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Research Article

Ginsenoside-Re-rich ethanol extract of Panax ginseng berry enhances healthspan extension via mitostasis and NAD metabolism

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ABSTRACT

Background: Ginseng Berry Concentrate (GBC) enhances exercise capacity in mice, but the effects of its key component, ginsenoside Re (G-Re), on aging and mitochondrial function are not well understood. This study investigates the impact of G-Re on mitophagy and its potential to promote healthy aging.
Methods: Experiments in C2C12 myocytes and HeLa-mitoKeima-PARKIN cells assessed GBC and G-Re's effects on mitophagy, supported by Gene Set Enrichment Analysis. G-Re was identified as the primary component of GBC via high-performance liquid chromatography. The influence of G-Re on lifespan and healthspan was examined in *Caenorhabditis elegans*, with a focus on mitophagy pathways.
Results: GBC and G-Re significantly induced mitophagy and enhanced mitochondrial gene expression, improving mitochondrial function. G-Re extended lifespan and healthspan in *C. elegans*, effects absent in mitophagy-impaired mutants.
Conclusion: G-Re enhances mitochondrial function and promotes healthy aging through mitophagy, suggesting its potential for mitigating age-related functional declines.

1. Introduction

Mitochondria are central to cellular energy metabolism and play a critical role in regulating cell survival and death [1]. Their function extends beyond energy production, encompassing roles in signaling, cellular differentiation, and the control of the cell cycle and cell growth [2–4]. However, mitochondrial dysfunction is a hallmark of aging and a plethora of age-associated diseases [5], including neurodegenerative disorders, cardiovascular diseases, and metabolic syndromes [6–11]. This dysfunction often arises from an imbalance between the production of reactive oxygen species (ROS) and the cell's antioxidant defenses, leading to oxidative stress, DNA damage, and impaired mitochondrial dynamics [12,13].

Mitochondrial homeostasis and quality control are pivotal for cellular health and longevity, underscored by the essential role of mitophagy in maintaining mitochondrial integrity [14,15]. Mitophagy, a specialized form of autophagy, targets damaged or superfluous mitochondria for degradation, thereby playing a crucial role in mitochondrial quality control [16]. It ensures the removal of dysfunctional mitochondria, preventing their accumulation and the subsequent disruption of cellular function. However, the efficiency of mitophagy declines with age, contributing to the accumulation of damaged

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mitochondria and exacerbating cellular dysfunction and disease progression [17].

The mitophagy pathway is regulated by both endogenously and exogenously produced metabolites, including dietary-derived compounds. For instance, natural compounds like urolithin A, nicotinamide riboside, and polyamine spermidine have been recognized for their capacity to induce mitophagy, offering therapeutic avenues for ameliorating mitochondrial dysfunction [9,18,19]. These substances promote mitochondrial quality control, delineating an effective approach to combat aging and mitochondrial disorders. By enhancing mitophagy, these compounds help maintain mitochondrial integrity, supporting cellular energy metabolism, and offering protection against the development of aging-related diseases and disorders.

Building on the potential of these mitophagy-inducing compounds, other natural substances, in particular the ginseng berry, have also garnered attention for their wide-ranging benefits in metabolic fitness and aging. Ginseng berry stands out for its extensive pharmacological benefits, including its role in disease prevention, aging mitigation, and mitochondrial function improvement [20–24]. Its active components significantly boost mitochondrial biogenesis and operational efficiency, influencing cellular energy processes. Additionally, ginseng berry's ability to stimulate sirtuins suggests a mechanism for extending lifespan and enhancing resilience against cellular stress [25,26]. While there has been considerable focus on various ginsenosides, G-Re has been somewhat underexplored. Recent research highlights that G-Re promotes autophagy, offers protection against myocardial fibrosis and ferroptosis, reduces oxidative stress, and mitigates neuroinflammation [27–31].

However, emerging evidence points to its significant health benefits, particularly in enhancing mitochondrial function. Additionally, we found that orally administering GBC improves endurance performance in mice by reducing post-exercise blood lactate and ammonia levels, which implies improved mitochondrial function [32]. Based on this, we hypothesize that ginsenoside Re, the major ingredient of GBC, contributes to mitochondrial homeostasis, encompassing both biogenesis and quality control mechanisms.

2. Materials and methods

2.1. Cell culture

HeLa-mitoKeima-PARKIN cells were acquired from Prof. Jeanho Yun's laboratory at Dong-A University, South Korea. HeLa-mitoKeima-PARKIN cells, engineered to express mitoKeima and PARKIN proteins, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with supplements. C2C12 myoblasts were similarly maintained, later inducing differentiation into myotubes with DMEM and horse serum. All cells were incubated at 37 °C with 5 % CO_2. Mitophagy was initiated using FCCP, along with oligomycin and antimycin A (OA), and bafilomycin A1 (BFA) was employed to assess mitophagy flux by inhibiting lysosomal degradation as described [18].

2.2. Western blotting

Western blotting was performed in accordance with established protocols [33]. Cells were washed with 1× PBS, lysed in 1× RIPA buffer (IBS-BR002, Intronbio) with added inhibitors, and centrifuged to remove debris. Protein concentrations were determined using the DC™ Protein Assay Kit (Bio-Rad, USA). Proteins were denatured, resolved on 8, 10, or 15 % SDS-PAGE, and transferred to PVDF membranes (Millipore, USA). After blocking with 3 % BSA, membranes were probed with primary and HRP-linked secondary antibodies (Invitrogen, USA).

2.3. Preparation of ginseng root, berry, seed raw materials and high-performance liquid chromatography (HPLC) analysis

Korean ginseng (Panax ginseng) and its berries, procured from Geumsan, Chungcheongnam-do, Korea, underwent thorough cleaning with seeds subsequently separated from the berries. Ginseng Berry Concentrate (GBC) was prepared by concentrating the berry juice to 20 Brix, further concentrating to 70 Brix, followed by sterilization at 90–95 °C. This extraction and concentration process was meticulously set up to comply with Good Manufacturing Practice (GMP) standards, ensuring high reproducibility and quality control. Ginseng seeds were washed, stratified with moisture, then ground and extracted with 50 % ethanol at 85 °C. The resultant extract was concentrated to 15 Brix and sterilized under the same conditions, producing Ginseng Seed Concentrate (GSC). Ginseng Root Concentrate (GRC) was obtained through a similar process with 70 % ethanol, concentrated to 70 Brix, and sterilized. Detailed protocols for the preparation of GBC, GSC, and GRC are provided in the Results section.

To identify the primary ginsenoside components of the ginseng extracts, we applied an HPLC-UV analysis method adapted from our previous study [32]. All extraction and analysis steps were conducted within a GMP-compliant setup, which includes the HPLC process, to ensure consistency and precision in detecting ginsenoside components. Extracts (500 mg) were dissolved in 100 mL of 50 % methanol and ultrasonicated for 20 min. The solutions were then filtered without further purification and directly injected into the HPLC system. Chromatographic separation was carried out using a Kinetex C18 column (100 mm × 4.60 mm, 2.6 μm, Phenomenex, USA) at 40 °C. UV detection was set at 203 nm. The mobile phase consisted of water (A) and acetonitrile (B) in a 20:80 ratio for B. The gradient elution was programmed as follows: 0–4 min, 78 % A; 4–11 min, 71 % A; 11–35 min, 67 % A; 35–47 min, 25 % A; 47–49 min, 0 % A; 49–49.5 min, 78 % A; 49.5–52 min, 78 % A. The flow rate was 1.5 mL/min, with an injection volume of 10 μL.

2.4. Oxygen consumption rate (OCR)

The oxygen consumption rate (OCR) was assessed using a Seahorse XFp Analyzer (Agilent Technologies), following the protocol described in Ref. [18]. The assay commenced under basal conditions, with subsequent injections of oligomycin A (1.5 μM), carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP, 0.5 μM), and a combination of rotenone with antimycin A (0.5 μM) administered in sequence.

2.5. Acquisition and analysis of fluorescence spectrum-based mitophagy data

Fluorescence spectrum-based mitophagy data were acquired utilizing the Synergy™ NEO HTS System (BioTek) system. The analysis was performed using Gen5™ Microplate Reader and Imager Software. We fully characterized the spectrum of HeLa-mitoKeima-PARKIN cells treated with mitophagy inducers such as OA and mitophagy flux inhibitors like BFA. Mitophagy scores were determined by the fluorescence intensity ratio at the excitation wavelengths of 550 nm–440 nm, reflecting the modulation of mitophagy by the compounds under investigation. The average fluorescence intensities were calculated for each well, following the application of mitophagy inducers and inhibitors for a duration of 16 h.

2.6. Single-cell-based mitophagy analysis via high-content screening (HCS)

HCS data were collected with the Cytation™ 5 System (BioTek) for the determination of mitophagy scores. The analysis of the data obtained from the Cytation 5 system was conducted using Gen5™ Software, specialized for imaging and microscopy. In the single-cell analysis, fluorescence intensities were measured using acridine orange (excitation 465 nm/emission 650 nm; intact mitochondria) and Texas red (excitation 586 nm/emission 647 nm; mitophagy) filter sets. These measurements facilitated the generation of scatter plots that illustrate

the mean values of the acridine orange channel on the x-axis, representing mitochondria in the cytoplasm, and the Texas red channel on the y-axis, indicating mitochondria within lysosomes. Each dot on the scatter plots corresponds to the mitophagy score of individual cells.

2.7. Mitophagy index

The Mitophagy Index is quantitatively calculated based on regions (i.e., triangles) marked on a scatter dot plot. This plot is generated from measurements taken using the Cytation™ 5 System (BioTek) on HeLa-mitoKeima-PARKIN cells, where each dot represents a single cell. These cells collect signals at two different wavelengths, resulting in x and y coordinates for each dot. The x-axis represents the signal from normal cytosolic mitochondria, while the y-coordinates represents the signal from mitochondria exposed to the acidic lysosomal environment, indicating mitophagy. The number of dots (cells) in the second quadrant (green regions) reflects the cells undergoing increased mitophagy, which we term the Mitophagy Index. The placement of triangles is based on relative, not absolute, values, adjusted according to the fluorescence signals that vary with cellular conditions, ensuring minimal mitophagy in the basal control.

In addition to the Mitophagy Index, we assess mitophagy flux by measuring the quantitative changes in FUNDC1 recovered after BFA treatment. These metrics together provide a comprehensive understanding of mitophagy activity and flux under various experimental conditions.

2.8. Healthy aging analysis in Caenorhabditis elegans

As detailed in our prior studies [34], we performed healthy aging assays on *C. elegans* to evaluate a spectrum of parameters reflective of physiological aging. These evaluations encompassed lifespan, motility (measured as movement speed over time), pharyngeal pumping rate, the accumulation of age-related biomarkers such as lipofuscin, progeny output, and resilience to environmental stressors, including heat and mitochondrial distress triggered by paraquat exposure. Each parameter offered insights into the health and aging dynamics of *C. elegans*, enhancing our comprehension of the variables that promote healthy aging.

2.9. RNA-sequencing and Gene Set Enrichment Analysis (GSEA)

RNA-sequencing and Gene Set Enrichment Analysis (GSEA) were performed as outlined [35]. Theragenbio, Inc. conducted RNA sequencing, including RNA isolation, library preparation, and FASTQ file generation. Data quality was assessed using FastQC, with reads aligned to the mouse genome (GRCm39/mm10) and expression quantified as TPM. Further analysis was conducted using log2 TPM values. GSEA was performed using the Broad Institute's Java application (www.gsea-msigdb.org), with visualizations created in RStudio using packages like ggplot2 and heatmap (www.r-project.org). Statistical significance was assessed with Student's t-test and Levene's test, with GSEA providing enrichment plots showing normalized enrichment scores (NES), p-values, and false discovery rate (FDR) q-values.

2.10. C. elegans strains maintenance and lifespan assay

All analyses with *C. elegans* were performed as described previously [34]. Wild-type N2 *Caenorhabditis elegans* and specific mutant strains were obtained from the Caenorhabditis Genetics Center (CGC), supported by NIH grant P40 OD010440. The strains used included Bristol N2, VC1024: *pdr-1*(gk448)III, and RG3133: *fndc-1*(ve633)II, all maintained at 20 °C. Age-synchronized populations were generated using established protocols, with L1-stage larvae grown on agar plates at 20 °C and fed *E. coli* OP50 until they reached the L4 stage. *E. coli* OP50 was cultured in LB media at 37 °C. For lifespan analysis, L4 larvae were transferred to NGM plates containing OP50 and test reagents added post-autoclaving. This day was designated as "day 0". To prevent progeny, 5-fluoro-2′-deoxyuridine (FUdR) was added 24 h before use. Worms were transferred every other day, noting survival and excluding those with anomalies such as vulval bursting. Lifespan assays were performed in triplicate and statistically analyzed using R software with the "*coin*" package, generating Kaplan-Meier survival curves and calculating p-values using the log-rank test. Additionally, indices of healthy aging, including spontaneous movement speed, pharyngeal pumping rate, relative lipofuscin accumulation, and progeny production, were conducted and analyzed as described [34].

2.11. Statistical analysis

Statistical analyses and visualizations, including bar graphs and scatter plots, were performed using GraphPad Prism 5 (GraphPad Software Inc.). Statistical significance was determined using unpaired t-tests or one- and two-way ANOVA, with a p-value of less than 0.05 considered significant.

3. Results

3.1. Enhanced gene expression profiles of mitochondrial homeostasis in GBC-treated C2C12 cells

To understand how orally administered GBC affects muscle function and adaptation, C2C12 myoblasts were treated with GBC for 24 h, followed by RNA isolation and sequencing. Gene Set Enrichment Analysis (GSEA) of the transcriptome revealed that GBC-treated cells showed enrichment in 5,786 gene sets compared to DMSO-treated controls (Fig. 1A). The enriched gene sets mainly involved pathways related to mitochondrial biogenesis, homeostasis, and quality control (Fig. 1B). Proteomics studies have indicated that around 1,000 proteins encoded by nuclear DNA (nDNA) and imported into mitochondria are functional within these organelles [36]. Notably, GBC treatment increased the expression of 561 genes coding for mitochondrial proteins, representing an upregulation of 50–60 % of nDNA-encoded mitochondrial genes (Fig. 1C). These upregulated genes are essential for mitochondrial biogenesis, oxidative phosphorylation (OXPHOS), and quality control mechanisms, including mitophagy (Fig. 1D). Additionally, mitochondrial DNA-encoded genes also showed increased expression, further highlighting GBC's broad influence on mitochondrial function and muscle adaptation (Fig. 1D). These findings suggest that the upregulation of mitochondrial genes by GBC may be a key mechanism behind the observed improvements in endurance exercise performance in mice, as reported in previous studies [32].

3.2. Establishing a single cell level assessment of mitophagy flux using a reporter line

The significant changes in mitochondrial gene expression following a single GBC treatment captured our attention. To accurately quantify GBC-induced mitophagy alterations, we utilized mitoKeima, a widely adopted reporter for monitoring mitophagy flux. We first confirmed the expression of the PARKIN protein—a key component of the mitophagy pathway, typically absent in standard HeLa cells—in our custom-engineered HeLa-mitoKeima-PARKIN cells (Supplemental Fig. 1A). Mitophagy flux was initially measured using the Synergy™ NEO HTS System (BioTek), a high-throughput multimode microplate reader, which showed no significant changes under FCCP or OA treatment conditions (Supplemental Fig. 1B). However, using the Cytation 5 System (BioTek), a cell imaging multimode reader, we observed a marked increase in mitophagy flux at the single-cell level following OA treatment compared to DMSO (Supplemental Fig. 1C). This method allowed clear detection of the mitophagy flux segment inhibited by BFA treatment.

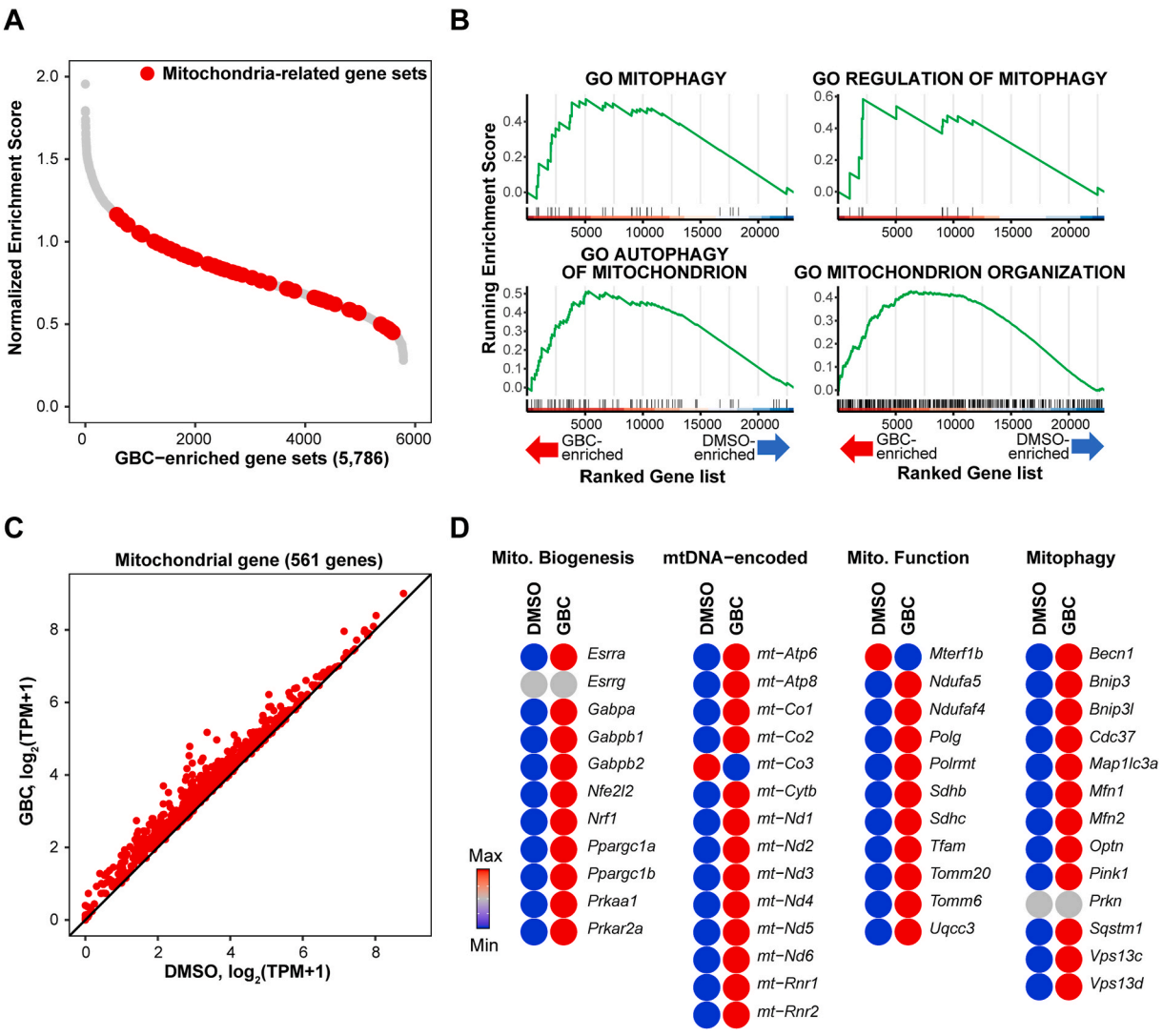


Fig. 1. Ginseng Berry Concentrate (GBC) treatment enhances gene expression related to mitochondrial homeostasis in C2C12 myoblasts. (A) Scatter plot showing mitochondria-related gene sets (49 red dots) among all GBC-enriched sets (gray dots). (B) GSEA enrichment plots highlighting mitochondrial biogenesis and quality control mechanisms. (C) Scatter plot showing the upregulation of 561 nuclear DNA-encoded mitochondrial genes. (D) Heatmaps of key genes in mitochondrial biogenesis, mitochondrial DNA, and mitophagy.

3.3. GBC induces mitophagy

Using an optimized method for mitophagy assessment, we investigated Ginseng Berry Concentrate's (GBC) ability to induce mitophagy, comparing it with Ginseng Seed Concentrate (GSC) and Ginseng Root Concentrate (GRC). Our analysis revealed that GBC, at concentrations of 25 and 100 μg/ml, effectively induced mitophagy after 24 h, while neither GSC nor GRC did (Fig. 2A). The mitophagy index from three independent experiments showed a significant increase with 100 μg/ml GBC, an effect not observed with GSC and GRC (Fig. 2B).

Western blot analysis showed a reduction in FUNDC1, a mitophagy receptor, after GBC treatment in mitoKeima reporter cells.

Although GBC treatment alone does not clearly reduce FUNDC1 levels, the accumulation of FUNDC1 observed with the additional BFA treatment suggests that GBC may have the potential to enhance mitophagy (Fig. 2C and D). Furthermore, quantification of mitophagy flux under basal conditions and with BFA co-treatment revealed a significant increase in mitophagy flux, confirming the enhanced mitophagy activity (Fig. 2E).

Additionally, mitochondrial functionality was assessed in C2C12 myotubes following mitophagy induction. While basal respiration remained unchanged, maximal respiration was enhanced after two consecutive 24-h treatments with GBC (Fig. 2F). This suggests that GBC not only promotes the removal of dysfunctional mitochondria via mitophagy but also stimulates mitochondrial biogenesis, as supported by RNA sequencing results (Fig. 1). Together, these findings indicate that GBC significantly increases mitophagy flux and enhances mitochondrial function.

3.4. G-Re identified as the predominant component in GBC

To elucidate how GBC enhances mitophagy and mitochondrial function, we analyzed its composition using high-performance liquid chromatography with ultraviolet detection (HPLC-UV), as detailed in the Materials and Methods (Fig. 3A). Our comparative analysis of Ginseng Berry Concentrate (GBC), Ginseng Root Concentrate (GRC), and Ginseng Seed Concentrate (GSC) identified ginsenoside Re as the predominant ginsenoside in GBC, with a concentration of 44.8 mg/g. This concentration significantly exceeded those of other ginsenosides, such as Rb1 (15.57 mg/g), Rb2 (3.47 mg/g), and Rc (4.18 mg/g) (Fig. 3B). This prompted us to investigate the effects of G-Re on mitochondrial homeostasis.

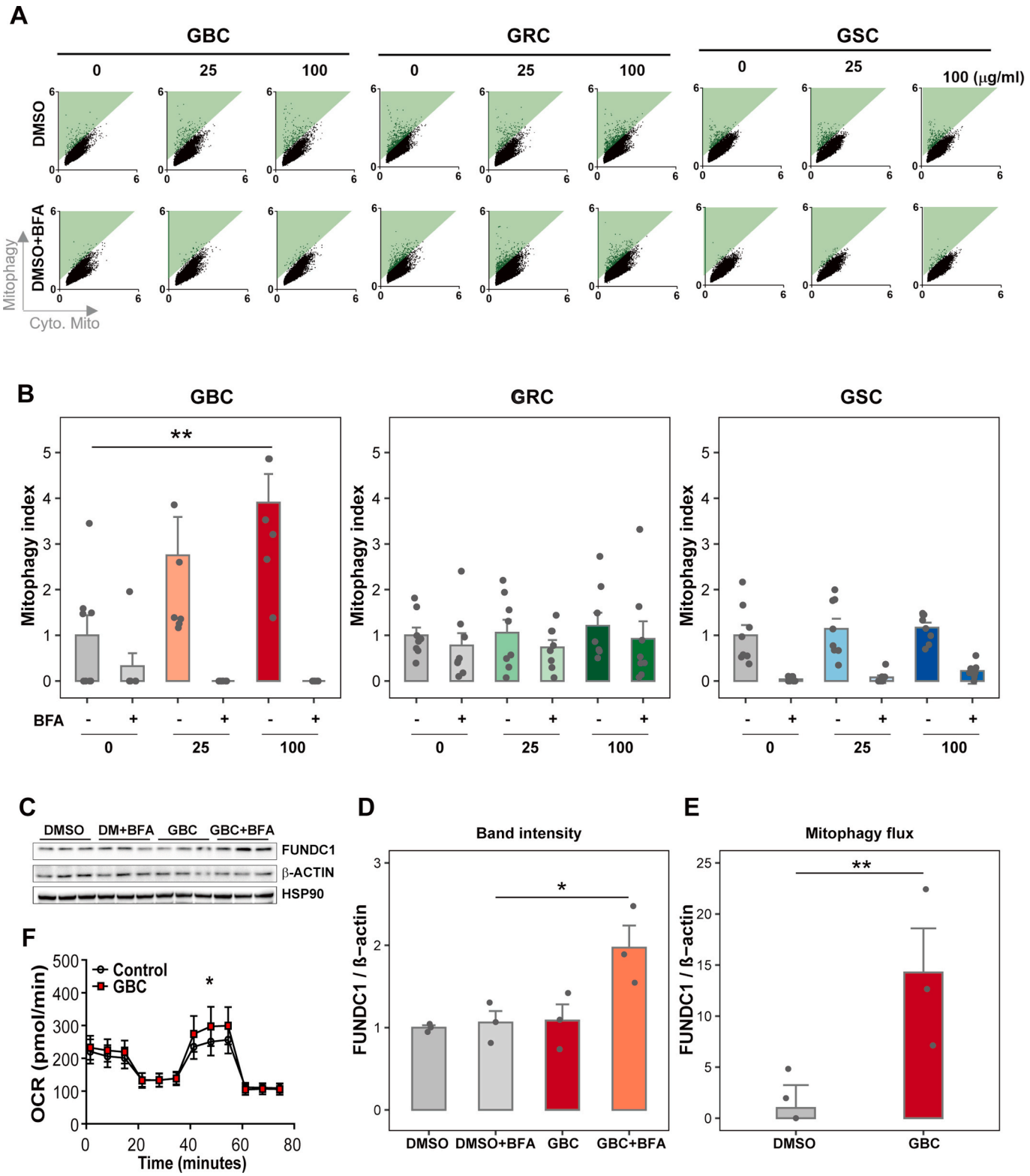
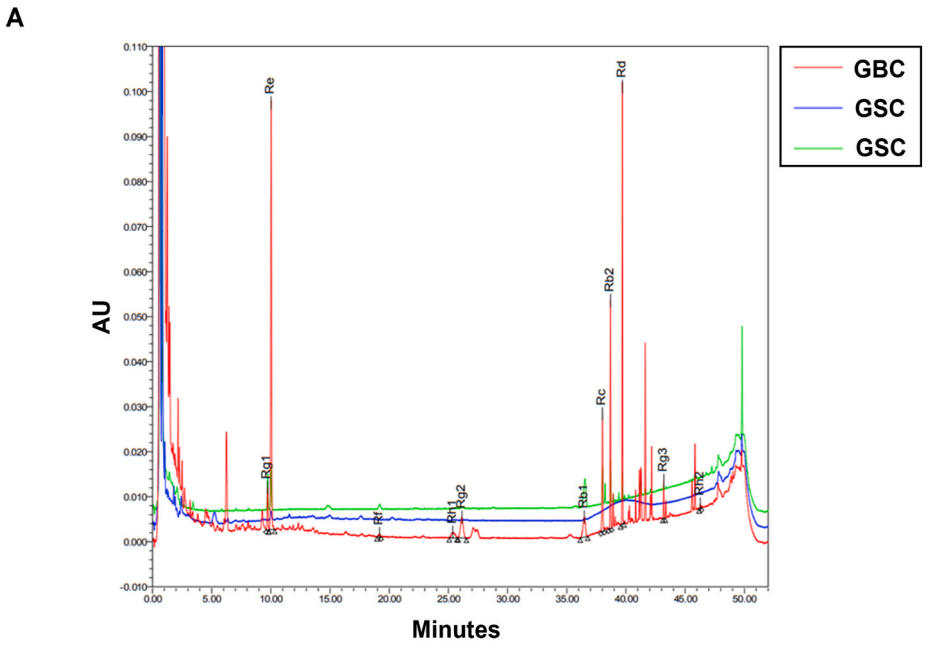
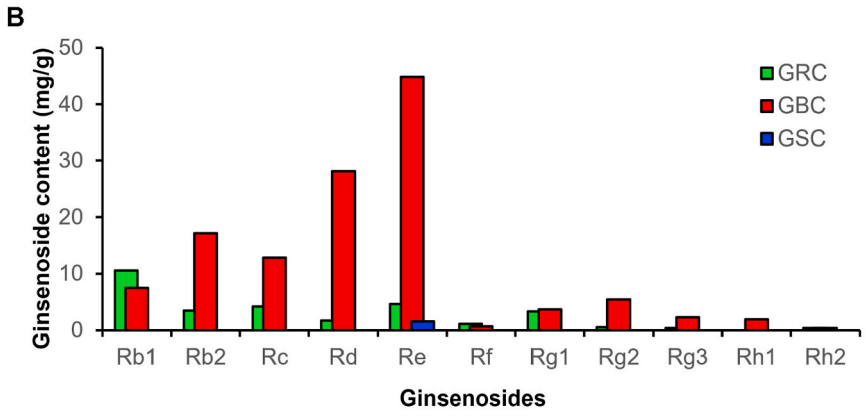


Fig. 2. GBC triggers mitophagy. (A) Scatter plots from high-content screening (HCS) showing mitophagy in HeLa-mitoKeima-PARKIN cells treated with GBC, Ginseng Root Concentrate (GRC), and Ginseng Seed Concentrate (GSC) (0, 25, 100 μg/ml). (B) Bar graph quantifying mitophagy from three HCS analyses. (C–E) Western blot for FUNDC1 and loading controls β-actin and HSP90, showing mitophagy flux via BFA-dependent FUNDC1 accumulation in C2C12 myotubes. (F) Oxygen consumption rate (OCR) in DMSO and GBC-treated C2C12 myotubes. All plots depict the mean and standard error of the mean (S.E.M.) from three separate high-content screening analyses for cytosolic mitophagy in C2C12 myotubes. Statistical significance is denoted by *$p < 0.05$ and **$p < 0.01$ when compared to the control group, as assessed by either a Student's *t*-test or ANOVA.





	Rb1	Rb2	Rc	Rd	Re	Rf	Rg1	Rg2	Rg3	Rh1	Rh2
GRC	11	3.5	4.2	1.7	4.6	1.1	3.3	0.5	0.4	ND	0.4
GBC	7.5	17	13	28	45	0.6	3.7	5.4	2.2	1.9	0.4
GSC	ND	ND	ND	ND	1.5	ND	ND	ND	ND	ND	ND

Fig. 3. Ginsenoside Re as the dominant component in GBC. (A) HPLC chromatogram of GBC, GSC, and GRC components. (B) Bar graph quantifying ginsenosides, highlighting ginsenosides Re as the principal component in GBC.

3.5. G-Re induces expression of mitochondrial genes

To explore G-Re's effects on mitochondrial homeostasis in a cellular context, we performed GSEA on the transcriptome of C2C12 myoblasts treated with G-Re for 24 h. This analysis revealed significant changes in gene sets related to mitochondrial function, autophagy/mitophagy, cellular aging, and NAD^+ biosynthesis in G-Re-treated cells (Fig. 4A and B). Notably, gene sets linked to mitochondrial homeostasis, including mitophagy—key for mitochondrial quality control—were strongly enriched under G-Re treatment. Additionally, gene sets associated with cellular senescence and NAD^+ biosynthesis were also enriched, highlighting G-Re's broad impact on cellular health and metabolism (Fig. 4C). Most of the genes in these sets were upregulated, indicating a robust enhancement of mitochondrial gene expression and function.

3.6. G-Re enhances mitophagy and mitochondrial homeostasis

Beyond transcriptome analysis, we used HeLa-mitoKeima-PARKIN cells to assess ginsenoside Re's ability to induce mitophagy and increase mitophagy flux. Treatment with 25 μM and 50 μM G-Re for 24 h significantly increased the population of mitophagy-positive cells, indicating enhanced mitophagy. Inhibition of lysosomal function with bafilomycin A1 (BFA) led to an increase of cells within the mitophagy zone, confirming G-Re's role in inducing mitophagy (Fig. 5A). Quantification from three independent experiments showed a dose-dependent increase in mitophagy (Fig. 5B). Similar to the observations in GBC-treated cells, G-Re treatment led to a reduction in the levels of the mitophagy receptor FUNDC1 protein in C2C12 myocytes. This reduction was reversed when BFA was co-administered, resulting in the accumulation of FUNDC1, indicative of active mitophagy flux (Fig. 5C). In parallel, the oxygen consumption rate (OCR), a key indicator of

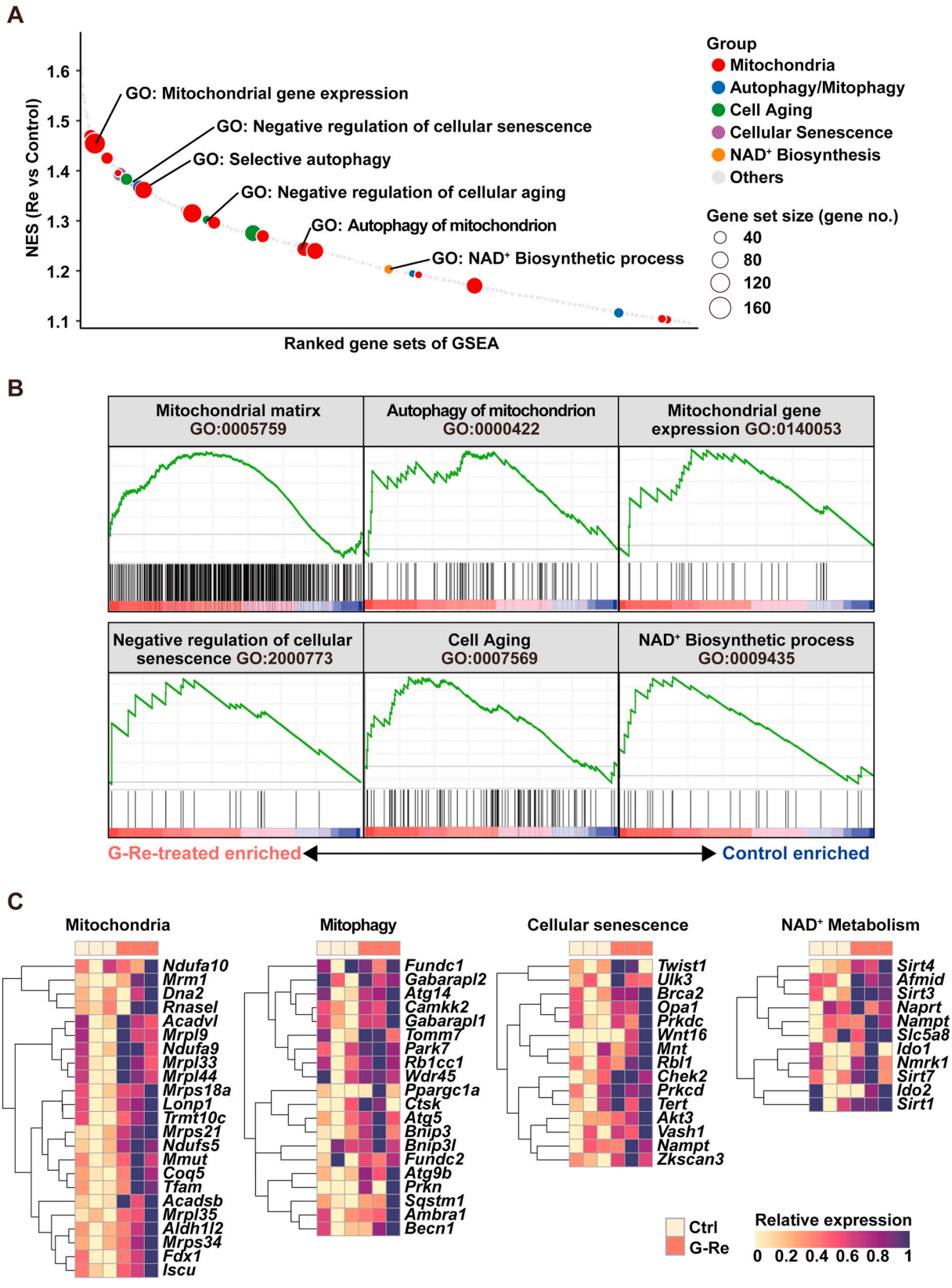


Fig. 4. G-Re's effects on mitochondrial, mitophagy, cellular senescence, and NAD^+ metabolism gene sets in C2C12 myoblasts. (A) Scatter plot of enriched gene sets related to mitochondrial homeostasis, senescence, and NAD^+ biosynthesis under G-Re treatment. (B) GSEA plots for mitochondrial homeostasis, senescence, and NAD^+ biosynthesis. (C) Heatmaps of key genes in mitochondrial homeostasis, mitophagy, senescence, and NAD^+ metabolism.

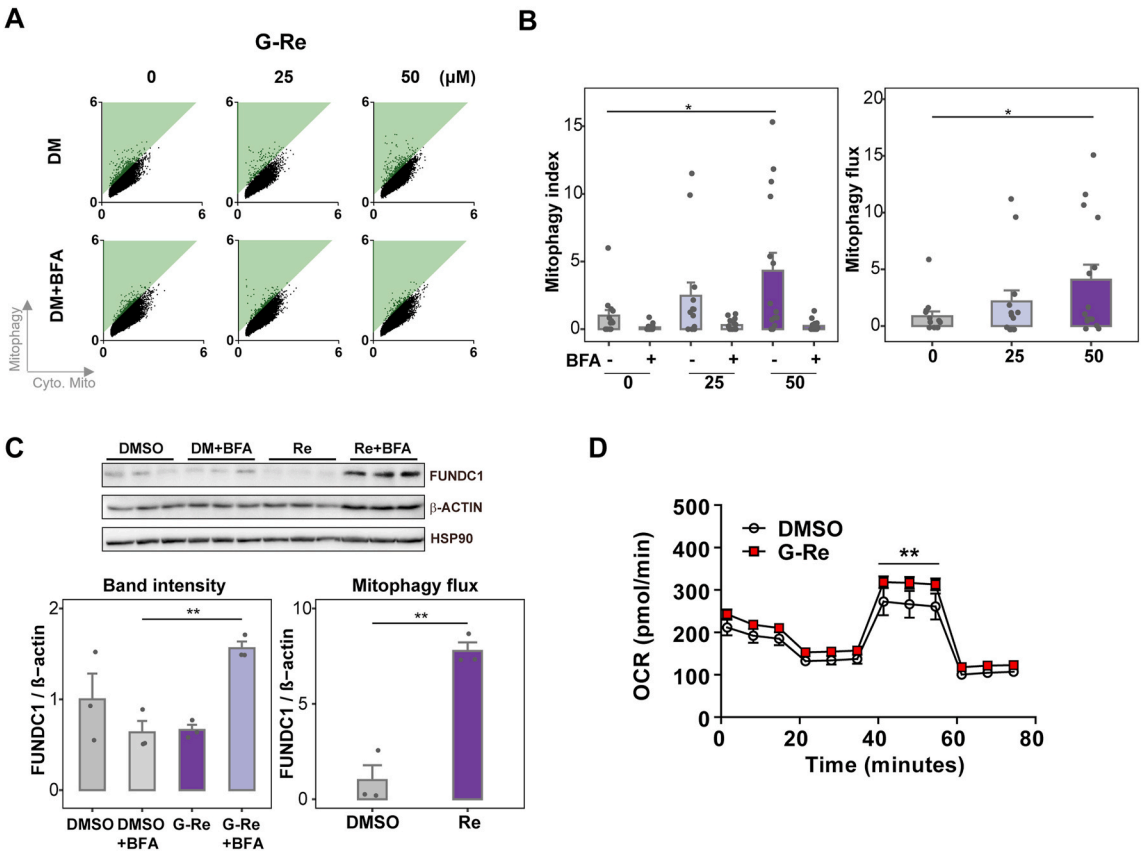


Fig. 5. G-Re activation of mitophagy. (A) HCS analysis showing mitophagy induction by G-Re (25, 50 μM) in HeLa-mitoKeima-PARKIN cells. (B) Quantitative HCS results from three experiments. (C) Western blot for FUNDC1, with β-actin and HSP90 as loading controls in C2C12 myotubes. The quantification of FUNDC1 and BFA-induced accumulation (mitophagy flux) is shown below. (D) OCR in DMSO and G-Re-treated C2C12 myotubes. All plots depict the mean ± S.E.M. from three separate high-content screening analyses for cytosolic mitophagy. Statistical significance is denoted by *p < 0.05 and **p < 0.01 when compared to the control group, as assessed by either a Student's *t*-test or ANOVA.

mitochondrial function, was significantly enhanced following 72 h of G-Re treatment in C2C12 myotubes (Fig. 5D). Given that both GBC and its primary ingredient, Re, commonly increased mitochondrial gene expression and functionally improved mitochondrial activity, we also attempted to assess the expression of PGC-1α via Western blotting to determine whether both compounds induce PGC-1α expression. PGC-1α is well-known for its role in regulating mitochondrial biogenesis and homeostasis through the modulation of nDNA-encoded mitochondrial gene expression. As anticipated, both GBC and G-Re potentiate PGC-1α protein levels (Supplementary Fig. 2). These findings collectively and conclusively demonstrate that G-Re treatment enhances mitochondrial homeostasis, including the induction of mitophagy. This evidence highlights the crucial role of ginsenoside Re, a primary component of GBC, in promoting mitochondrial health and function, thereby contributing to cellular homeostasis and potentially extending cellular longevity.

3.7. The administration of G-Re promotes healthy aging in *C. elegans*

Previous research has shown that GBC enhances exercise capacity in mice [32], but the specific effects of G-Re have not been thoroughly explored. To address this, we investigated whether the cell-based effects of G-Re on mitophagy and mitochondrial function translate to in vivo benefits in *C. elegans*. Ryu D et al. previously demonstrated that enhancing mitophagy promotes healthy aging in nematodes [18,37]. We tested whether G-Re could extend healthspan in *C. elegans*. Lifespan assays showed that 10 μg/ml GBC extended lifespan, but higher concentrations did not offer additional benefits (Fig. 6A). G-Re supplementation increased longevity at 10 and 20 μM, with no further effects at 50 μM (Fig. 6B).

In addition to lifespan extension, we observed improvements in healthy aging metrics, including average speed, pumping rate, lipofuscin accumulation, progeny count, and stress resilience (Fig. 6C–H). G-Re significantly increased average speed at 20 and 50 μM and improved survival against mitochondrial and oxidative stress at 10, 20, and 50 μM (Fig. 6G and H). These results suggest that ginsenoside Re, the main component of GBC, drives its beneficial effects on healthy aging.

Finally, to verify that G-Re's promotion of healthy aging is attributable to the induction of mitophagy, we evaluated lifespan in the loss-of-function state of key mitophagy factors. In the absence of the *pdr-1* gene, the *C. elegans* homolog of *PARKIN*, and the *fndc-1* gene, the *C. elegans* homolog of *FUNDC1*, G-Re supplementation failed to extend lifespan (Fig. 6I and J). This indicates that ginsenoside Re-induced longevity is mediated through mitophagy.

4. Discussion

Our study significantly advances the understanding of G-Re's role in modulating mitochondrial homeostasis, which is key to promoting healthy aging. Our findings show that a single treatment with GBC or G-Re upregulates most nuclear DNA-encoded mitochondrial genes, with a notable enrichment in gene sets related to mitochondrial homeostasis, cellular senescence, and NAD^+ metabolism—key processes in aging [5]. These pathways are crucial for maintaining mitochondrial integrity, regulating aging, and supporting energy metabolism [2,14,16–19].

This study marks a significant stride in elucidating the role of G-Re in the modulation of mitochondrial homeostasis, crucial for promoting

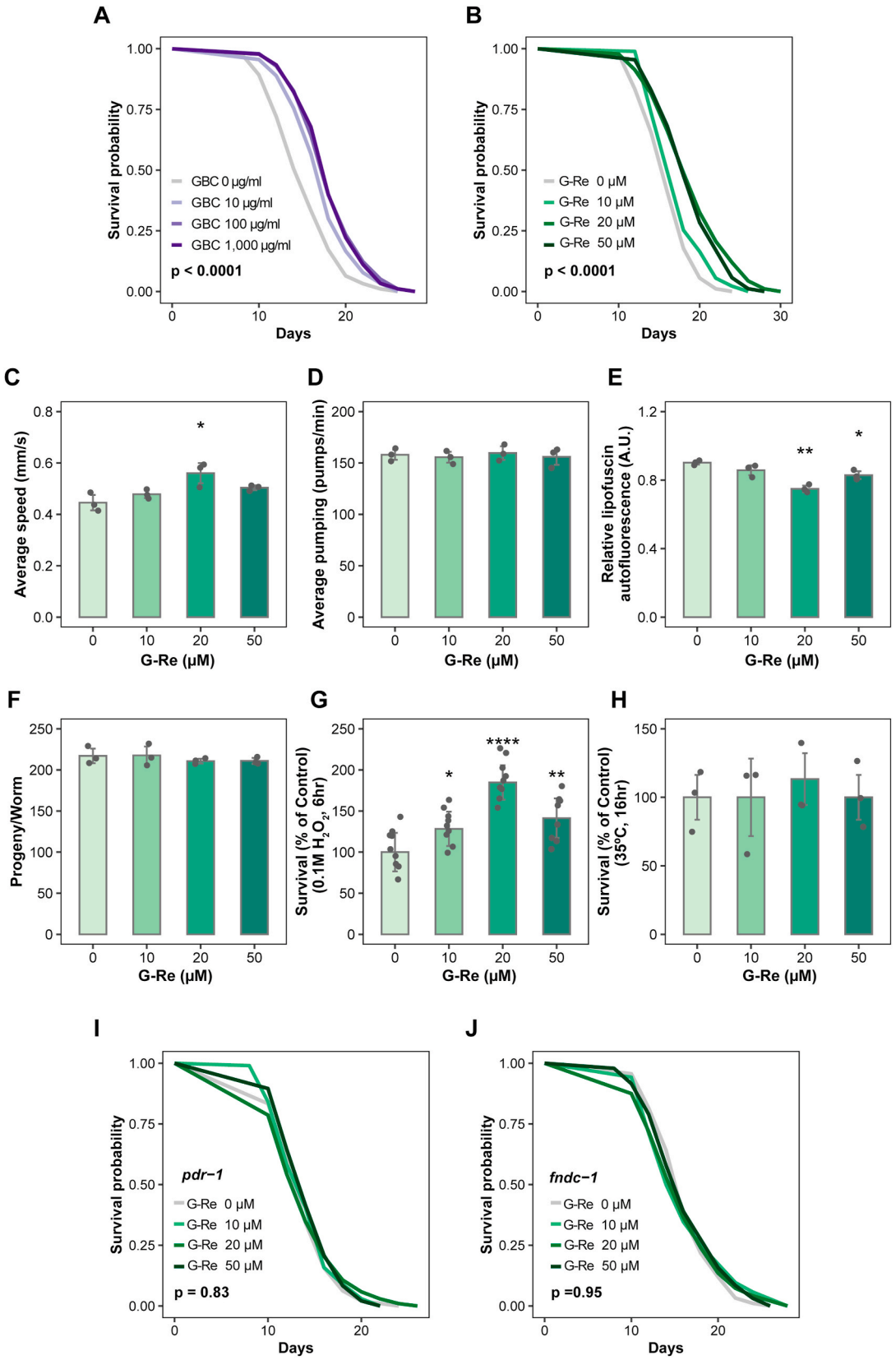


Fig. 6. G-Re enhances healthy aging via mitophagy-dependent mechanisms. (A, B) Lifespan extension effects of GBC (10, 100, 1000 μg/ml) and G-Re (10, 20, 50 μM) versus vehicle control (log-rank test). (C–H) G-Re's impact on healthspan metrics in *C. elegans*, including movement speed, pharyngeal pumping, lipofuscin accumulation, progeny production, and stress resilience under oxidative and thermal stress. (I, J) Lifespan effects of G-Re on *pdr-1(gk448)* and *fndc-1(ve633)* mutants, homologs of human PARKIN and FUNDC1, respectively (log-rank test).

healthy aging. A notable advance from our results indicates that a single treatment with GBC or G-Re alone leads to an upregulation of the majority of nuclear DNA-encoded mitochondrial genes. This upregulation transcends a mere quantitative increase in expression; it is distinguished by a significant enrichment of gene sets integral to essential cellular functions: mitochondrial homeostasis, cellular senescence, and NAD^+ metabolism—each a hallmark of aging. These pathways are well-documented as central to the maintenance of mitochondrial integrity, regulation of aging processes, and energy metabolism, respectively.

GBC, particularly G-Re, induces mitophagy and enhances mitochondrial gene expression, leading to significant improvements in muscle function and adaptability. These cellular changes translate into extended lifespan and improved healthspan in *C. elegans* treated with G-Re, evidenced by enhanced neuromuscular performance and increased stress resilience. The absence of lifespan extension in mitophagy-deficient mutants underscores that G-Re's beneficial effects are mitophagy-dependent. This directly links mitochondrial quality control to the aging process, highlighting G-Re's potential to mitigate age-related deterioration.

Our findings in *C. elegans* show that G-Re supplementation extends lifespan and enhances markers of healthy aging, such as neuromuscular function and stress resilience. These results are consistent with previous studies that link enhanced mitophagy to healthy aging in nematode models [18,37]. By boosting mitophagy, G-Re helps maintain mitochondrial function and integrity, which are crucial for cellular health and longevity, suggesting its potential as a therapeutic agent in promoting healthy aging through mitochondrial modulation.

Natural compounds like urolithin A [18,37] and spermidine [9], known to induce mitophagy, have shown promising clinical outcomes despite the complexity of identifying their precise protein targets [38–41]. Similarly, ginsenosides have been shown to induce mitophagy in various studies [42–44]. For instance, ginsenoside Rg3 promotes mitophagy through a PARKIN-dependent pathway [45], which differs from our findings. Ginsenoside Re may interact with the hypoxia response pathway [46], as indicated by changes in the FUNDC1 mitophagy receptor observed in our unpublished data. This suggests that G-Re might mediate mitophagy through FUNDC1, possibly involving hypoxia pathways [47]. Further research is needed to validate this hypothesis and clarify the underlying mechanisms.

Despite these advances, our study has limitations. The specific molecular targets of G-Re affecting mitochondrial gene expression remain unidentified, highlighting the need for future research to map its molecular influence. This understanding is essential for determining therapeutic dosages and refining G-Re for clinical use. Additionally, while upregulation of mitochondrial genes related to homeostasis, senescence, and NAD^+ metabolism is promising, translating these findings to human health and aging poses significant challenges. Future research must focus on validating these mechanisms in human cells and clinical settings to develop effective anti-aging therapies.

In summary, our study highlights the potent effects of G-Re on mitochondrial gene expression and function, with implications for aging and longevity. While promising, further investigation is required to explore the molecular mechanisms and validate G-Re's therapeutic potential in human models.

5. Conclusion

This study provides strong evidence that ginsenoside Re promotes healthy aging by inducing mitophagy and enhancing mitochondrial homeostasis. These findings deepen our understanding of ginseng-derived compounds, particularly G-Re, and their role in modulating cellular processes linked to aging. The ability of G-Re to improve mitochondrial function and extend lifespan in *C. elegans* highlights its potential as a therapeutic agent for improving healthspan and combating age-related diseases.

Author contributions

M.K., S.H.K., Y.J.C., S.M.J., and D.R. designed the project. M.K., S.H.K., and D.R. wrote the manuscript with input from co-authors. E.J.J., D.C., and W.S. conducted the wet lab experiments, and Y.J. performed dry lab analyses with assistance from C.M.O. and K.T.H. S.H.K. and D.C. prepared the ginseng berry extract. M.K., Y.J., S.M.J., and D.R. handled statistical analysis and visualization. Mitophagy activity was assessed by M.K., with technical support from E.J.J., D.C., and W.S. J.K. performed the *C. elegans* experiments.

Data availability statement

The datasets used in this study are available on the Gene Expression Omnibus (GEO) database under accession number GSE259316.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors declare no competing financial interests, except for S.H.K., J.H.R., W.G.K., and D.C., who are employed by AMOREPACIFIC Corporation.

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Abbreviations

BFA	bafilomycin A1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FDR	false discovery rate
GBC	ginseng berry concentrate
GRC	ginseng root concentrate
GSC	ginseng seed concentrate
GSEA	gene set enrichment analysis
HCS	high content screening
HTS	high throughput screening
NES	normalized enrichment score
OA	oligomycin A and antimycin A
OCR	oxygen consumption rate
G-Re	ginsenoside Re
RIPA	radioimmunoprecipitation assay
TPM	transcripts per million

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2024.11.002.

References

[1] Nguyen TT, Wei S, Nguyen TH, Jo Y, Zhang Y, Park W, et al. Mitochondria-associated programmed cell death as a therapeutic target for age-related disease. Exp Mol Med 2023;55(8):1595–619.
[2] Lima T, Li TY, Mottis A, Auwerx J. Pleiotropic effects of mitochondria in aging. Nat Aging 2022;2(3):199–213.
[3] Chen W, Zhao H, Li Y. Mitochondrial dynamics in health and disease: mechanisms and potential targets. Signal Transduct Targeted Ther 2023;8(1):333.
[4] Jin S, Lee CJ, Lim G, Park S, Lee SH, Chung JH, et al. C-reactive protein accelerates DRP1-mediated mitochondrial fission by modulating ERK1/2-YAP signaling in cardiomyocytes. BMB Rep 2023;56(12):663–8.
[5] Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. Hallmarks of aging: an expanding universe. Cell 2023;186(2):243–78.
[6] Yun J, Finkel T. Mitohormesis. Cell Metabol 2014;19(5):757–66.
[7] Chen H, Chan DC. Mitochondrial dynamics–fusion, fission, movement, and mitophagy–in neurodegenerative diseases. Hum Mol Genet 2009;18(R2):R169–76.
[8] Lemasters JJ. Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. Rejuvenation Res 2005;8(1):3–5.
[9] Eisenberg T, Abdellatif M, Schroeder S, Primessnig U, Stekovic S, Pendl T, et al. Cardioprotection and lifespan extension by the natural polyamine spermidine. Nat Med 2016;22(12):1428–38.
[10] Montgomery MK, Turner N. Mitochondrial dysfunction and insulin resistance: an update. Endocr Connect 2015;4(1):R1–15.
[11] Shin WH, Chung KC. Tollip negatively regulates mitophagy by promoting the mitochondrial processing and cytoplasmic release of PINK1. BMB Rep 2022;55(10):494–9.
[12] Amorim JA, Coppotelli G, Rolo AP, Palmeira CM, Ross JM, Sinclair DA. Mitochondrial and metabolic dysfunction in ageing and age-related diseases. Nat Rev Endocrinol 2022;18(4):243–58.
[13] Chistiakov DA, Sobenin IA, Revin VV, Orekhov AN, Bobryshev YV. Mitochondrial aging and age-related dysfunction of mitochondria. BioMed Res Int 2014;2014:238463.
[14] Andreux PA, Houtkooper RH, Auwerx J. Pharmacological approaches to restore mitochondrial function. Nat Rev Drug Discov 2013;12(6):465–83.
[15] Um JH, Yun J. Emerging role of mitophagy in human diseases and physiology. BMB Rep 2017;50(6):299–307.
[16] Picca A, Faitg J, Auwerx J, Ferrucci L, D'Amico D. Mitophagy in human health, ageing and disease. Nat Metab 2023;5(12):2047–61.
[17] Bakula D, Scheibye-Knudsen M. MitophAging: mitophagy in aging and disease. Front Cell Dev Biol 2020;8:239.
[18] Ryu D, Mouchiroud L, Andreux PA, Katsyuba E, Moullan N, Nicolet-Dit-Felix AA, et al. Urolithin A induces mitophagy and prolongs lifespan in C. elegans and increases muscle function in rodents. Nat Med 2016;22(8):879–88.
[19] Fang EF, Hou Y, Lautrup S, Jensen MB, Yang B, SenGupta T, et al. NAD(+) augmentation restores mitophagy and limits accelerated aging in Werner syndrome. Nat Commun 2019;10(1):5284.
[20] Park HW, Cho SY, Kim HH, Yun BS, Kim JU, Lee SJ, et al. Enantioselective induction of SIRT1 gene by syringaresinol from Panax ginseng berry and Acanthopanax senticosus Harms stem. Bioorg Med Chem Lett 2015;25(2):307–9.
[21] Nam Y, Ko SK, Sohn UD. Hepatoprotective effect of ultrasonicated ginseng berry extract on a rat mild bile duct ligation model. J Ginseng Res 2019;43(4):606–17.
[22] Li Z, Kim HJ, Park MS, Ji GE. Effects of fermented ginseng root and ginseng berry on obesity and lipid metabolism in mice fed a high-fat diet. J Ginseng Res 2018;42(3):312–9.
[23] Choi HS, Kim S, Kim MJ, Kim MS, Kim J, Park CW, et al. Efficacy and safety of Panax ginseng berry extract on glycemic control: a 12-wk randomized, double-blind, and placebo-controlled clinical trial. J Ginseng Res 2018;42(1):90–7.
[24] Kim M, Yi YS, Kim J, Han SY, Kim SH, Seo DB, et al. Effect of polysaccharides from a Korean ginseng berry on the immunosenescence of aged mice. J Ginseng Res 2018;42(4):447–54.
[25] Kim J, Cho SY, Kim SH, Cho D, Kim S, Park CW, et al. Effects of Korean ginseng berry on skin antipigmentation and antiaging via FoxO3a activation. J Ginseng Res 2017;41(3):277–83.
[26] Choi W, Kim HS, Park SH, Kim D, Hong YD, Kim JH, et al. Syringaresinol derived from Panax ginseng berry attenuates oxidative stress-induced skin aging via autophagy. J Ginseng Res 2022;46(4):536–42.
[27] Jeong G, Shin SH, Kim SN, Na Y, Park BC, Cho JH, et al. Ginsenoside Re prevents 3-methyladenine-induced catagen phase acceleration by regulating Wnt/beta-catenin signaling in human dermal papilla cells. J Ginseng Res 2023;47(3):440–7.
[28] Sun J, Wang R, Chao T, Peng J, Wang C, Chen K. Ginsenoside Re inhibits myocardial fibrosis by regulating miR-489/myd88/NF-kappaB pathway. J Ginseng Res 2023;47(2):218–27.
[29] Madhi I, Kim JH, Shin JE, Kim Y. Ginsenoside Re exhibits neuroprotective effects by inhibiting neuroinflammation via CAMK/MAPK/NF-kappaB signaling in microglia. Mol Med Rep 2021;24(4).
[30] Lee GH, Lee WJ, Hur J, Kim E, Lee HG, Seo HG. Ginsenoside Re mitigates 6-hydroxydopamine-induced oxidative stress through upregulation of GPX4. Molecules 2020;25(1).
[31] Ye J, Lyu TJ, Li LY, Liu Y, Zhang H, Wang X, et al. Ginsenoside Re attenuates myocardial ischemia/reperfusion induced ferroptosis via miR-144-3p/SLC7A11. Phytomedicine 2023;113:154681.
[32] Jin EJ, Wei S, Jo Y, Nguyen TT, Ji M, Paik MJ, et al. Oral administration of ginseng berry concentrate improves lactate metabolism and increases endurance performance in mice. BMB Rep 2023;56(6):353–8.
[33] Kim K, Ryu D, Dongiovanni P, Ozcan L, Nayak S, Ueberheide B, et al. Degradation of PHLPP2 by KCTD17, via a glucagon-dependent pathway, promotes hepatic steatosis. Gastroenterology 2017;153(6):1568–1580 e10.
[34] Kim J, Jo Y, Cho D, Ryu D. L-threonine promotes healthspan by expediting ferritin-dependent ferroptosis inhibition in C. elegans. Nat Commun 2022;13(1):6554.
[35] Kim J, Lee H, Jin EJ, Jo Y, Kang BE, Ryu D, et al. A microfluidic device to fabricate one-step cell bead-laden hydrogel struts for tissue engineering. Small 2022;18(1):e2106487.
[36] Rath S, Sharma R, Gupta R, Ast T, Chan C, Durham TJ, et al. MitoCarta3.0: an updated mitochondrial proteome now with sub-organelle localization and pathway annotations. Nucleic Acids Res 2021;49(D1):D1541–7.
[37] Zhang Y, Wei S, Zhang H, Jo Y, Kang JS, Ha KT, et al. Gut microbiota-generated metabolites: missing puzzles to hosts' health, diseases, and aging. BMB Rep 2024;57(5):207–15.
[38] Schwarz C, Benson GS, Horn N, Wurdack K, Grittner U, Schilling R, et al. Effects of spermidine supplementation on cognition and biomarkers in older adults with subjective cognitive decline: a randomized clinical trial. JAMA Netw Open 2022;5(5):e2213875.
[39] Singh A, D'Amico D, Andreux PA, Fouassier AM, Blanco-Bose W, Evans M, et al. Urolithin A improves muscle strength, exercise performance, and biomarkers of mitochondrial health in a randomized trial in middle-aged adults. Cell Rep Med 2022;3(5):100633.
[40] Liu S, D'Amico D, Shankland E, Bhayana S, Garcia JM, Aebischer P, et al. Effect of urolithin A supplementation on muscle endurance and mitochondrial health in older adults: a randomized clinical trial. JAMA Netw Open 2022;5(1):e2144279.
[41] Andreux PA, Blanco-Bose W, Ryu D, Burdet F, Ibberson M, Aebischer P, et al. The mitophagy activator urolithin A is safe and induces a molecular signature of improved mitochondrial and cellular health in humans. Nat Metab 2019;1(6):595–603.
[42] Li Y, Li J, Yang L, Ren F, Dong K, Zhao Z, et al. Ginsenoside Rb1 protects hippocampal neurons in depressed rats based on mitophagy-regulated astrocytic pyroptosis. Phytomedicine 2023;121:155083.
[43] Wang N, Yang J, Chen R, Liu Y, Liu S, Pan Y, et al. Ginsenoside Rg1 ameliorates Alzheimer's disease pathology via restoring mitophagy. J Ginseng Res 2023;47(3):448–57.
[44] Wang X, Ling G, Wei Y, Li W, Zhang Y, Tan N, et al. Activation of ULK1 to trigger FUNDC1-mediated mitophagy in heart failure: effect of Ginsenoside Rg3 intervention. Phytomedicine 2023;120:155042.
[45] Sun X, Hong Y, Shu Y, Wu C, Ye G, Chen H, et al. The involvement of Parkin-dependent mitophagy in the anti-cancer activity of Ginsenoside. J Ginseng Res 2022;46(2):266–74.
[46] Sun H, Ling S, Zhao D, Li J, Li Y, Qu H, et al. Ginsenoside Re treatment attenuates myocardial hypoxia/reoxygenation injury by inhibiting HIF-1alpha ubiquitination. Front Pharmacol 2020;11:532041.
[47] Liu L, Feng D, Chen G, Chen M, Zheng Q, Song P, et al. Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells. Nat Cell Biol 2012;14(2):177–85.