tested antiaging drugs could modulate betaine metabolism in aged nematodes. Previous studies have reported that betaine is linked to aging and can be influenced by various interventions. Shi reported a positive correlation between longevity-related dietary fiber content and betaine levels in humans (29). McCarty found that a betaine-rich diet might reduce Alzheimer's disease risk by enhancing hydrogen sulfide synthesis in the brain (30). Therefore, we hypothesized that an increase in betaine is crucial for the antiaging benefits of senolytics and that drugs that delay aging and have senolytic properties may affect aging by influencing betaine metabolism.

Based on the above results, we speculated that betaine plays a crucial role in extending lifespan and that betaine supplementation may increase lifespan. Survival analysis was conducted using different concentrations of betaine. The survival curves revealed that betaine at different concentrations exhibited various impacts on extending the lifespan of nematodes compared to the DMSO group. The most significant effect was observed at a concentration of 2 mM betaine (Figure 3C), where the lifespan of nematodes increased by 114% compared to controls ($p < .0001$, Supplementary Table 3), indicating that betaine supplementation effectively increases lifespan. Notably, the 2 mM betaine concentration significantly differed from the 500 μM, 1 mM, and 50 mM concentrations (Supplementary Figure 7). This finding aligns with previous findings suggesting that many antiaging treatments exhibit dose effects at intermediate levels that diminish at higher doses due to saturation (31,32). It is plausible that using a lower dose of betaine could have yielded results resembling a typical dose–response curve, which needs further exploration. The accumulation of the autofluorescent pigment lipofuscin, which is mainly composed of oxidized proteins resistant to proteolytic degradation and lipid peroxidation products that accumulate over time in cells, was used as a marker. Consistent with our hypothesis, the relative fluorescence intensity of nematodes was significantly decreased in the betaine group (2 mM; Figure 3D), which suggested that betaine had effects similar to those of other antiaging drugs in delaying lipofuscin accumulation. To identify the mechanism underlying the life-prolonging effects of betaine, we identified the top 20 metabolic pathways with significant changes (Figure 3E, Supplementary Figure 8). The metabolic pathways regulated by betaine were similar to those regulated by antiaging drugs, such as the FoxO signaling pathway, MAPK signaling pathway, autophagy pathway, longevity regulating pathway, and mTOR signaling pathway, suggesting that betaine may play a role in longevity regulation and physiological activities such as autophagy and oxidative stress. Therefore, we assumed that autophagy and oxidative stress are involved in the protective effects of betaine.

Autophagy is Stimulated in Response to Betaine Treatment

Most antiaging interventions depend on autophagy to exert their protective effects, and betaine has been reported to stimulate autophagy (33). Accumulating evidence highlights the pivotal role of autophagy in regulating lifespan through essential physiological processes. Enhanced autophagy has health benefits and plays a significant role in slowing the aging process (34). Chen reported that betaine attenuates age-related suppression of autophagy via the Mettl21c/p97/VCP axis to delay muscle loss (35). Using high-resolution ^{1}H NMR spectroscopy, Snytnikova reported that chloroquine treatment modulated autophagy by affecting betaine levels in senescent-accelerated rats (36). According to the above pathway results, which showed that betaine may play a role in autophagy, we hypothesized that autophagy is related to the ability of betaine to delay aging. The mRNA expression of several autophagy-related upstream target genes, such as *aak-2*, *rheb-1*, and *let-363*, was measured by RT-qPCR. Compared with those in the older control group, the mRNA expression levels of *rheb-1* and *let-363* in nematodes treated with betaine (2 mM) decreased 2.9-fold ($p < .0001$) and 3.9-fold ($p < .001$), respectively (Figure 4A), whereas *aak-2* increased 4.9-fold ($p < .01$). These results suggested that autophagy-related genes regulate *C elegans* longevity in response to betaine. We further investigated the formation of autophagosomes in the older control and betaine groups using electron microscopic analysis. An increase in the number of autophagosomes was observed following betaine feeding (Figure 4B). These results indicated that the beneficial effects of betaine might be partially due to the activation of autophagy through the AMPK/mTOR signaling pathway.

Betaine Reduced Oxidative Stress in *C elegans*

A key feature of aging is increased oxidative stress, characterized by elevated ROS (37). Mohammadi demonstrated that betaine protects against cell senescence by increasing the activity of antioxidant enzymes and downregulating aging-related signaling pathways (27). Li reported that dietary betaine reduced oxidative stress induced by a high-carbohydrate diet in mandarin fishes (38). Based on the pathway analysis indicating that betaine may influence oxidative stress, we hypothesize that oxidative stress is linked to betaine's ability to delay aging. We analyzed the changes in the mRNA expression of oxidative stress-related genes (*akt-1*, *akt-2*, *sgk-1*, and *daf-16*) following treatment with 2 mM betaine. After betaine treatment, the mRNA expression of these genes showed notable alterations, shifting toward a resemblance to that of the young group rather than that of the older group, in contrast to the control group (Figure 4C, $p < .05$). Betaine is a trimethylglycine with antioxidant properties, and it has been reported to attenuate oxidative stress in rat models of Alzheimer's disease (39). We monitored ROS production in the older control and betaine groups using H2DCF-DA. A significant reduction in ROS accumulation in nematodes treated with betaine, whose DCF fluorescence intensity was 30% lower than that in the older controls, was observed ($p < .0001$; Figure 4D). The antioxidant enzyme superoxide dismutase (SOD) is directly linked to intracellular defense against ROS. We examined the effect of betaine on SOD activity. The betaine group exhibited more significant SOD activity, which was 1.3-fold more remarkable than that of the control group ($p < .05$; Figure 4E). These results indicated that betaine could inhibit ROS accumulation in *C elegans*. The free radical theory of aging points out that the formation of ROS and the subsequent proliferation of damaged macromolecules are primary contributors to aging (40). Therefore, we propose that the ability of betaine to retard the aging process may be partially attributed to its ability to scavenge ROS. Overall, the prolongation of the nematode lifespan by betaine might be closely related to its antioxidant activity, which enhances the resistance of *C elegans* to oxidative stress.

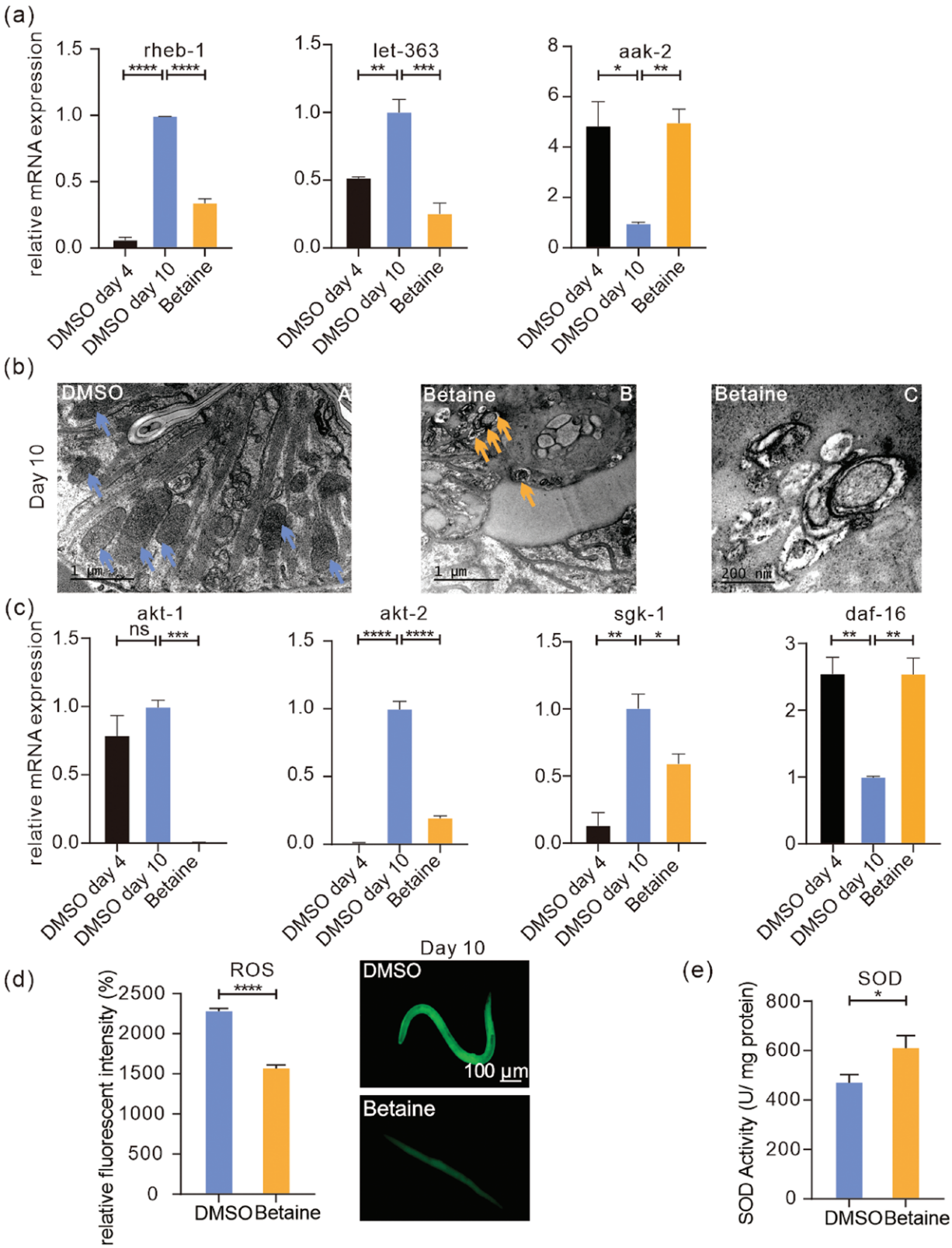

Figure 4. Effects of betaine exposure on autophagy and oxidative stress responses in nematodes. (A) Comparison of the expression patterns of genes related to autophagy in young (DMSO day 4), aged (DMSO day 10), and betaine-treated (day 10) nematodes. The outcomes are presented as the relative expression levels of the tba-1 reference gene and target genes (*rehb-1*, *let-363*, and *aak-2*). (B) The structure of the autophagy pathway was analyzed by electron microscopy. Transmission electron microscopy images of nematodes treated with DMSO (A) or betaine (B), and higher magnification images of the boxed region in B are shown in C. The blue arrows indicate mitochondria, and the yellow arrows indicate autophagosomes. Scale bars are 1 μm in A and B and 200 nm in C. (C) Comparison of the expression patterns of genes related to oxidative stress responses in young (day 4), older (day 10), and betaine-treated (day 10) nematodes. The results are presented as the relative expression ratio between the tba-1 reference gene and target genes (*akt-1*, *akt-2*, *sgk-1*, *daf-16*). (D) The left side shows the relative fluorescence intensity comparison between the control and betaine-exposed groups. The right panels are images of the nematodes showing the generation of ROS. The scale bar is 100 μm. (E) Comparison of antioxidant enzyme activities between the control group and betaine-exposed group. The data are expressed as the mean ± SEM of 3 independent experiments. *$p < .05$, **$p < .01$, ***$p < .001$, and ****$p < .0001$ indicate a significant difference compared to the older group (DMSO day 10), and n.s. indicates no significant difference.

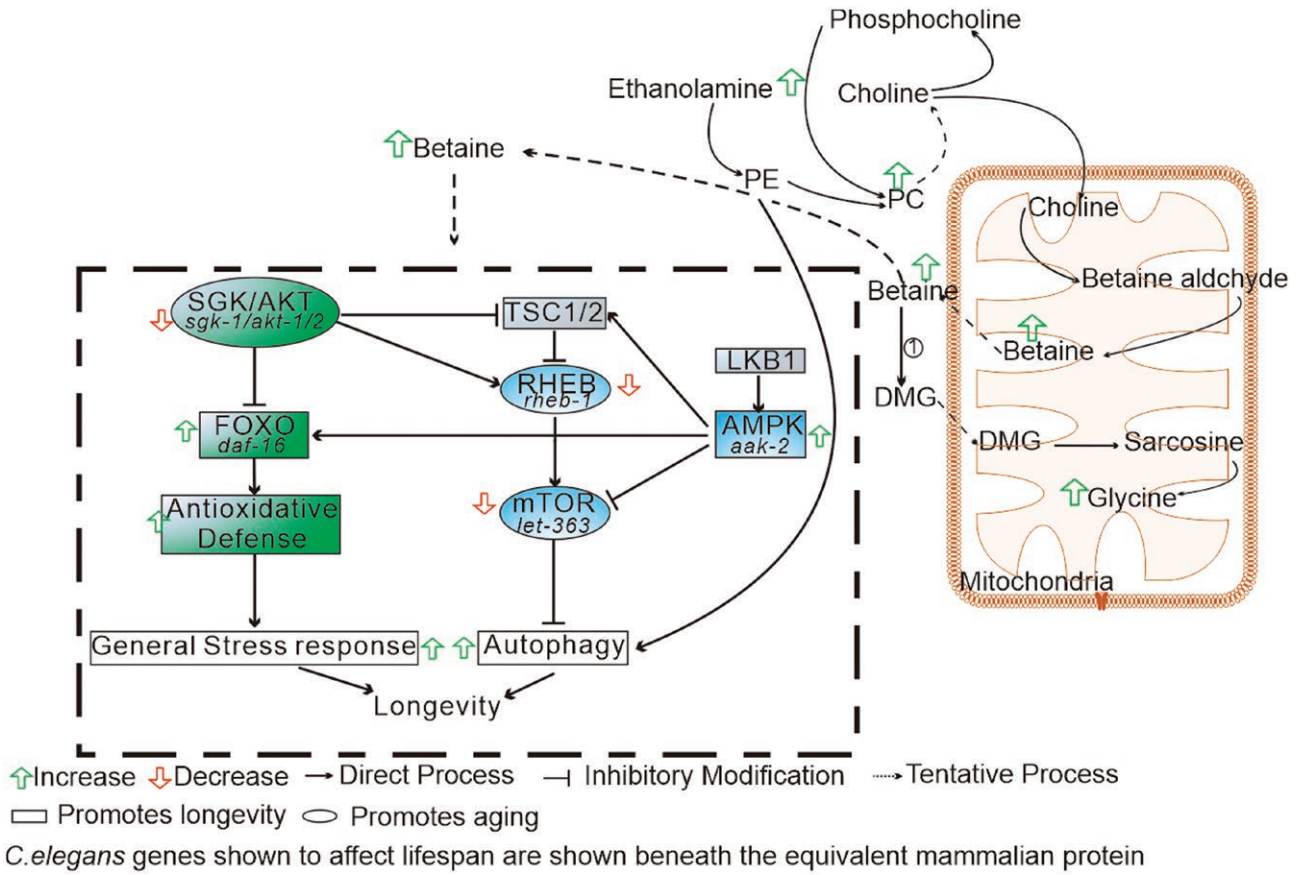

Figure 5. An abridged view of the synthesis and metabolism of betaine and its role in the age-related pathway in *Caenorhabditis elegans*. The enzymes mentioned are displayed and labeled in the cycle with individual numbers: 1. Genes of nematodes that affect lifespan are shown below equivalent mammalian proteins. Proteins not yet shown to influence aging are shown in gray. BHMT = betaine-homocysteine methyltransferase; DMG = *N,N*-dimethylglycine; PE = phosphatidylethanolamine; PC = phosphatidylcholin.

Discussion

During aging, the metabolite levels of the nematodes significantly changed. According to our results, the levels of amino acids, such as methionine, alanine, betaine, and tryptophan, decreased in the older samples compared to the young samples. Among the differentially abundant metabolites, betaine was particularly noteworthy, as its levels markedly decreased with age in both nematode models and human samples. Several studies have proposed a role for betaine in aging; therefore, our study hypothesized that betaine metabolism plays a crucial role in the aging process of nematodes. In contrast, glycerophospholipids such as choline and ethanolamine were increased in older individuals. Notably, methionine, choline, and betaine act as methyl donors, and it has been proposed that age-associated DNA methylation be modified and subsequently alter age-associated physiological and pathological processes (41). A clear negative correlation between tryptophan levels and age was observed, consistent with trends seen in human serum samples (42). Previous research has reported that tryptophan levels are lower in aging-related disorders (40), supporting our findings. Strikingly, metabolites related to the tricarboxylic acid (TCA) cycle, such as succinic acid, citric acid, and fumaric acid, were significantly altered in nematodes of different ages. This decrease in TCA cycle metabolites with aging is likely due to mitochondrial impairment caused by mitochondrial ROS (43).

In this study, we identified betaine as a key metabolite that functions both as a common marker of natural aging and as a crucial component of a metabolic pathway regulated by antiaging drugs, essential for extending nematode lifespan. Our results suggest that betaine is integral to the antiaging effects of these drugs by mitigating various age-related changes, including those in lipofuscin accumulation, autophagy, SOD activity, and free radical levels. In addition, several metabolites involved in the betaine metabolic pathway also exhibited significant alterations in response to the treatments.

A biological model for the betaine metabolic pathway was constructed to elucidate the underlying mechanism (Figure 5). In one critical branch of this pathway, betaine is synthesized from choline derived from several essential compounds, including ethanolamine, phosphatidylethanolamine (PE), and PC. We focused on these components because choline, ethanolamine, PE, and PC are crucial in delaying aging (44). The level of ethanolamine (Etn), a precursor of PE, was found to increase during the antiaging process, which subsequently led to higher levels of PC (Supplementary Figure 6). Despite this, stable choline concentrations were observed in nematodes treated with antiaging drugs, likely due to its conversion to betaine (Supplementary Figure 6). Early studies have shown that PE is crucial for the autophagic process, and a higher concentration of PE can help delay aging (45). PC, derived from the diet, is the primary source of choline, and PC supplementation has been shown to slow aging and extend the

lifespan of nematodes (46). Interestingly, the level of glycine, a downstream metabolite of the pathway, also tended to increase after antiaging treatment, especially in the metformin group ($p < .001$; Supplementary Figure 6), indicating the upregulation of this pathway. Recent studies have revealed that glycine supplementation contributes to extending the life of nematodes (47).

The other branch is related to longevity, including autophagy and the oxidative stress response. In our study, betaine stimulated autophagy by inhibiting the mTOR pathway. Early research indicated that while autophagy occurs at baseline levels under normal growth conditions, it is quickly re-regulated in response to various conditions (48). For instance, betaine-controlled expression levels of *rheb-1*, *let-363*, and *aak-2* were consistent with those in the young group; these genes play a crucial role in prolonging the lifespan of creatures and have been shown to stimulate autophagy to eliminate senescent cells (49). Our study also revealed that the expression of these autophagy-related genes was altered in response to betaine. In worms, *let-363* encodes an orthologue of mTOR, a highly conserved kinase that regulates autophagy (50). Studies on *C elegans* have shown that suppressed expression of mTOR/*let-363* was associated with an increase in the lifespan of almost twofold (51). The activity of TOR complex 1 (TORC1) switches between anabolism and catabolism in the presence of *rheb-1*, thus controlling lifespan, development, and autophagy (52). Honjoh et al. reported that a decrease in the level of *rheb-1* prolongs the lifespan of worms (53). *ak-2* encodes the α-subunit of AMPK, which is critical for autophagy, stress responses, energy metabolism, and life span (54,55). Onken et al. indicated that metformin could increase the expression of *akk-2* and extend lifespan through the AMPK/*akk-2* pathway (24,56). However, the exact mechanism by which autophagy extends lifespan is not yet fully understood.

In addition, our study showed that the antioxidant capacity of nematodes improved under betaine treatment by promoting the FoxO pathway. The expression of oxidative stress-related genes, including *akt-1*, *akt-2*, *sgk-1*, and *daf-16*, was significantly altered in the young group. The *daf-16* gene encodes the FoxO transcription factor, which has been reported to significantly regulate the aging process in both *C elegans* and mammals (57). Liu et al. indicated that lentinan could increase the expression of *daf-16* and dramatically prolong the lifespan of *C elegans* (58). The *akt-1/2* genes encode the kinase cascade in the insulin signaling pathway, which is required to control longevity (59). Liu et al. observed decreased *akt-1* and *akt-2* expression after paeoniflorin administration, which benefitted lifespan (60). *sgk-1* encodes a threonine/serine protein kinase that is crucial in the cellular stress response and is also believed to function similarly to *akt-1/2* in lifespan regulation by phosphorylating and inhibiting the nuclear translocation of DAF-16/FoxO (61). A study on *C elegans* revealed that suppressed expression of *sgk-1* resulted in increased stress resistance and an extension of lifespan (62).

Despite the metabolic patterns associated with aging, some limitations to this study exist. Our study included 5 dishes (10 000 worms per dish), and the limited sample size has hindered the detection of differences with smaller effect sizes. Although strong associations were observed between betaine, lipofuscin, autophagy, and oxidative stress, the lack of experiments that block related genes weakens the evidence supporting the idea that changes in these factors are necessary for betaine-induced lifespan extension. In addition, as we used FUDR to inhibit nematode oviposition, the results of this study may be affected by the presence of FUDR, which inhibits DNA synthesis and alters metabolism by inhibiting thymidylate synthase. Although FUDR is commonly used in *C elegans* research, including in our study, it's important to acknowledge that its presence introduces a technical confound. Many mutants and drugs show effects that are influenced by FUDR (63–69). Given that FUDR is used in chemotherapy to inhibit cell division (70), which could limit the applicability of our findings to broader biological contexts.

In conclusion, based on this study, we identified the metabolic reprogramming of nematodes due to natural aging. In addition, our study revealed that antiaging drugs significantly affect antiaging by regulating betaine levels and related target genes. Moreover, the beneficial regulation of betaine relies on the participation of autophagy and antioxidative activity (Figure 5). Our findings reveal the relationship among senolytics, betaine, and aging. At the same time, this study provides a reference for subsequent clinical trials, and hopefully, betaine is a promising intervention against aging.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

None.

Data Availability

All data supported by this work are available from the corresponding author on reasonable request.

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Author Contributions

W.L.: conceptualization, methodology, investigation, data curation, formal analysis, visualization, writing–original draft, and writing–review and editing. X.X.: investigation, data curation, formal analysis, visualization, and writing–review and editing. J.N.: investigation. Z.W.: visualization. X.Z.: investigation. Y.W.: investigation and formal analysis. D.Z.: investigation and formal analysis. J.C.: investigation

and formal analysis. W.B.: methodology. C.L.: methodology. Y.Z.: conceptualization, methodology, data curation, resources, supervision, project administration, and funding acquisition. A.Z.: conceptualization, methodology, data curation, resources, supervision, project administration, and funding acquisition. F.Z.: conceptualization, methodology, formal analysis, resources, data curation, writing—review and editing, supervision, project administration, and funding acquisition.

Ethical Statements

This study was approved by the Ethics Committee of Mengchao Hepatobiliary Hospital of Fujian Medical University.

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