Animal Science Papers and Reports vol. 43 (2025) no. 4, 385-404
DOI: 10.2478/aspr-2025-0026
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Ginger supplementation supports liver health during aging through regulation of lysosomal function and molecular markers

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(Accepted September 30, 2025)

Aging is marked by hepatic structural deterioration, inflammation, and impaired autophagy. This study evaluated the effects of dietary ginger (*Zingiber officinale*) on liver aging in Swiss Webster mice across three age groups (3, 6, and 12 months) supplemented with 0.6% or 1.8% ginger powder for three months. Liver samples were assessed for lysosomal enzyme activity, expression of PI3K and TNF-α, histology, and ultrastructure of hepatocytes.

Aging induced hepatocellular hypertrophy, mitochondrial damage, and increased lysosomal enzyme activity, particularly arginyl aminopeptidase and acid phosphatase, and β-galactosidase - a widely recognized marker of aging, particularly cellular senescence. Ginger supplementation preserved liver morphology in young and middle-aged mice, while high doses showed mild subcellular stress in older mice. Ginger also suppressed age-related PI3K upregulation and influenced TNF-α expression, indicating potential anti-inflammatory effects.

These findings suggest that ginger modulates hepatic aging through lysosomal, inflammatory, and metabolic pathways in an age- and dose-dependent manner. Moderate intake appears most beneficial, supporting its potential as a nutraceutical for liver health during aging.

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KEY WORDS: hepatic aging / ginger / lysosomal enzymes / PI3K / nutraceuticals

Aging is a complex biological process characterized by the progressive loss of physiological integrity, leading to organ dysfunction with an increased vulnerability to chronic diseases, including metabolic, neurodegenerative, and inflammatory conditions [López-Otín *et al*. 2013]. At the cellular level, aging is defined by various well-established hallmarks, including genomic stability, cellular senescence, altered intercellular communication, mitochondrial dysfunction, impaired autophagy, and loss of proteostasis [Kennedy *et al*. 2014]. Among these, chronic inflammation and impaired autophagic-lysosomal function play central roles in hepatic aging by deteriorating the turnover of damaged organelles and promoting oxidative stress [Rubinsztein *et al*. 2011]. The liver, as a primary metabolic and detoxifying organ, is highly susceptible to age-related changes, was selected in this study for comprehensive analysis due to its central role in systemic aging and metabolic regulation. Thus, exploring therapeutic strategies that can modulate age-related pathways could lead to promising interventions for promoting healthy aging.

In recent years, the use of natural compounds with antioxidant and anti-inflammatory properties has shown a growing interest as potential agents to counteract the effects of aging [Bensz *et al*. 2025, Cătană *et al*. 2018]. Among these, ginger *Zingiber officinale* stands out as a widely consumed medicinal plant with a wide range of bioactive compounds such as gingerols, shogaols, and paradols, demonstrating anti-inflammatory, antioxidant, and hepatoprotective effects in various experimental models [Mao *et al*. 2019, Matin *et al*. 2024b, Shanmugam *et al*. 2011]. We previously demonstrated in a mouse model that dietary ginger (0.6 and 1.8%) modulates hepatic antioxidant defenses in a clear dose- and age-dependent manner, showing increased Glutathione level (GSH) and total antioxidant capacity, and lowered Malondialdehyde level (MDA), with the strongest benefits in older animals [Matin *et al*. 2025]. Emerging studies also suggest that the ginger compounds can modulate autophagy, reduce oxidative damage, and impede pro-senescent signaling cascades [Matin *et al*. 2024a]. However, the potential effect of ginger supplementation to influence age-associated cellular and molecular hallmarks, particularly on lysosomal dysfunction, gene expression, and ultrastructure in the context of aging mice in liver tissue, remains underexplored. Given the rising interest in nutraceuticals for healthy aging, it is critical to understand how dietary ginger might possess a key influence on the age-related pathways in a tissue-specific and age-dependent manner.

To address this gap, the present study was designed to investigate the potential effect of ginger supplementation to modulate the hallmarks of aging in the liver of mice across different age groups. This age-stratified approach allows for the evaluation of age- and dose-dependent effects of ginger on the physiology of the liver and molecular signaling. Specifically, an investigation on the activity of lysosomal enzymes (aminopeptidases and glycosidases) was undertaken to determine how ginger affects the lysosomal system across different age groups. Furthermore, gene expression analyses of tumor necrosis factor-alpha TNF-α (a pro-inflammatory cytokine linked

to age-related inflammation and immune dysfunction) and phosphoinositide 3-kinase PI3K (a critical node in the insulin/IGF-1 signaling pathway, implicated in longevity and metabolic homeostasis) were conducted to explore the impact of ginger on key inflammatory and metabolic pathways. Of relevance, a prior study by Kakino et al. (2018) reported increased hepatic TNF-α mRNA expression in aged mice, which was associated with enhanced NF-κB activation and inflammatory gene expression [Kakino *et al*. 2018]. Furthermore, aging is associated with decreased PI3K activity in liver tissues, leading to impaired downstream signaling and glucose metabolism [Braccini *et al*. 2015].

In addition to biochemical and molecular analyses, histological and ultrastructural studies on mice liver were performed in our study to visualize the morphological changes associated with aging and their modulation by ginger. Ultrastructure analyses have previously revealed that aging hepatocytes exhibit significant morphological alterations, including mitochondrial swelling, increased lipofuscin accumulation, and disrupted endoplasmic reticulum [Łysek-Gładysińska *et al*. 2021]. Thus, a study by Terman and Brunk [2004] highlighted such changes suggesting the impaired autophagy contributes to the accumulation of damaged organelles in aged liver [Terman and Brunk 2004]. By correlating these microscopic findings with biochemical and molecular markers, our study aims to investigate age- and dose-dependent effects of ginger influencing hepatic aging at multiple biological levels.

Material and methods

Experimental setup

In this study, 144 male Swiss Webster mice were used, categorized into three age groups (Tab. 1).

Table 1. Experimental animal groups

Ginger dose/age	3-month-old mice	6-month-old mice	12-month-old mice
Standard diet	control 3 months old, n = 16	control 6 months old, n = 16	control 12 months old, n = 16
0.6% ginger diet	ginger 0.6%, 3 months old, n = 16	ginger 0.6%, 6 months old, n = 16	ginger 0.6%, 12 months old, n = 16
1.8% ginger diet	ginger 1.8%, 3 months old, n = 16	ginger 1.8%, 6 months old, n = 16	ginger 1.8%, 12 months old, n = 16

Only male mice were used to avoid variations in liver physiology and enzyme activities associated with hormonal changes throughout the female estrous cycle. This strategy was intended to reduce biological variability and support clearer interpretations of the age- and dose-dependent outcomes of ginger supplementation.

Animals were housed in groups of four per standard cage under controlled environmental conditions (22°C, 12-hour light/dark cycles), with food and water

available ad libitum. Over the course of the 3-month supplementation, animals were fed either a standard diet (Control group) or the same base diet enhanced with 0.6 or 1.8% dried ginger powder. The ginger powder, standardized to contain 5% gingerols, was sourced from Sabinsa Corporation (20 Lake Drive, East Windsor, NJ 08520, USA). Fresh supplemented feed was prepared twice weekly. Upon completion of the supplementation period, fresh liver tissues of mice were taken following the American Veterinary Medical Association (AVMA) Guidelines (2020) and processed for further investigation of biochemical and analytical procedures.

All procedures involving animals were conducted in the frame of animal handling permission No. 14186201 in accordance with the relevant institutional and national regulations (including the EU Directive 2010/63/EU for animal experiments and National Research Council's Guide for the Care and Use of Laboratory Animals). The chosen dietary inclusion rates of ginger (0.6 and 1.8%) were informed by earlier rodent studies demonstrating the efficacy of ginger in improving liver function. Prior research has reported relevant bioactivities with dietary ginger concentrations ranging from 1 to 2% [Ahmed *et al*. 2008, Mallikarjuna *et al*. 2008, Shanmugam *et al*. 2011].

Tissue preparation for morphological examination

Liver fragments were promptly excised from mice upon the feeding experiment termination. The samples were finely chopped and immersed in a fixative consisting of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for two hours at 4°C. Following primary fixation, the tissue fragments were rinsed in the same buffer, followed by post-fixation in a 1% osmium tetroxide solution prepared in 0.1 M cacodylate buffer for two hours at ambient temperature, protected from light. Subsequently, the specimens were washed with buffer and dehydrated using a graded ethanol series, with each step lasting 10 minutes. Dehydrated tissues were infiltrated and embedded in epoxy resin (Agar 100) using propylene oxide as an intermediary solvent, following standard protocols. The embedded samples were polymerized in silicone molds at 60°C for 48 hours, resulting in hardened resin blocks containing the preserved tissue.

Sectioning and staining for light and electron microscopy

Embedded liver samples were first trimmed into semithin sections to survey broader tissue architecture under a light microscope, followed by the preparation of ultrathin sections for detailed ultrastructural analysis using transmission electron microscopy (TEM). Semithin sections, ranging from 0.5 to 1 μm in thickness, and ultrathin sections of approximately 50-60 nm were obtained using a Reichert-Jung ultramicrotome equipped with either dry or wet glass knives.

Semithin slices were floated onto water droplets on glass slides and then dried by placing the slides on a hot plate at 60°C to facilitate adhesion. These sections were stained at 80°C with Sudan III and aniline blue. After staining, slides were thoroughly rinsed under running water, air-dried, mounted with Histokitt, and examined under a

light microscope. Selected fields from each section were photographed using a Nikon Eclipse 80i microscope equipped with Nikon NIS Elements D 3.10 imaging software (Nikon Instruments, Inc., NY, USA).

Ultrathin sections, characterized by their grey-to-silver interference color and 50-60 nm thickness, were transferred to 300-mesh copper grids. These grids were then double-stained with saturated aqueous uranyl acetate and lead citrate. The stained samples were observed using a TESLA BS500 transmission electron microscope. High-resolution images were captured with a Variospeed 1K-SSCCD slow scan camera (TRS, Moorenweis, Germany) and processed using the associated image acquisition software.

Determination of enzyme activities related to carbohydrate metabolism, aminopeptidases, and acid phosphatase

The activities of enzymes involved in carbohydrate metabolism, lysosomal function, and protein degradation were analyzed using specific substrates obtained from Sigma-Aldrich (St. Louis, MO, USA). Lysosomal enzyme activity measurements were performed following the methodology established by Barrett and Heath (1977). Specifically, assays were conducted for β-glucuronidase (BGRD; EC 3.2.1.31), α-galactosidase (AGAL; EC 3.2.1.22), β-glucosidase (BGLU; EC 3.2.1.21), α-glucosidase (AGLU; EC 3.2.1.20), β-galactosidase (BGAL; EC 3.2.1.23), β-N-acetylhexosaminidase (HEX; EC 3.2.1.52), and mannosidase (MAN; EC 3.2.1.24). Each enzyme was incubated with its corresponding chromogenic substrate at 37°C: p-nitrophenyl-β-D-glucuronide, p-nitrophenyl-α-D-galactopyranoside, p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-N-acetyl-β-D-glucosaminide, p-nitrophenyl-β-D-mannopyranoside, and p-nitrophenyl-α-D-glucopyranoside [Barrett and Heath 1977]. Acid phosphatase (ACP; EC 3.1.3.2) activity was evaluated by monitoring the conversion of a 4-nitrophenyl phosphate derivative. The absorbance was measured at 420 nm using a Cary 50 Bio UV-VIS spectrophotometer (Varian, part of Agilent Technologies, Mulgrave, VA, Australia), following the same protocol described by Barrett and Heath [Barrett and Heath 1977].

The activities of three aminopeptidases - alanyl aminopeptidase (AlaAP; EC 3.4.11.2), leucyl aminopeptidase (LeuAP; EC 3.4.11.1), and arginyl aminopeptidase (ArgAP; EC 3.4.11.6) were quantified in the prepared supernatants using the method reported by McDonald and Barrett [McDonald and Barrett 1986]. These assays employed Fast Blue BB salt (4-benzoylamino-2,5-diethoxybenzene-diazonium chloride) as the chromogenic reagent, and absorbance was measured at 540 nm with the same Cary 50 Bio spectrophotometer. All enzyme activities were standardized and reported as nanomoles per milligram of total protein per hour (nmol/mg protein/h).

RNA extraction, cDNA synthesis, and quantitative Real-Time PCR (qRT-PCR)

Liver tissue samples were processed to extract total RNA using the RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's guidelines. The RNA concentration and purity were assessed using a NanoDrop™ 2000

Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) by measuring absorbance at 230, 260, and 280 nm. Isolated RNA samples were stored at -80°C until further processing. For cDNA synthesis, 1 µg of total RNA from each sample was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. The reverse transcription reaction (20 µL total volume) was conducted with the following thermal profile: 5 minutes at 25°C, 20 minutes at 46°C, and 1 minute at 95°C.

Quantitative real-time PCR (qRT-PCR) was carried out using the CFX Opus 96 thermal cycler (Bio-Rad, Richmond, CA, USA). Each 20 µL PCR reaction contained 10 µL of 2X SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 500 nM of primers specific to phosphoinositide 3-kinase (PI3K) or tumor necrosis factor alpha (TNF), and 10 ng of cDNA template. The qPCR cycling conditions were initial denaturation at 95°C for 30 seconds, followed by 40 cycles consisting of 95°C for 15 seconds and 60°C for 30 seconds for annealing and extension. A Ct value greater than 37 was considered indicative of a negative result for target gene expression.

A negative control from a healthy mouse in the 3-month control group was designated as the calibrator. Gene expression levels were normalized to the housekeeping gene GAPDH and calculated using the 2-ΔΔCT method as described by Ruiz-Villalba *et al*. [2017] and He *et al*. [2024]. Data were normalized and analyzed using CFX Maestro V2.2 software (Bio-Rad). Each gene expression measurement was conducted in technical triplicate, and biological replicates were derived from independent cell cultures.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism version 9.0.0 (GraphPad Software, Boston, MA, USA). A significance level of $p \leq 0.05$ was considered statistically significant throughout all the analyses. Multi-factorial ANOVA (multiplate ANOVA) was used to evaluate the effects of age and ginger dosage on enzyme activity and gene expression. When significant differences were identified, Duncan's multiple range test was used for post hoc comparisons.

Fisher's test was applied to assess the variance among experimental groups. Pearson correlation analysis was conducted to determine associations between biochemical and molecular parameters. Additionally, linear regression models were used to evaluate age- and dose-dependent effects on hepatic outcomes. Results are presented as least squares means±standard deviation (LSM±SD), and group differences are annotated using superscript letters indicating statistical significance.

Results and discussion

Morphological analysis

As evident from Figure 1 and 2 histological and ultrastructural analysis of liver tissue revealed progressive age-dependent changes in hepatocyte morphology and subcellular organization. In 3-month-old (3M) control mice, hepatocytes appeared

uniformly polygonal with centrally located nuclei, abundant cytoplasmic organelles, and small lipid droplets. Sinusoids were patent, and hepatic architecture was well-preserved. Transmission electron microscopy (TEM) (as shown in Fig. 2), confirmed the presence of nuclei with normal distribution of eu- and heterochromatin, with an active nucleolus and correct nuclear envelope, numerous oval mitochondria with normal matrix density, abundant rough endoplasmic reticulum (RER), numerous peroxisomes, primary lysosomes, and glycogen granules, indicating robust metabolic activity.

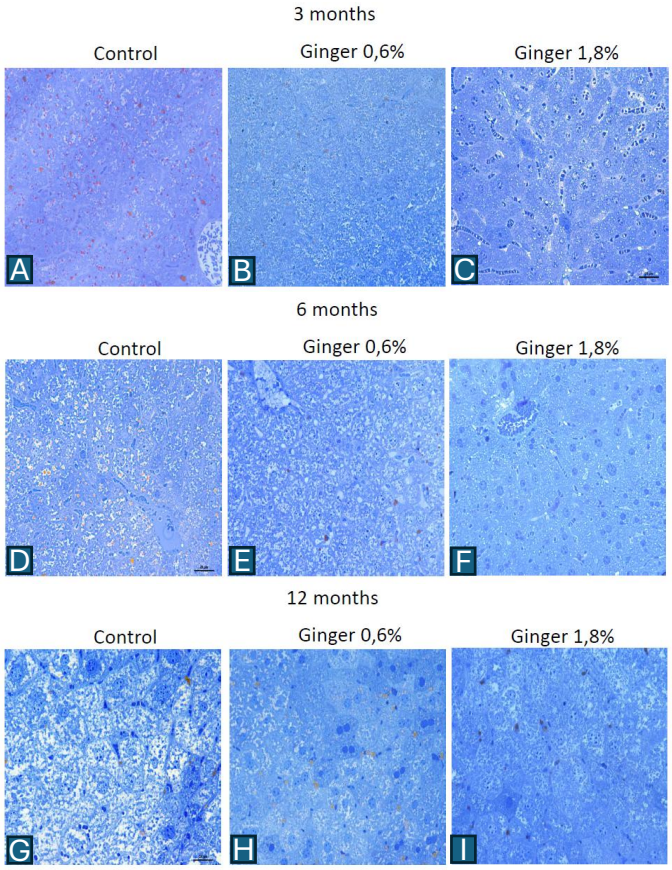

Fig. 1. Histological evaluation of liver tissue architecture in mice across age and dietary groups. Representative light microscopy images of liver sections stained with Sudan III and aniline blue, highlighting morphological features across experimental conditions. The upper panel shows 3-month-old mice: (A) control, (B) 0.6% ginger-supplemented, (C) 1.8% ginger-supplemented. The middle panel depicts 6-month-old mice: (D) control, (E) 0.6% ginger-supplemented, (F) 1.8% ginger-supplemented. The lower panel includes 12-month-old mice: (G) control, (H) 0.6% ginger-supplemented, (I) 1.8% ginger-supplemented. Notable features include hepatocyte morphology, lipid droplet accumulation, and sinusoidal organization. Scale bar = 20 μm.

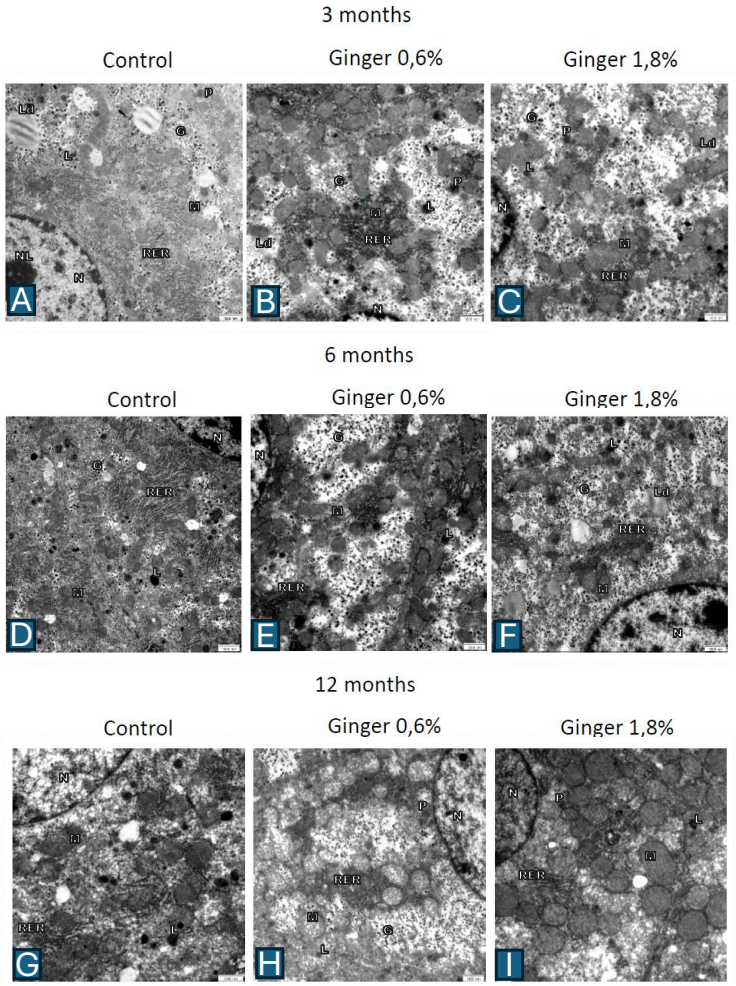

Fig. 2. Transmission electron micrographs showing age- and diet-associated ultrastructural alterations in hepatocytes. Electron micrographs of liver sections from mice at different ages and treatment groups. The upper panel illustrates 3-month-old mice: (A) control, (B) 0.6% ginger-supplemented, (C) 1.8% ginger-supplemented. The middle panel shows 6-month-old mice: (D) control, (E) 0.6% ginger-supplemented, (F) 1.8% ginger-supplemented. The lower panel presents 12-month-old mice: (G) control, (H) 0.6% ginger-supplemented, (I) 1.8% ginger-supplemented. Labeled organelles include lipid droplets (Ld), lysosomes (L), mitochondria (M), damaged mitochondria (dM), nucleus (N), nucleolus (NL), peroxisomes (P), rough endoplasmic reticulum (RER), and vacuoles (V). Scale bar = 1000 nm.

By 6 months (6M), the control livers maintained their general structure, but early signs of cellular aging were evident. Some hepatocytes displayed lightly transparent cytoplasm with fewer visible organelles, though mitochondria and RER remained structurally intact. At 12 months (12M), age-associated changes became

more pronounced. Hepatocytes were often enlarged, with variable nuclear sizes and increased binucleation. Lipid droplets were more numerous, especially visible in Ito cells, and glycogen reserves were reduced. Ultrastructural analysis revealed mitochondrial enlargement and swelling, shortened cristae, and reduced RER, all signs of declining biosynthetic and energy-related functions. Primary lysosomes exhibit an increase in both number and size compared to those of younger mice.

Ginger supplementation influenced these age-related patterns in an age- and dose-dependent manner. In young mice, both 0.6 and 1.8% ginger diets preserved normal hepatocyte morphology, with minimal ultrastructural deviations. Occasional cells in the high-dose group displayed mildly enlarged mitochondria and slightly reduced RER content, but overall organelle integrity was maintained. At 6M, low-dose ginger maintained cellular architecture similar to controls, while high-dose ginger resulted in greater variability in hepatocyte size and cytoplasmic clarity. Ultrastructurally, RER was slightly diminished, and glycogen accumulation was more localized.

In aged mice (12M), the 0.6% ginger diet was associated with moderate ultrastructural alterations, including mitochondrial enlargement and swelling and RER depletion, although lipid droplet accumulation was not exacerbated. The 1.8% ginger group exhibited more substantial changes: mitochondria appeared greatly enlarged, swollen and vacuolated, RER was scarce, and cytoplasmic areas devoid of organelles were common, filled mainly with residual glycogen. The number of primary lysosomes was reduced compared to a control. Despite these subcellular changes, overall tissue architecture remained intact in all groups, suggesting that while high-dose ginger may intensify age-related cellular stress, it does not induce overt hepatocellular damage.

Enzyme activity

Lysosomal enzyme activity was significantly influenced by both age and diet (Tab. 2). Aging alone resulted in elevated activity of several key lysosomal enzymes. Among them, arginyl aminopeptidase (ArgAP) showed a marked and consistent increase with age, particularly evident in 12M controls. Acid phosphatase (ACP), a general marker of lysosomal hydrolysis, and α-galactosidase (AGAL), involved in glycoside metabolism, also exhibited clear age-dependent increases. These patterns suggest an upregulation of degradative lysosomal function as a compensatory response to accumulating macromolecular waste in aging cells.

Not all enzymes changed with age. Leucyl aminopeptidase (LeuAP), for instance, remained stable across time points, indicating that age effects are enzyme-specific. Other glycosidases, such as β-galactosidase (BGAL) and β-glucosidase (BGLU) exhibited increases by 12M, though less consistently across groups.

Ginger supplementation modulated enzyme activity in complex ways that depended on both dose and age. At 3M, 0.6% ginger significantly increased the activity of enzymes such as ArgAP and AGAL compared to controls. This suggests that moderate ginger intake may stimulate lysosomal turnover and enhance protein and glycoside catabolism early in life. However, increasing the dose to 1.8% did not

Table 2. Impact of ginger supplementation on the liver enzyme activities (nmol/mg of protein/hour) and gene expression across the different age groups

Factors		3M			6M			12M			F-test	p-value
		control	EX1	Ex2	control	EX1	Ex2	control	EX1	Ex2		
AlaAP	LSM	308.92^{A}	483.39^{B}	258.18^{A}	$223.15^{A}c$	404.00^{Bd}	267.72^{Ac}	364.16^{BEd}	337.97^{ABEcd}	313.66^{ABEcd}	18.754	0.000
	SD	60.03	100.20	53.28	34.51	32.56	39.52	51.85	26.88	30.93		
LeuAP	LSM	407.97^{A}	397.39^{A}	340.38^{A}	293.37^{A}	386.30A	343.37^{A}	529.65^{b}	478.26^{Abc}	419.66^{Abc}	6.759	0.039
	SD	106.39	55.13	44.90	36.30	67.37	70.85	122.29	99.29	58.93		
ArgAP	LSM	259.03^{A}	253.88^{A}	225.25^{A}	204.43^{A}	239.97	219.12^{A}	500.29^{B}	442.08^{BC}	410.11^{BCD}	68.818	0.003
	SD	39.70	29.84	33.81	21.17	14.90	24.64	46.05	75.05	23.23		
ACP	LSM	2825.75^{A}	3443.68^{A}	3005.52^{A}	2271.12^{AB}	3606.84^{A}	2932.32^{AB}	3561.16^{AB}	2780.34^{AB}	2710.24^{AB}	4.388	0.000
	SD	323.12	613.73	312.20	135.93	1393.14	599.19	352.63	394.23	237.77		
HEX	LSM	710.72^{A}	703.30^{A}	702.15^{A}	594.91^{A}	749.23^{A}	613.30^{A}	820.35^{AD}	1001.58^{B}	917.71^{ABCD}	4.388	0.000
	SD	47.67	76.04	57.66	117.98	210.32	81.11	93.42	44.72	86.02		
AGAL	LSM	70.85^{a}	92.23^{b}	70.95^{a}	76.57^{ab}	119.76^{C}	89.33^{ab}	130.79^{CD}	122.14^{CDE}	129.41^{CDEF}	32.49	0.000
	SD	5.17	11.20	6.27	10.49	11.85	7.58	13.68	19.84	18.31		
BGAL	LSM	258.41^{A}	308.67^{A}	229.97^{E}	198.10^{Eb}	288.78^{A}	238.42^{AEFb}	349.06^{AC}	329.27^{ACD}	306.51^{ACD}	18.186	0.000
	SD	20.53	31.82	43.22	22.57	45.54	37.94	29.57	38.29	17.99		
AGLU	LSM	211.90^{a}	267.05^{b}	199.29^{a}	175.21^{a}	269.36^{bc}	230.10^{abc}	211.40^{abc}	197.89^{abc}	229.30^{abc}	7.202	0.000
	SD	27.45	33.06	34.84	30.34	40.50	41.35	12.21	47.21	12.10		
BGLU	LSM	59.77^{A}	78.63^{B}	52.86^{A}	47.71^{Ac}	64.92^{d}	49.71^{Ac}	67.76^{ABd}	64.65^{ABde}	65.26^{ABdef}	15.237	0.000
	SD	9.14	5.86	5.24	6.55	6.83	8.10	5.13	8.30	7.86		
BGRD	LSM	223.69^{A}	197.92^{A}	196.44^{A}	143.00^{B}	168.82^{ABC}	155.21^{ABCD}	209.35^{A}	193.03^{ABC}	177.17^{ABCDE}	13.691	0.002
	SD	30.98	19.94	15.12	13.15	13.03	9.78	22.20	31.42	7.98		
MAN	LSM	257.16^{A}	299.42^{A}	208.11^{b}	206.50^{bc}	$291.27A^{bc}$	208.83^{Abcd}	337.65^{Abc}	295.46^{Abc}	314.13^{Abc}	7.068	0.006
	SD	48.49	48.41	55.92	69.56	78.84	66.83	30.48	29.03	25.76		
PI3K	LSM	0.976^{A}	0.954^{A}	1.014^{A}	2.179^{B}	0.869^{AC}	0.684^{D}	2.409^{E}	0.606^{DF}	0.191^{G}	1363.694	0.000
	SD	0.028	0.019	0.021	0.095	0.026	0.019	0.118	0.047	0.008		
TNF	LSM	1.02^{A}	1.06^{A}	1.01^{A}	1.70^{B}	0.96^{A}	3.42^{C}	2.13^{D}	0.95^{A}	2.68^{E}	420.202	0.000
	SD	0.040	0.020	0.110	0.130	0.020	0.170	0.110	0.050	0.250		

LSM – least squares mean; SD – standard deviation; control – control group; EX1 – experimental group 1 (0.6%); EX2 – experimental group 2 (1.8%); F-test – Fisher test; p-value – statistically significant value.

$^{Aa...}$Statistically significant differences: small letters – $p \leq 0.05$; capitals –$p \leq 0.01$; groups that share the same letter (e.g., both marked A or a) are not significantly different from one another.

further enhance these enzyme activities, and in some cases, levels returned closer to control values indicative of a threshold or adaptive response.

At 6M, low-dose ginger increased ACP and AGAL activities while reducing ArgAP, implying a selective enhancement of glycosidase over protease activity. The 1.8% dose elicited similar but less pronounced changes, possibly reflecting metabolic compensation or saturation. In older mice (12M), enzyme activities in ginger-fed animals were generally lower or equal to those in age-matched controls. For example, ArgAP and ACP were slightly reduced in both ginger groups compared to 12M controls. This may reflect ginger's potential to normalize or moderate overactive lysosomal function in aged tissue, potentially by reducing inflammatory or oxidative stress signals that drive enzyme upregulation. Increased activity of specific lysosomal enzymes enhances some autophagy, which plays a protective role by preventing the onset of inflammation and oxidative stress [Butler and Bahr, 2006].

Taken together, the data indicate that ginger exerts both stimulatory and regulatory effects on hepatic lysosomal enzymes. These effects are more pronounced in younger animals and tend to stabilize or reduce enzyme activity in older mice, especially at the higher dose. The 0.6% dose often had a more favorable impact, supporting the idea that moderate ginger intake may help maintain lysosomal function without over activation.

Gene expression

Gene expression analyses of PI3K and TNF-**α** (Tab. 2) provided insight into the molecular pathways affected by aging and ginger supplementation. In control mice, PI3K mRNA levels increased in with the advancement of age in the control animals, and this effect was counteracted in a dose-dependent manner from ginger supplementation. TNF-**α** expression, a marker of inflammatory signaling, increased by 12 months, consistent with a low-grade, chronic inflammatory state in aging livers.

Ginger supplementation induced more distinct changes. PI3K expression was significantly suppressed in a dose-dependent manner, especially in mice receiving the 1.8% diet. This suppression was consistent across ages, indicating a robust dietary effect on this key metabolic regulator. Although the physiological implications remain to be fully elucidated, reduced PI3K signaling could reflect altered autophagic or insulin-responsive pathways.

In contrast, TNF-α expression was consistently reduced in ginger-fed mice, with the most significant suppression observed in the 6M and 12M groups receiving the lower dose. These findings support ginger's known anti-inflammatory effects and suggest a potential role in attenuating age-related inflammatory signaling in hepatic tissue.

The aging process is influenced by an increase in PI3K gene expression, which may be linked to liver injury and the decrease in function of the liver cell [Dostert *et al*. 2012]. While PI3K activity supports cellular survival mechanisms, its over activation can lead to detrimental effects, including apoptosis of the cell and damage of the organelles. Research suggests that reducing PI3K activity can promote longevity and potentially delay aging-related processes [Hedges *et al*. 2023].

Regression analysis

Linear regression confirmed aging as a major driver of changes in lysosomal enzyme activity. In Table 3 ArgAP and AGAL showed the strongest correlations with age (R^2 values of 0.70 and 0.60, respectively), consistent with their steady increase across the lifespan. ACP, HEX, and BGAL also demonstrated moderate positive associations with age. TNF-α had a weaker but significant correlation with age (R^2 ~0.13), while PI3K expression showed no statistically significant age-related trend.

Table 3. Linear Regression (influences of aging)

Factor	R_squared	p_value	Slope	Intercept
AlaAP	0.0	0.961	-0.142	330.013
LeuAP	0.206	0.0	12.158	314.488
ArgAP	0.7	0.0	24.971	131.222
ACP	0.001	0.818	-5.17	3051.409
HEX	0.375	0.0	26.0	575.023
AGAL	0.595	0.0	5.476	61.893
BGAL	0.28	0.0	8.021	222.427
AGLU	0.018	0.258	-1.545	232.092
BGLU	0.025	0.184	0.484	57.865
BGRD	0.002	0.739	-0.329	187.263
MAN	0.175	0.0	7.705	214.789
PI3K	0.001	0.851	0.004	1.069
TNF	0.128	0.002	0.082	1.086

Regression with ginger dose as the predictor (Tab. 4) revealed that PI3K expression decreased significantly with increasing ginger concentration ($R^2 = 0.61$, $p<0.001$), making it the most diet-sensitive parameter. AGLU activity also rose modestly with dose, while other enzymes showed no consistent linear dose response, reflecting the previously observed non-linear or age-specific effects of ginger. These results confirm that aging is a broad modulator of lysosomal and inflammatory markers, while ginger exerts more targeted, pathway-specific effects.

Table 4. Linear Regression (influences of diet)

Factor	R_squared	p_value	Slope	Intercept
AlaAP	0.057	0.044	45.41	298.744
LeuAP	0.006	0.527	-16.105	410.329
ArgAP	0.009	0.42	-22.85	321.249
ACP	0.017	0.274	193.813	2886.01
HEX	0.046	0.069	72.554	708.656
AGAL	0.04	0.093	11.235	92.735
BGAL	0.016	0.294	15.078	268.524
AGLU	0.129	0.002	32.657	199.506
BGLU	0.031	0.14	4.259	58.413
BGRD	0.026	0.174	-10.585	192.016
MAN	0.0	0.89	2.433	267.103
PI3K	0.607	0.0	-1.135	1.855
TNF	0.001	0.776	0.062	1.618

Correlation analysis

As evident from Figure 3 correlation mapping revealed networks of co-regulated biochemical markers. Lysosomal enzymes such as ArgAP, AGAL, and ACP were positively correlated with one another, forming a cluster indicative of synchronized age-related upregulation. TNF also showed moderate positive correlations with this cluster (with the exception of ACP), suggesting that inflammatory signaling may accompany lysosomal activation in the aging liver.

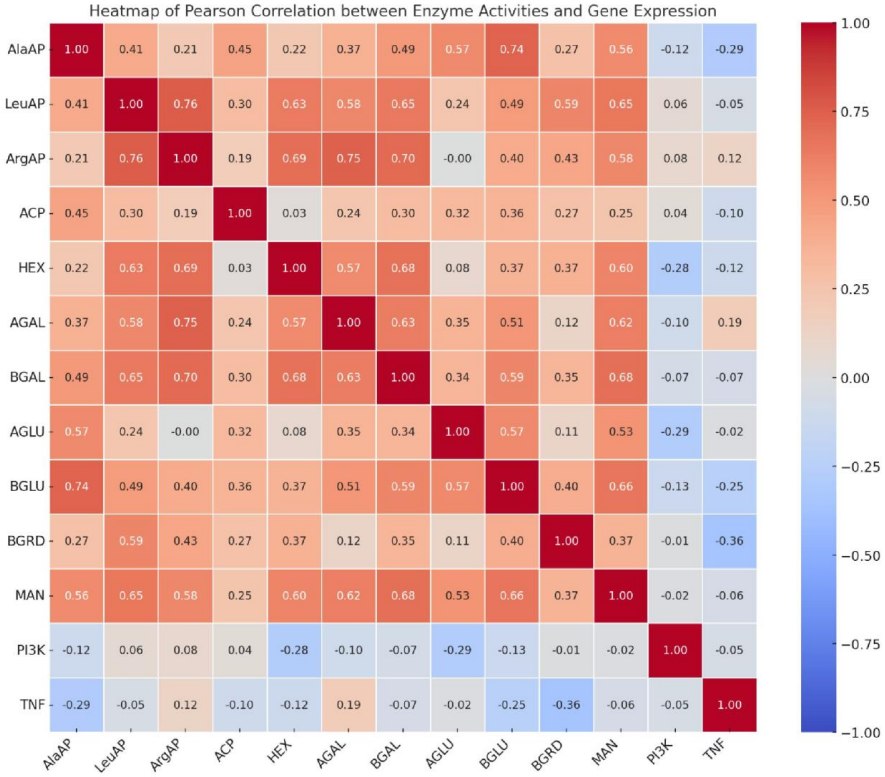

Fig. 3. Correlation heatmap of biochemical and molecular markers in hepatic tissue. Heatmap illustrating Pearson correlation coefficients among key lysosomal enzymes (e.g., ArgAP, AGAL, ACP), inflammatory marker TNF-α, and PI3K gene expression. Positive correlations are depicted in red and negative correlations in blue, with intensity reflecting correlation strength. The map highlights co-regulated enzymatic activities and the inverse relationships between PI3K expression and both lysosomal and inflammatory markers, suggesting coordinated responses in aging and ginger-mediated modulation.

PI3K expression, in contrast, negatively correlated with many enzymes and with TNF, highlighting an opposing regulatory pattern. This suggests that high PI3K levels are associated with lower inflammatory and catabolic activity, and that ginger's suppression of PI3K aligns with increased lysosomal turnover and reduced

inflammation. These correlation patterns reinforce the broader view that aging enhances a network of inflammatory and degradative functions, while ginger modulates this system, suppressing PI3K and TNF-**α** and stabilizing lysosomal activity in a dose- and age-dependent fashion.

Hepatic aging is a complex, multifactorial process marked by various morphological and cellular changes in hepatocytes [Hunt *et al*. 2019]. Our present study offers novel insights into the effects of ginger supplementation on age-related alterations in hepatic structure and function. By employing a stratified approach across three different age groups, we investigated the influence of two doses of ginger on lysosomal enzyme activity, inflammatory and metabolic gene expression, and histological features of liver tissue. The present study confirms that aging is associated with progressive histological changes, including hepatocellular hypertrophy, increased nuclear pleomorphism, enhanced lipid droplet accumulation, and reduction in glycogen storage. At the subcellular level, transmission electron microscopy revealed mitochondrial enlargement and swelling, cristae shortening, and reduction in rough endoplasmic reticulum (RER), which were particularly prominent, indicating impaired energy metabolism and protein biosynthesis capacity in senescent liver cells. These observations are consistent with prior studies reporting similar features of hepatocellular aging [Terman and Brunk, 2004]. Our findings provide substantial evidence that ginger exerts age- and dose-dependent regulatory effects on liver aging processes, primarily through modulating autophagy, inflammation, and metabolic signaling.

Interestingly, ginger supplementation demonstrated a dose- and age-dependent ability to partially modulate these age-related hepatic changes. Moderate ginger supplementation (0.6%) effectively preserved hepatocellular morphology in both young and middle-aged mice, while the older animals receiving a higher dose (1.8%) led to greater morphological changes, suggesting a potential adaptive effect, where higher doses may induce mild cellular stress. Such dose-sensitive behavior is increasingly recognized in nutraceutical research, emphasizing the need to identify optimal intake levels for long-term benefit [Bondy 2023, Caruso *et al*. 2024].

Lysosomal enzyme activity, which plays an important role in autophagic degradation and turnover of macromolecules, was significantly influenced by both age and ginger supplementation. In younger mice, ginger enhanced the activity of enzymes such as ArgAP and AGAL. These findings align with the concept that aged cells increase the function of lysosomal enzymes as a compensatory response to macromolecular damage and impaired autophagy [Cuervo and Dice 2000]. This suggests that ginger stimulates degradative capacity early in life, potentially preventing the buildup of damaged proteins and organelles. In contrast, aged mice exhibited naturally elevated lysosomal enzyme activity likely as a compensatory response to cellular damage and ginger moderated this increase, potentially stabilizing lysosomal function. Such nuanced regulation is beneficial, excessive lysosomal activation in aging can be deleterious, leading to leakage and further stress, whereas targeted

enhancement or normalization helps maintain cellular homeostasis [Gómez-Sintes *et al*. 2016, Tan and Finkel 2023]. In our study, the activity of lysosomal enzymes generally corresponds to the number of primary lysosomes present in the hepatocytes of the mice studied groups.

At the molecular level, ginger influenced key regulators of metabolic and inflammatory signaling. Expression of phosphoinositide 3-kinase (PI3K), a critical mediator in insulin/IGF and mTOR pathways [Harrington *et al*. 2005], increased with age in control livers but was significantly suppressed by ginger, especially at higher doses. This downregulation may enhance autophagy and reduce metabolic stress. Dysregulation of PI3K/Akt/mTOR is a hallmark of aging, and inhibition of this pathway has been linked to lifespan extension in various models [Abdellatif *et al*. 2022, Castillo-Quan *et al*. 2019]. Our findings are consistent with studies reporting that ginger and its active constituents, such as 6-gingerol and 6-shogaol, suppress PI3K/Akt activity and promote autophagy [Pei *et al*. 2021, Wang *et al*. 2016].

The anti-inflammatory effects of ginger were corroborated by the significant reduction in TNF-α expression across ginger-supplemented groups, particularly at the lower dose (0.6%) in older mice (both 6-and 12-month groups). Tumor necrosis factor-alpha (TNF-α), is a key pro-inflammatory cytokine that is often elevated with aging (a phenomenon known as inflammaging) [Muhammad 2020] and is known to disrupt insulin signaling and promote oxidative stress. Interestingly, the most notable effects observed in the 6- and 12-month-old mice at the lower dose, aligns with ginger's known anti-inflammatory properties through suppression of NF-κB and COX-2 signaling [Kim *et al*. 2004]. Reducing chronic hepatic inflammation is critical for maintaining tissue integrity and preventing age-associated liver pathologies such as fibrosis or steatosis.

Together, these data demonstrate that ginger targets multiple hallmarks of hepatic aging. Its effects include preservation of ultrastructure, modulation of lysosomal activity, and suppression of inflammatory and metabolic signaling. This multifaceted action profile is characteristic of other phytochemicals known to promote healthy aging, such as resveratrol, curcumin, and green tea catechins, all of which act through overlapping pathways like Nrf2 activation, mTOR inhibition, or AMPK signaling [Chestnut *et al*. 2021].

While these findings are encouraging, further research is needed to determine the long-term functional outcomes of ginger supplementation. Future studies should assess whether these molecular and histological changes translate into improved liver performance, resistance to disease, or extended health span. Additionally, direct measurement of autophagy markers (e.g., LC3, p62, Beclin-1) and profiling of hepatic immune cells would clarify the underlying mechanisms.

Importantly, our study reveals a dose-sensitive response to ginger. While 0.6% ginger frequently exhibited beneficial effects across all parameters, the 1.8% dose, though effective in gene expression modulation, showed signs of subcellular stress in older animals. This aligns with the concept of hormesis, where low-to-moderate

doses of bioactive compounds elicit adaptive beneficial responses, while higher doses may impose stress [Mattson 2008]. Therefore, optimizing ginger dosage is crucial for therapeutic efficacy in aging interventions. From a translational perspective, the observed dose-dependent effects highlight the importance of defining optimal intake levels. Although ginger is widely consumed and generally safe, excessive doses could pose risks, especially in older individuals with reduced stress resilience. Clinical studies are warranted to test ginger's efficacy in mitigating liver aging in humans, particularly in the context of metabolic disorders or age-associated hepatic decline.

From our study, the linear regression and correlation analyses provided further insight into the relationship between age, ginger dose, and molecular changes. Aging was a strong predictor of increased lysosomal enzyme activity and TNF expression, whereas ginger dose was more tightly associated with PI3K suppression. These findings emphasize that while aging globally impacts cellular catabolism and inflammation, ginger exerts targeted effects on specific pathways, particularly autophagy and inflammatory regulation.

Nevertheless, the study has limitations. The effects of ginger were only evaluated in male mice; sex-specific responses should be addressed in future research. Additionally, long-term studies are needed to evaluate whether the observed molecular and structural changes translate into improved liver function or lifespan extension.

Conclusion

In conclusion, this study demonstrates that dietary ginger supplementation exerts beneficial effects on key biological processes associated with liver aging in a dose- and age-dependent manner. Ginger significantly exerted protective effects on key hallmarks of hepatic aging, including stabilized lysosomal enzyme activity, inflammation, and metabolic signaling pathways in moderate doses. Moderate supplementation (0.6%) effectively preserved hepatocellular ultrastructure and improved lysosomal function, particularly in young and middle-aged mice, while also suppressing age-related increases in PI3K and TNF-α expression. Although high-dose ginger (1.8%) also influenced gene expression, it was associated with mild subcellular stress in aged mice, suggesting an adaptive response and underscoring the importance of dose optimization. These findings contribute to the growing body of evidence supporting the potential of ginger as a safe and natural nutraceutical for promoting healthy liver aging and warrant further investigation in clinical settings.

Data availability statement

The authors declare that all data and materials supporting the findings of this article are available from the corresponding authors upon request.

Acknowledgment. *The authors acknowledge Sabinsa Corporation (East Windsor, NJ, USA) for providing Dry Ginger Powder containing 5% gingerols used in this study.*

Funding Statement

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Ethics declaration

All procedures involving animals were conducted in the frame of animal handling permission No. 14186201 in accordance with the relevant institutional and national regulations (including the EU Directive 2010/63/EU for animal experiments and National Research Council's Guide for the Care and Use of Laboratory Animals).

Declaration of competing statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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