

PRECLINICAL STUDY

Photobiomodulation therapy mitigates cardiovascular aging and improves survival

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Abstract

Background: Photobiomodulation (PBM) therapy, a form of low-dose light therapy, has been noted to be effective in several age-associated chronic diseases such as hypertension and atherosclerosis. Here, we examined the effects of PBM therapy on age-associated cardiovascular changes in a mouse model of accelerated cardiac aging.

Methods: Fourteen months old Adenylyl cyclase type VIII (AC8) overexpressing transgenic mice ($n = 8$) and their wild-type (WT) littermates ($n = 8$) were treated with daily exposure to Near-Infrared Light (850 nm) at 25 mW/cm² for 2 min each weekday for a total dose of 1 Einstein (4.5 pJ/cm² or fluence 3 J/cm²) and compared to untreated controls over an 8-month period. PBM therapy was administered for 3.5 months (Early Treatment period), paused, due to Covid-19 restrictions for the following 3 months, and restarted again for 1.5 months. Serial echocardiography and gait analyses were performed at monthly intervals, and serum TGF- β 1 levels were assessed following sacrifice.

Results: During the Early Treatment period PBM treatments: reduced the age-associated increases in left ventricular (LV) mass in both genotypes ($p = 0.0003$), reduced the LV end-diastolic volume (EDV) in AC8 ($p = 0.04$); and reduced the left atrial dimension in both genotypes ($p = 0.02$). PBM treatments substantially increased the LV ejection fraction ($p = 0.03$), reduced the aortic wall stiffness ($p = 0.001$), and improved gait symmetry, an index of neuro-muscular coordination ($p = 0.005$). The effects of PBM treatments, measured following the pause, persisted. Total TGF- β 1 levels were significantly increased in circulation (serum) in AC8 following PBM treatments ($p = 0.01$). We observed a striking increase in cumulative survival in PBM-treated AC8 mice (100%; $p = 0.01$) compared to untreated AC8 mice (43%).

Conclusion: PBM treatment mitigated age-associated cardiovascular remodeling and reduced cardiac function, improved neuromuscular coordination, and increased longevity in an experimental animal model. These responses correlate with increased TGF- β 1 in circulation. Future mechanistic and dose optimization studies are necessary to assess these anti-aging effects of PBM, and validation in future controlled human studies is required for effective clinical translation.

KEYWORDS

AC8, aging, heart, mice, photobiomodulation, TGF- β 1

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INTRODUCTION

Biological aging results in a protracted loss of form or function characterized by tissue senescence. This leads to the development of human diseases such as cancer, stroke, heart failure, diabetes, and dementia that create major medical, socioeconomic, and health policy issues. Thus, approaches to ameliorate or delay the effects of aging would significantly impact the substantial burden of chronic diseases on the current healthcare system. Accumulating evidence has shown the importance of light in various conditions, including cardiovascular health.¹

The use of low-dose light treatment is termed photobiomodulation (PBM) therapy.^{2,3} PBM treatment utilizes light sources, such as lasers or LEDs, in the visible and infrared spectrum. It is a nonionizing, nonthermal treatment that elicits photophysical and photochemical therapeutic responses at various biological scales. The simplicity, safety, and effectiveness of PBM in human health and wellness are promoting its popularity as an emerging new therapy. These therapeutic PBM responses have been broadly attributed to three major photoresponsive pathways, namely mitochondrial energy homeostasis, cell membrane receptors or transporters, and extracellular growth factor, TGF- β 1 activation.² PBM has been noted to activate endogenous latent TGF- β 1 to promote repair and regeneration.⁴ Interestingly, PBM appears to invoke a delicate balance between the pathophysiological roles of endogenous TGF- β 1 as evident in its ability to promote tissue healing versus scarring.⁵⁻⁷ There have also been significant recent insights into PBM dosimetry, safety, and efficacy biomarkers enabling its rationalized clinical implementation.⁸

With respect to cardiovascular diseases, the therapeutic benefits of PBM therapy against ischemia-perfusion injury, myocardial infarction, hypertension, stroke, myocardial infarction, and aortic aneurysm formation have been reported.⁹⁻¹⁴ The benefits of PBM treatments on histological and cardiovascular changes are summarized in excellent reviews.^{15,16} There have been no studies, to our knowledge, on the benefits of effects PBM in mice mitigating the effects of aging. Based on this evidence, the present study examined its effects on cardiovascular aging. Prior longitudinal studies in nongenetically manipulated C57 black mice indicate that left ventricle (LV) structure and function and heart rate begin to substantially deteriorate beyond the age of 21 months and accelerate between 21 and 30 months of age.¹⁷ Here we examined the effects of PBM treatments in a well-characterized, transgenic mouse model of accelerated aging, i.e. mice in which adenylyl cyclase type VIII (AC8) is overexpressed in a cardiac-specific manner. At 3 months of age AC8 mice manifest a significant increase in heart rate (HR) and left ventricular (LV) ejection fraction (EF) in response to elevated cAMP-PKA-Ca²⁺ signaling.¹⁷⁻¹⁹ But after 12 months of age, AC8 mice exhibit significantly accelerated cardiac remodeling, chamber dilation, and functional deterioration and predominantly die due to dilated heart failure at around 19 months of age.¹⁹

In this proof-of-concept study, we performed PBM treatments in AC8 mice at 14-19 months of age to target the most dramatic phase of accelerated cardiac aging. We hypothesized that PBM treatments during the critical stage of cardiac remodeling in this accelerated aging model might attenuate the cardiac chamber dilation and functional deterioration, improve gait, and possibly extend lifespan. TGF- β s play central roles in cardiovascular homeostasis; disrupting its intricate signaling functions leads to cardiac dysfunction and failures.^{20,21} We also examined the putative roles of TGF- β signaling in mediating global PBM responses.

METHODS

Animal studies

All animal studies were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication no. 85-23, revised 1996). Experimental protocols were approved by the Animal Care and Use Committee of the National Institutes of Health (IACUC protocol #441LCS2022). A breeder pair of AC8 overexpression mice, generated by ligating the murine α -myosin heavy chain promoter to a complementary DNA coding for human AC8, was a gift from Nicole Defer & Jacques Hanoune (Hôpital Henri Mondor).²² AC8 mice and wild-type siblings, male, 14 months old, were housed in a climate-controlled room with a 12-h light cycle and free access to food and water.

Photobiomodulation therapy

Mice were randomly divided into four groups: (1) AC8 mice without any treatment/controls (AC group; $n = 7$); (2) AC8 mice treated with PBM (AC + T group; $n = 8$); (3) wild-type mice without any treatment/controls (WT group; $n = 9$); and (4) wild-type mice treated with PBM (WT + T group; $n = 8$). Mice in AC + T and WT + T groups received near-infrared LED light exposure at 25 mW/cm² for 120 s at 850 nm for 1 Einstein (4.5 pJ/cm² photonic fluence or the conventional dose of 3 J/cm²) for 5 days per week.²³ Mice were placed under a LED light source (Bio900, Platinum LED Inc) at a fixed distance in their original cages without cage covers, and light irradiance was calibrated using an optical power meter (ThorLabs Inc). The mice were subjected to a whole-body treatment while they were awake and freely moving in their cage. As the PBM device was placed on the top of the cage, the dorsum (back) surface of the mice was directly exposed to the light. While shaving and restraining animals would have been ideal for PBM dosimetry, it was not considered appropriate from an animal use and care perspective due to the repeated, extensive, long-term treatment regimen. To ensure suitable dosimetry, average irradiance in ambulatory animals at a median anatomical plane was calculated. Mice

were treated simultaneously between 10:00 and 11:00 a.m. each day. Control groups were similarly handled, but without light treatments.

Cardiovascular assessments

All mice underwent serial (monthly) echocardiography (Echo) examination as previously described.¹⁹ All mice underwent monthly echocardiography (Echo) examination (40-MHz transducer; Visual Sonics 3100; Fuji Film Inc) under light anesthesia with isoflurane (2% in oxygen) via nosecone and body temperature was maintained at 37°C with a heating pad. Mice were placed in the supine position; ventral fur was shaved. Standard electrocardiogram (ECG) electrodes were placed on the limbs, and Lead II ECG were recorded with Echo images. Each Echo examination was completed within 10 min. Body weight (BW) and HR were recorded. Parasternal long-axis views of the LV were obtained and recorded to ensure that the mitral and aortic valves and the LV apex were visualized.

M-mode tracing of the left atrium and basal aorta were recorded at the aortic valve level. Left atrium end-systolic dimension (LAD) and the Aortic end-systolic lumen dimension (AoD) were measured, and LAD/AoD was calculated. Parasternal short-axis views of the LV were recorded at the mid-papillary muscle level. From the parasternal short-axis view of the LV, M-mode tracings of the LV were obtained. Thicknesses of the LV Posterior Wall (PW) and intraventricular septum (IVS) were measured from M-mode tracing of LV. Endocardial area tracings, using the leading-edge method, were performed in the 2D mode (short-axis and long-axis views) from digital images captured on a cine loop to calculate the end-diastolic and end-systolic LV areas. LV End-diastolic volume (EDV) and end-systolic volume (ESV) were calculated by a Hemisphere Cylinder Model method. LV ejection fraction (EF) was derived as $EF = 100 \times (EDV - ESV)/EDV$. Stroke volume (SV) was the net difference between EDV and ESV. LV mass (LVM) was calculated from EDV, IVS, and PW. Four chamber view of the heart was obtained from the apex.

Mitral valve blood flow velocity was recorded at the tip of the mitral valves using a pulsed Doppler. E and A wave velocities and E/A ratios were measured offline. Pulse wave velocity (PWV) was measured at the lower part of the abdominal aorta. Doppler flow velocity was acquired at two points of the abdominal aorta: just above to the iliac bifurcation and 6~10 mm proximal to the iliac bifurcation. The distance between two sampling points was measured from 2D images using Image J software (NIH). The time between R wave of ECG and starting point of the Doppler flow velocity wave was measured offline for both sampling points. Their net difference was used as the wave traveling time between two points. PWV was calculated as $PWV = \text{distance}/\text{traveling time}$. All measurements were made by a single observer who was blinded to the identity of the tracings, an average of five consecutive cardiac cycles

covering at least one respiration cycle (100 tpm on average). The reproducibility of measurements was assessed by one repeated measurement (?) week apart in randomly selected images, and the repeated-measure variability was less than 5%.

Gait analysis

All mice underwent monthly Gait examination (DigiGait System, Mouse Specifics Inc). To assess gait symmetry, an unrestrained mouse was placed on the treadmill belt for 1 min for acclimatization period and forced to run at a belt speed of 5 cm/s for 2 min, 15 cm/s for 5 min, and then 18 cm/s while recording gait. If a mouse were to have difficulties running at this belt speed, gait was measured at the maximum belt speed it tolerated. After obtaining a 4~6 s of video recording of steady run, the mouse was returned to its original cage. Gait parameters were analyzed off-line using DigiGait Analysis Program (Mouse Specifics Inc) as described previously.²⁴ $\text{Gait symmetry} = [\text{right forelimb step frequency} + \text{left forelimb step frequency}] / [\text{right hind limb step frequency} + \text{left hind limb step frequency}]$.

Animal sacrifice, tissue collection, and TGF- β 1 enzyme-linked immunosorbent assay (ELISA)

All surviving animals were sacrificed at the end of the 8-month observation period. Serum, left ventricle, gastrocnemius, quadriceps, and skin samples were collected and stored at -80°C. To assess TGF- β 1, tissue samples were pulverized with a sonicator (QSonica) in RIPA buffer with proteinase inhibitors (Complete Mini, Sigma-Aldrich) on ice. Total protein was assessed with a BCA assay (Pierce, Thermo Fisher Scientific), and equal amounts of protein were used for TGF- β 1 ELISA (R&D Systems). To evaluate total TGF- β 1 in these samples, the sum of basal and chemically-activated samples was individually assessed. To perform chemical activation, an aliquot of the sample was treated with 1 N HCl (1:10 v/v) for 20 min followed by neutralization with 1 N NaOH-HEPES (1:10 v/v) for 20 min before assessment as per the manufacturer's instructions. Samples were incubated in microplate wells coated with an anti-TGF- β 1 monoclonal capture antibody that binds the physiologically-active TGF- β 1, but not latent TGF- β 1. This is followed by incubation with a secondary antibody, detection with a colorimetric substrate, and absorbance was assessed with microplate reader (SpectraMax, Molecular Devices).

Study design

The original study was designed based on the period (14~19 months) when the transgenic mice demonstrated the most prominent deterioration of cardiac functions

(Supporting Information: Figure 1). Our intent was to perform PBM treatments continuously over an 8-month period. However, the study was interrupted by COVID pandemic restrictions in the workplace. Hence, the mice in this study received PBM treatments for first 3.5 months (Early Treatment), following a 3-month pause during which monthly assessment of study variables could not be performed, and carcasses of mortalities were unavailable for evaluation. Following the pause, study variables were assessed again (Post Pause) and PBM treatments were resumed for 1.5 months (Late Treatment).

Statistical analyses

All data was organized in Excel (Microsoft) and analyzed using RStudio (RStudio). The effects of age, genotype, and PBM therapy were assessed in a three-way mixed analysis of variance (ANOVA) analysis during each treatment period and over the entire (8 months) study duration. In addition to the three main effects, age,

genotype, and PBM treatments, interaction terms in the analysis inform on whether the PBM therapy effects differ by age and genotype. Statistical significance was assumed at $p < 0.05$. Backward elimination of non-significant terms, starting with the highest order terms, yields the final model for each parameter. For TGF- β 1 ELISA, one-way ANOVAs were used to compare the four groups for active and total TGF- β 1. When significant, a post-hoc comparison with FDR correction for multiple testing was used to identify differences. For survival data, Kaplan–Meier survival curve was computed for each of the four groups and a log-rank test was applied to determine if the survival curves differ.

Statistical assessment of repeated measure variables

Means and standard deviations of all serially measured parameters throughout the 8-month study period are listed in Supporting Information: Table S1. The results of statistical analyses (mixed ANOVAs) for the effects of age,

TABLE 1 Mixed ANOVA analyses by study phase (Early treatment).

Variable	Early Treatment						
	PBM	Age	Genotype	PBM:Age	PBM:G	Age:G	P:A:G
Heart Structure							
LVM	0.02	0.0003	0.91				
Body Weight	0.60	0.42	0.53				
LVM/BW	0.03	0.001	0.91				
EDV	0.07	0.09	0.04	0.05			
PW	0.50	0.06	0.07				
IVS	0.19	0.04	0.01				
LAD	0.02	0.41	0.15				
AoD	0.10	0.64	0.27				
LAD/AoD	0.02	0.64	0.15	0.22	0.50	0.50	0.04
Heart Function							
ESV	0.04	0.27	0.003				
EF	0.03	0.87	1.E-05				
HR	0.24	0.05	0.003				
A	0.09	0.06	0.64				
E/A	0.12	0.16	0.01				
PWV	0.001	0.57	0.15	0.004			
Gait Symmetry	0.005	0.69	0.83				

Note: p values from Mixed ANOVAs.

Significant: $p \leq 0.05$.

Marginally Significant: $0.05 < p \leq 0.10$.

TABLE 2 Mixed ANOVA analyses by study phase (Post-Pause).

Variable	Post Pause			PBM:Age	PBM:G	Age:G	P:A:G
	PBM	Age	Genotype				
Heart Structure							
LVM	0.03	0.11	0.44				
Body Weight	0.54	0.08	0.78				
LVM/BW	0.09	0.11	0.44				
EDV	0.13	0.002	0.63				
PW	0.17	0.92	0.05				
IVS	0.21	0.14	0.19				
LAD	0.04	0.27	0.07				
AoD	0.37	0.31	0.94				
LAD/AoD	0.03	0.52	0.02		0.04		
Heart Function							
ESV	0.07	0.003	0.79		0.04		
EF	0.07	0.001	0.30		0.02	0.03	
HR	0.003	0.28	0.0005			0.01	
A	0.01	0.73	0.52				
E/A	0.04	0.26	0.07				
PWV	0.0001	0.45	0.08				
Gait Symmetry	0.02	0.11	0.80	0.84	0.07	0.22	0.01

Note: *p* values from Mixed ANOVAs.

Significant: $p \leq 0.05$.

Marginally Significant: $0.05 < p \leq 0.10$.

genotype, and PBM treatments during the Early Treatment period, Post Pause, and the Late Treatment period for all measured variables are listed in Table 1 and Supporting Information: S2. The mixed-ANOVAs of the effects of age, genotype, and PBM treatments for all measured variables over the combined periods are in Table 2 and Supporting Information: S3. The effects of age, PBM treatments, and genotype on selected serially measured variables are presented in Figures 1–4, and statistical analyses of these variables, for the convenience of the reader, are listed in Tables 1 and 2. The confidence intervals in Figures 1–4 refer to variabilities around a single point with respect to treatment and genotype at each time point (age). The robust statistical analyses employed, not only test for main effects of age, treatment, or genotype, but also for interactions among the main effects. Loess smooth curves that use local regression to smooth the data to allow the overall trajectory to be viewed with confidence bands, have been provided in Supporting Information: Figure S2.

To clarify genotype-age, and genotype-treatment interactions in the mixed ANOVA analyses, we applied linear mixed-effects models to analyze this repeated-

measures data (lmer in R). Since lmer uses a likelihood-based approach to fit the model, no data are excluded and data “missing at random” (e.g., death in specific animal groups) still allow for the assessment of any statistical differences among them. Age, genotype, and treatment are treated as categorical factors, and interactions of these factors are included in the full model. There is a random term for the intercept (allowing mice to be consistently above/below the average). The random effect also allows for repeated measures within mice to be correlated.

RESULTS

Early Treatment period

Effects of age, genotype, and PBM treatments on LV structure and function

Although cardiac structure and function differ remarkably between AC8 and WT, there were no baseline differences

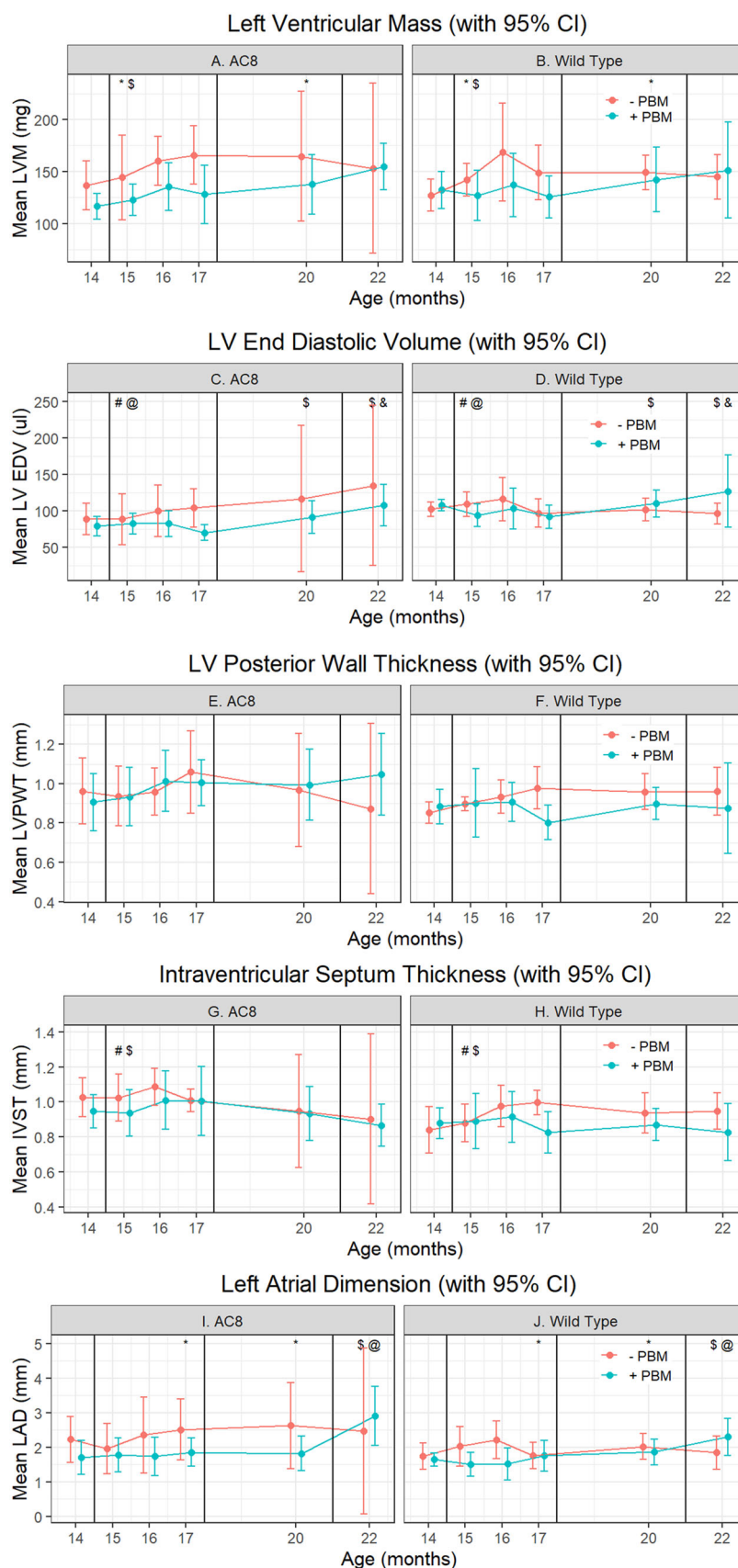


FIGURE 1 (See caption on next page)

between echo-assessed structure and function in AC8 versus WT at the beginning age of this study (14 months) (Figure 1 and Supporting Information: Table S1). Further, within either genotype there were no baseline differences between mice to be treated or not treated for any measured variable (Supporting Information: Table S1).

In the absence of PBM therapy, LVM, determined by the thickness of the LV chamber walls and the LV chamber volume at end diastole (i.e., just before the beginning of the next heart beat) increased with age in both AC8 and WT (by 19%) during the Early Treatment period (Figure 1A,B and Table 1). BW did not decline in either genotype during the Early Treatment period and was not affected by PBM therapy (Supporting Information: Table S2).

Effects of PBM treatments with age in both genotypes

During the Early Treatment period, LV EDV increased in untreated AC8 (by 17%) but did not increase in PBM treated mice or in WT mice (Figure 1C,D and Table 1), explaining the age-treatment interaction effect in Table 1. The thickness of LV posterior wall (PW) marginally increased in both genotypes with age (Figure 1E,F and Table 1), and the thickness of intraventricular septum (IVS) increased modestly with age while PBM treatments did not significantly alter the LV PW and IVS thickness (Figure 1E,H and Table 1). Thus, age-associated increase in LVM in untreated AC8 and the PBM treatment effect is attributable to a reduction in LV size without impact on LV wall thickness. PBM therapy also reduced the left atrial dimension (LAD) in both genotypes (Figure 1I,J and Table 1).

Effects of PBM treatments on heart function

The LV ESV, that is, the volume of the LV at the end of the heartbeat, which informs on the amount of blood left in the heart at the end of each heartbeat, was reduced by PBM treatments (Figure 2). The heart rate and LV EF, the fraction of the EDV ejected from the heart during each beat, was higher in AC8 than in WT; while PBM treatments did not affect HR, EF was substantially increased (Figure 2C,F and Table 1). The amplitude of the E-wave was not affected by PBM therapy (Supporting Information: Table S3). The amplitude of the atrial contraction (A-wave) was modestly ($p = 0.09$) reduced by PBM therapy (Table 1).

Effects of age, PBM treatments, and genotype on aortic wall stiffness

Aortic PWV informs on aortic wall stiffness. Before PBM treatments, no statistically significant differences were observed between the genotypes and the untreated and treated groups. PWV was substantially reduced by PBM treatments during the Early Treatment period (Figure 3, Table 1). The significant age-treatment interaction indicates that the effect of PBM treatment on PWV depends on age.

Effects of age, PBM treatment, and genotype on gait

Gait symmetry is determined by numerous parameters measured during locomotion that is based on overall neuro-muscular coordination (Supporting Information: - Figure S3). Before PBM treatments, no statistically significant differences were observed between the genotypes and the untreated and treated groups. PBM treatments significantly improved gait symmetry in both genotypes (Figure 4, Table 1).

Post Pause measurements

Effects of age, genotype, and PBM treatments

The study was interrupted by COVID pandemic restrictions in the workplace. Hence, the mice in this study received PBM therapy for the first 3.5 months, following a 3-month pause during which monthly assessment of study variables could not be performed. Following the pause, study variables were assessed again at 20 months of age.

The effects of PBM treatments following the 3-month pause are shown in Figures 1–4, Table 2, Supporting Information: Table S3. Note that following the pause the significance of effects of PBM therapy or lack thereof, were essentially the same as those following the early period. This suggests that the effects of PBM therapy on some of these variables remained for this period of time following the termination of Early Treatments. Note, that even though the effect for treatment is not significant overall, a statistically significant three-way interaction term means there is an effect of treatment that differs by genotype and age in a nonadditive manner. For gait

FIGURE 1 Mean echocardiography parameters for heart structure by age and genotype. (A, B) Left ventricular mass; (C, D) left ventricular end-diastolic volume; (E, F) left ventricular posterior wall thickness; (G, H) intraventricular septal thickness; (I, J) left atrial dimensions. Statistical significance: The Early Treatment period looks at changes from age 14 to 17. For the Post Pause, the analyses are for ages 17 and 20, and for Late Treatment for ages 20 and 22 in the surviving animals. For a significant ($p \leq 0.05$) main effect for a variable, the symbols are: Treatment, *; genotype, #; time, \$. Symbols for significant interactions in the final model are: Treatment by genotype, %; treatment by Time @; genotype by time, &; and the three way interaction is represented by!. Note that the confidence intervals are from the data points for each group at each time. The mixed ANOVA examines all the data together, see methods for statistical details. AC, AC-8 mice (left); WT, Wild type mice (right).

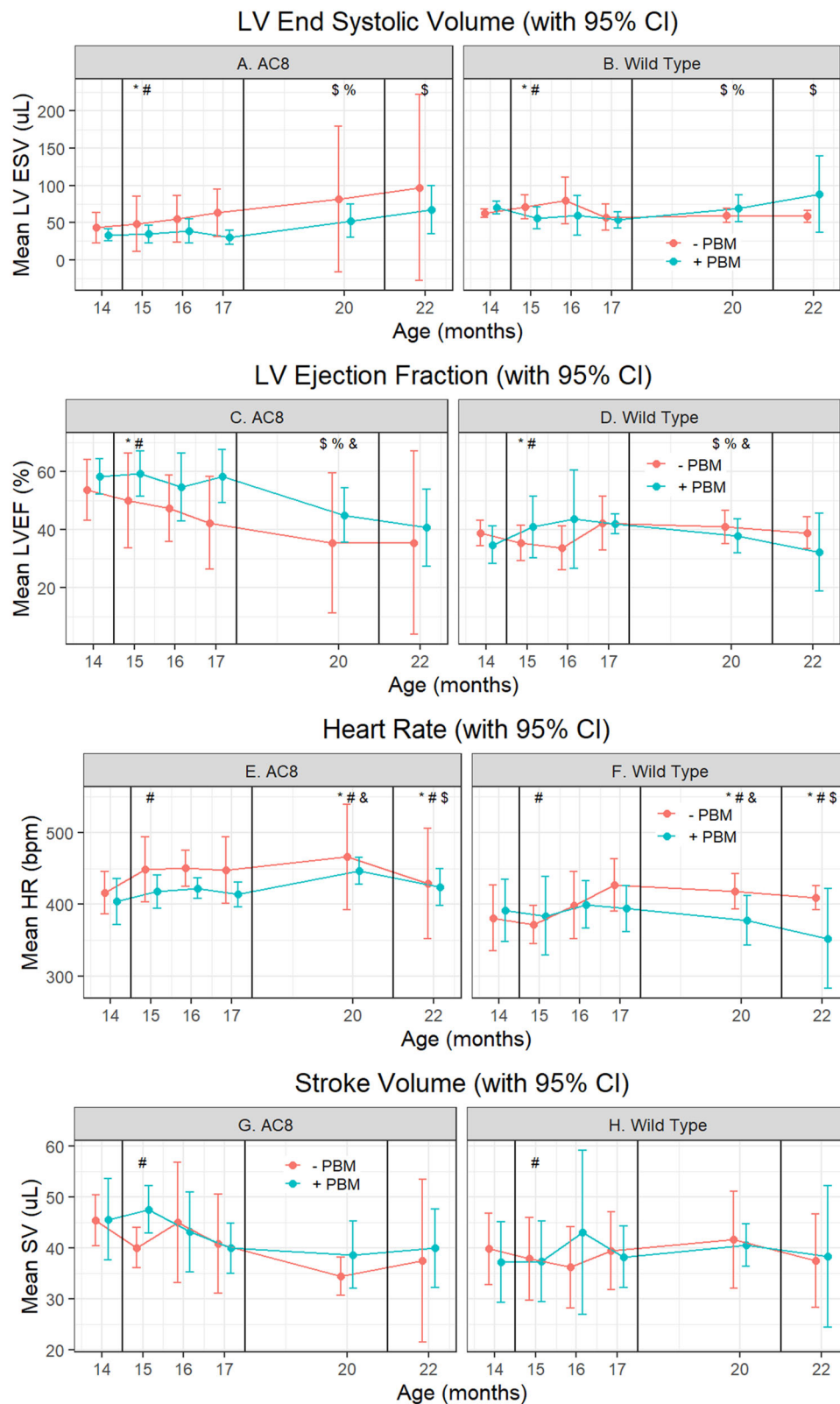


FIGURE 2 Mean echocardiography parameters for heart function by age and genotype. (A, B) Left ventricular end-systolic volume; (C, D) left ventricular ejection fraction; (E, F) heart rate; (G, H) stroke volume. Statistical significance: The Early Treatment period looks at changes from age 14 to 17. For the Post Pause, the analyses are for ages 17 and 20, and for Late Treatment for ages 20 and 22 in the surviving animals. For a significant ($p \leq 0.05$) main effect for a variable, the symbols are: Treatment, *; genotype, #; time, \$. Symbols for significant interactions in the final model are: Treatment by genotype, %; Genotype by time, &. Note that the confidence intervals are from the data points for each group at each time. The mixed analysis of variance examines all the data together, see Methods for statistical details. AC, AC-8 mice (left); WT: Wild type mice (right).

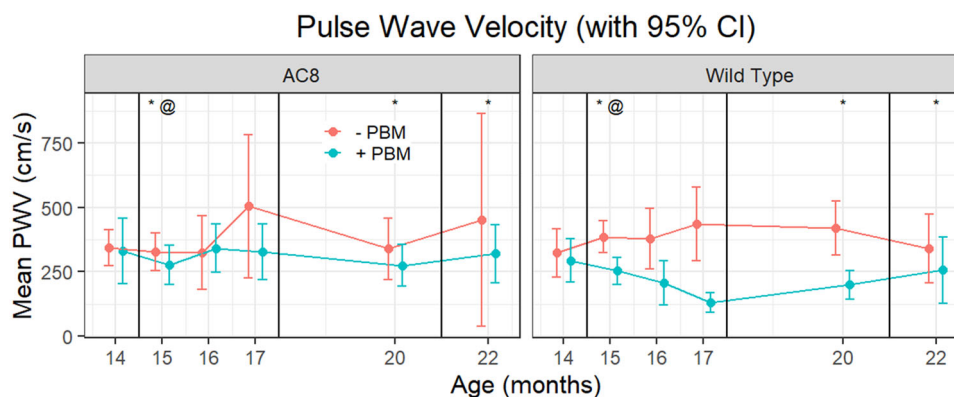


FIGURE 3 Mean parameters for aortic wall stiffness by age and genotype assessing aortic pulse wave velocity. Statistical significance: The Early Treatment period looks at changes from age 14 to 17. For the Post Pause, the analyses are for ages 17 and 20, and for Late Treatment for ages 20 and 22 in the surviving animals. For a significant ($p \leq 0.05$) main effect for a variable, the symbols are: Treatment, *. Symbols for significant interactions in the final model are: Treatment by Time @. Note that the confidence intervals are from the data points for each group at each time. The mixed analysis of variance examines all the data together, see Methods for statistical details. AC, AC-8 mice (left); WT, Wild type mice (right).

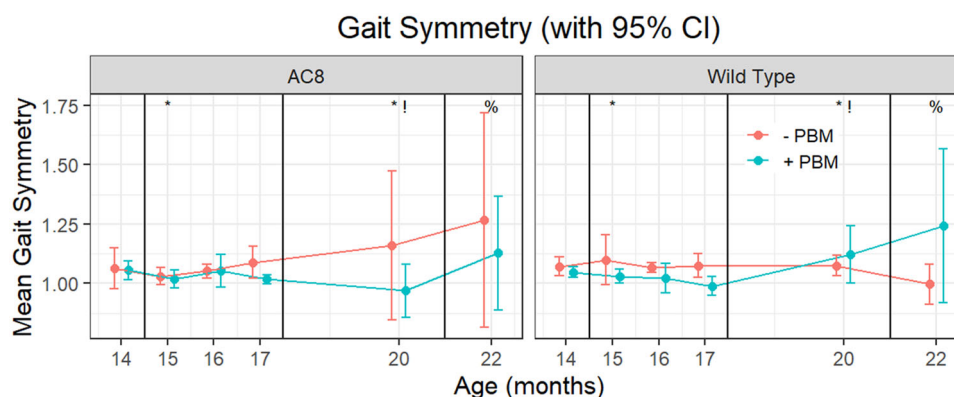


FIGURE 4 Mean parameters for Gait by age and genotype. Statistical significance: The Early Treatment period looks at changes from age 14 to 17. For the Post Pause, the analyses are for ages 17 and 20, and for Late Treatment for ages 20 and 22 in the surviving animals. For a significant ($p \leq 0.05$) main effect for a variable, the symbols are: Treatment, *. Symbols for significant interactions in the final model are: Treatment by genotype, %; and the three way interaction is represented by !%. Note that the confidence intervals are from the data points for each group at each time. The mixed analysis of variance examines all the data together, see Methods for statistical details. AC, AC-8 mice (left); WT, Wild type mice (right).

symmetry, there is a triple interaction term which is significant. Unlike other measured variables, HR was significantly reduced in treated versus untreated mice at the Post Pause assessment (Figure 2E,F and Table 2).

Late Treatment measurements

Effects of age, genotype, and PBM treatments

Following the 3-month pause, mice had reached 20 months of age and treatment was resumed for one additional month. HR, A-wave, and PWV remained significantly lower in the PBM treated versus nontreated mice in the surviving animals (Figures 2E,F, and 3, Table 3 and 4).

Effects of age, genotype, and PBM treatment over the entire study period

In addition to the statistical analysis by study phase, it was important to analyze the results over the entire study duration of 8 months to ascertain age effects over a more prolonged period of time and identify additional age-treatment, age-genotype, and genotype-treatment interactions. During the 8-month period between 14 and 22 months of age, age had a significant effect on body weight and heart structure parameters which became substantially significantly altered (Figures 1–4, Table 3 and Supporting Information: Table S2). Specifically, body weight decreased in AC8 and treated WT while it remained stable in untreated WT. LV mass increased and PW increased in treated AC8 but decreased in untreated

TABLE 3 Mixed ANOVA analyses by study phase (Late treatment).

Variable	Late Treatment					
	PBM	Age	Genotype	PBM:Age	PBM:G	Age:G
Heart Structure						
LVM	0.52	0.61	0.57			
Body Weight	0.30	0.0003	0.90			
LVM/BW	0.91	0.03	0.42			0.05
EDV	0.87	0.02	0.84			0.02
PW	0.96	0.86	0.34			
IVS	0.24	0.20	0.66			
LAD	0.91	0.04	0.13	0.002		
AoD	0.17	0.73	0.87			
LAD/AoD	0.67	0.06	0.11	0.02		
Heart Function						
ESV	0.83	0.02	0.79			
EF	0.82	0.11	0.65			
HR	0.01	0.01	0.0001			
A	0.03	0.80	0.99			
E/A	0.20	0.58	0.10			
PWV	0.003	0.56	0.24			
Gait Symmetry	0.88	0.16	0.71		0.01	

Note: *p* values from Mixed ANOVAs.

Significant: $p \leq 0.05$.

Marginally Significant: $0.05 < p \leq 0.10$.

AC8 and increased in untreated WT. IVS decreased in AC8 but increased in untreated WT. The PBM treatment effects on body weight, EDV, and LVM/BW depended upon age and the effects of PBM treatments on EDV depended on both age and genotype (interaction terms in Table 2, Supporting Information: Table S1). Age had a significant effect on and heart function for ESV, EF, and HR. ESV increased while EF decreased with Age in AC8. There were modest differences in HR with Age and PBM treated mice tended to have lower HR.

TGF- β 1 levels

TGF- β 1 levels were measured in serum and LV tissue in mice that survived the entire 8-month study period. Active TGF- β 1 levels did not significantly vary in serum and heart (left ventricle) in either AC8 or WT mice in controls or following PBM therapy (Figure 5A). However, total TGF- β 1 levels were significantly increased in circulation (serum) in AC8 following PBM treatments

(Figure 5B). TGF- β 1 levels were not significantly different in other tissues such as skin and skeletal muscles (Gastrocnemius and Quadriceps) in any group irrespective of PBM therapy. We examined the statistical significance of the association between elevated serum and LV total TGF- β levels against all other outcomes and noted a significant correlation with EDV, and LAD (Supporting Information: Table S4).

Survival analyses

The effects of PBM treatments on survival was assessed both within each genotype and in all four groups (two genotypes, PBM treated and untreated) combined. During the Early Treatment period, survival did not vary among the four groups ($p = 0.77$). After the Post Pause period, survival differed ($p = 0.04$) due to the fact that 2 untreated AC8 mice died during the post-pause compared to none in the WT group. When all four groups are compared over the entire 8-month study

TABLE 4 Mixed ANOVA analyses over the entire study period.

Variable	Age	Genotype	PBM	Age:Genotype	Age:PBM	Genotype:PBM	Age:Genotype:PBM
Heart Structure							
LVM	1.E-06	0.83	0.04		0.03		
BW	2.E-08	0.61	0.85	0.41	0.02	0.58	0.01
LVM/BW	4.E-07	0.74	0.08		0.02		
EDV	8.E-07	0.42	0.10	0.003	0.28	0.15	0.02
PW	0.19	0.11	0.70				
IVS	0.03	0.04	0.12				
LAD	0.0003	0.07	0.08		0.003		
AoD	0.01	0.32	0.34				
LAD/AoD	0.04	0.07	0.06		0.04		
Heart Function							
ESV	1.E-06	0.23	0.06	0.0008	0.34	0.11	0.04
EF	1.E-04	0.003	0.06	0.0002			
HR	0.02	7.E-05	0.04				
A	0.13	0.88	0.02		0.02		
E/A	0.19	0.01	0.10				
PWV	0.85	0.07	4.E-05		0.02		
Gait Symmetry	0.003	0.83	0.17	0.51	0.29	0.06	0.001

Note: Final Models (include non-significant main effects).

p values from Mixed ANOVAs.

Significant: $p \leq 0.05$.

Marginally Significant: $0.05 < p \leq 0.10$.

period, the effect of PBM treatments significantly improved survival ($p = 0.04$) (Figure 6). When survival was analyzed within genotype, PBM treatments improved survival in AC8 mice, (100% in treated vs. 43% in untreated; $p = 0.01$). In contrast, PBM therapy did not have a significant effect (64% in treated vs. 89% in untreated; $p = 0.29$) on survival in the WT mice.

DISCUSSION

This is the first in vivo long-term study, to the best of our knowledge, that demonstrates the remarkable effects of PBM therapy in the context of aging. Our results show, for the first time, the beneficial effects of long-term whole-body exposure of low dose Near-Infrared light treatments on cardiovascular health and lifespan in a mouse model of accelerated aging. Specifically, 3 months of uninterrupted PBM treatments showed reduced age-associated increases in LV mass in both genotypes, reduced the LV EDV in

AC8; and reduced the left atrial dimension in both genotypes. PBM treatments substantially increased the LV ejection fraction, reduced the aortic wall stiffness, and improved gait symmetry, an index of neuro-muscular coordination. These effects of PBM therapy persisted following discontinuation of the therapy. At the end of the study, total TGF- β 1 levels were significantly increased in the serum in AC8 following PBM treatments along with a striking increase in their cumulative survival.

This study targeted this specific age range (14–19 month) in the AC8 mice to begin PBM treatments that significantly attenuated several age-associated adverse remodeling events and strikingly improved survival. Regular treatments over 3 months with a brief 2 min per day, 1 Einstein (4.5 pJ/cm² photonic fluence at 850 nm) exposure to near-infrared light significantly mitigated cardiovascular and neuro-musculoskeletal functions. The discrete improvements in several parameters such as EDV, ESV, HR, PW, PWV, and gait symmetry showed significant improvements after the initial 3-month Early

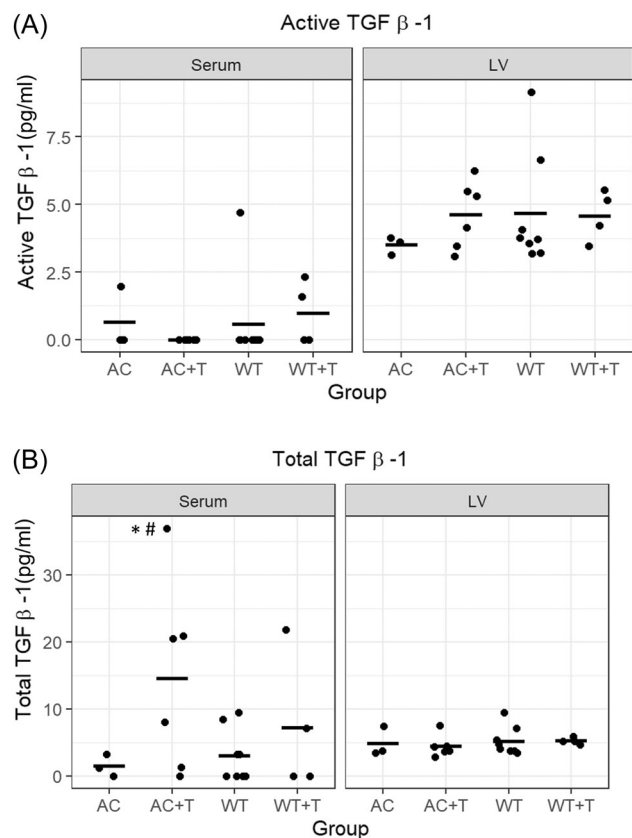


FIGURE 5 TGF- β 1 levels were assessed with an enzyme-linked immunosorbent assay. Active TGF- β 1 levels were assessed with following chemical activation (A) and total (B) in serum and heart at the end of experiment in the surviving animals. Left panel shows TGF- β 1 concentration in serum; Right panel shows TGF- β 1 concentration in the left ventricle. The horizontal line indicates the mean. AC, AC-8 mice; AC + T, PBM-treated AC-8 mice; WT, Wild type mice; WT + T, PBM-treated wild type mice; statistical significance: Treatment, *; Genotype, #.

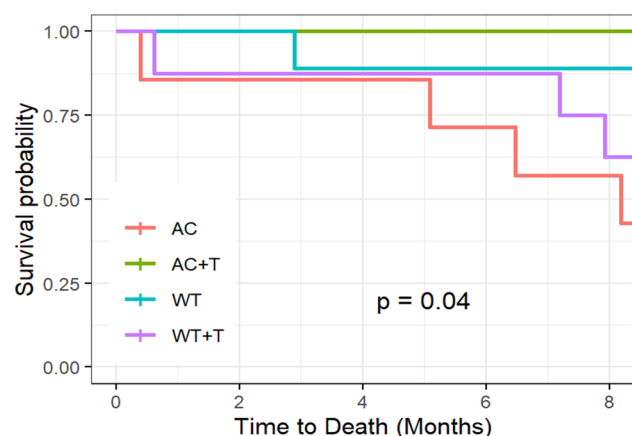


FIGURE 6 Kaplan-Meier cumulative survival curves. There are significant different among the four groups. AC, AC-8 mice; AC + T, PBM-treated AC-8 mice; WT, Wild type mice; WT + T, PBM-treated wild type mice; Statistical significance: * $p = 0.04$.

Treatment phase. PBM therapy also improved gait symmetry in both genotypes. Gait Symmetry, determined by numerous parameters measured during locomotion, informs on overall neuro-muscular coordination. It is worth highlighting that the values hovering around unity reflect the preserved gait symmetry, while higher values indicate gait symmetry deterioration. The AC8 mice are a well-established heart disease model developed by Lipskaia et al. that overexpresses cardiac-specific adenylyl cyclase type VIII (AC8), resulting in significantly increased heart rate (30%) and cardiac contractility at younger age.¹⁸ This constant stress in the cardiac contractile machinery leads to myocardial dysfunction and failure.^{18,19} Functional analysis of the AC8 mice exhibit characteristics of accelerated cardiovascular aging phenotypes starting from 9 to 12 months of age, such as significant left ventricular dilatation, functional deterioration, hypertrophy and diastolic dysfunction that leads to a significantly shortened lifespan.

This study also observed significant perturbations in TGF- β 1 levels in the cardiac tissues and serum (circulatory), especially after PBM treatments. TGF- β has a central role in human health and disease, especially in senescence and aging.^{25–27} The complexity of its biological functions stems from its cell type, dose, and context-dependent signaling that contributes to its broad effects on cellular degeneration, fibrosis, inflammation, regenerative capacity, and metabolic functions.^{28–30} The protective roles of TGF- β in maintaining tissue homeostasis have been attributed to both its immune-modulatory signaling and pro-regenerative responses.³¹ The ability to activate latent TGF- β 1 using PBM treatments provides an unprecedented tool to explore its endogenous roles with exquisite spatiotemporal precision.⁷ This study observed significant perturbations in TGF- β 1 levels in the serum (circulatory), especially after PBM treatments in AC8 mice. The precise roles of TGF- β 1 in these pathophysiological contexts remain to be fully elucidated. However, recent work has noted that PBM treatments induced both TGF- β dependent and independent anti-inflammatory pathways.³² This suggests other PBM-induced biological signaling, such as ATF-4, NF κ B, VEGF, and PI3K signaling in a cell-tissue-organ context dependent manner, may contribute to cardiovascular tissue recovery and homeostasis that requires further investigations.^{8,33,34}

Although COVID-19 pandemic restrictions faltered our original experimental plan, the pausing and re-introduction of PBM treatments provided a unique perspective of the observed outcomes. The effect of PBM therapy on LVM, LVM/BW, LAD, LAD/AoD, ESV, EF, A-wave, PWV, and gait symmetry was preserved following the 3-month pause suggesting that the effect of PBM therapy on these variables remained for this period of time following the termination of Early Treatment.

Following the 3-month pause, mice had reached 20 months of age and treatment was resumed for one additional month. Although the earlier beneficial effects of PBM treatments on most parameters were not observed during this short treatment resumption phase, beneficial effects on some parameters (HR, A-wave, and PWV) were still significant. Most strikingly, there was a significant improvement in survival in AC8 mice compared to untreated mice, which has 43% mortality at the age when the study was finally terminated.

During the past decade, there has been tremendous progress in the clinical applications and molecular mechanisms of PBM in a broad range of human diseases.³⁵ PBM treatments have been noted to promote several cardioprotective effects such as improved cell viability and restoration of functions following ischemia-perfusion injury, functional cardiovascular recovery following myocardial infarction, promotes angiogenesis and prevented the aortic aneurysm formation and progression.^{9–16} In a series of studies using animal models and human clinical trials, several investigators have demonstrated the utility of PBM therapy in improving cardiovascular functional recovery after cardiac infarcts.^{22,36–55} The benefits of PBM treatments in these acute MI models demonstrated reduced infarct size (33% to 77%), positive modulation of cardiovascular functions (LV mass and functions), and modulation of inflammatory cytokines (IL1 β , IL-6, VEGF) and oxidative stress (SOD, NO, GPX) factors affecting overall improvement in cardiac functions. The acute infarct model represents an immediate, aggravated disruption of cardiac functions in contrast to the subtle, chronic damage in AC8 mice. But there were a few parallels in the benefits demonstrated by PBM treatments in our study namely improved left ventricular functions. Most strikingly, a significant improvement in the overall survival (100%) in AC8 mice that usually demonstrate 43% mortality due to cardiac dysfunctions.

PBM represents a noninvasive, nonthermal, and nonpharmacological approach that requires optimal intensity, wavelength, and treatment time, including repetitions capable of eliciting analgesia, anti-inflammation, tissue healing, and regeneration.^{56–60} PBM therapy has demonstrated efficacy in a broad range of neurocognitive and musculoskeletal diseases such as Parkinson's disease, depression, age-related dry macular degeneration, chronic wounds, arthritis, knee osteoarthritis, tendinopathy, and oral mucositis among many others.^{61–72} However, the reported PBM parameters used in these studies have varied considerably. There is a clear need for the rigorous device and delivery parameters and precise rationale for biological targets to address the inconsistent clinical outcomes. In this study, PBM parameters were chosen based on the reports noting PBM exposure with near-infrared light could directly activate latent TGF- β 1 and optimal dosing from several prior studies.⁴ Further, several careful light

dosimetry studies, specifically for deeper anatomical sites such as intracranial delivery, have outlined efficacious PBM dosing.^{73,74} This study extended these concepts to generate a median irradiance field enabling delivery of a whole body PBM treatment in live, awake animals to ensure minimal interventional variations with these long-term and repetitive therapy. It is worth emphasizing that the subtlety and simplicity of these low dose light treatments raise exciting future possibilities for nonobtrusive PBM delivery with ambient lighting fixtures, timed illuminations within lamps, digital phone apps, and electronic (tablets, computer or television) displays.

There are some limitations of the current investigation that could be addressed carefully in future studies. (1) This is a small study that measures many outcomes over multiple time points. Thus, when correcting for multiple comparisons, subtle changes are likely rendered insignificant. (2) The long-term results are based on surviving animals and hence, may be biased asymmetrically between the two groups. However, the mortality issue should not be considered to be a limitation because the analyses applied linear mixed-effects models to analyze this repeated-measures data (lmer in R) and lmer uses a likelihood-based approach to fit the model so no data are excluded and data “missing at random” (e.g., death in specific animal groups) still allow for the assessment of any statistical differences among them (see methods).

PBM treatments in AC8 mice improved left ventricular functions (EF) and reduced myocardial hypertrophy (LVM) and aortic wall stiffness (PWV), which mitigated some of the deteriorating aging-related effects noted in nontreated mice. The improvements in gait symmetry in these mice also suggest there is improved brain-limb coordination, which is an early indicator of adverse cerebral aging.^{75,76} While there were some improvements in these aging-related parameters in wild type mice, most of them were not statistically significant. Lack of robust age-associated changes in the cardiovascular system which is typical for this age-range in normal C57 mice might be the cause of such outcome. Finally, another possibility is that more careful (lower) PBM dosimetry is likely necessary to impact noncompromised, aging-related responses in wild type mice scenarios. Overdosing PBM therapy is construed to be a major factor in its inconsistent clinical benefits. Nonetheless, the results of this study outline the effectiveness of PBM therapy in counteracting the accelerated aging process in a rigorous transgenic animal model.

As proof-of-concept, this study focused on the changes in the cardiovascular system and, to a lesser extent, on brain-limb coordination that observed discrete benefits of PBM therapy. The correlation with both physiological and functional parameters during the active PBM treatment phases and weaning during the rest phase suggests that continued maintenance therapy is necessary. While the precise biological mechanisms

responsible for this phenomenon remain to be fully investigated, these observations further highlight the central roles of light in human health and wellness that is gaining much attention.⁷⁷ These nonvisual phototransduction pathways have exciting new implications for ambient lighting showcasing the putative roles of light as an essential supplement for optimal human health. Thus, several limitations in this study include disruption of the planned treatment routine, a limited number of animals per group, lack of dose escalation for more rigorous PBM dosing, and a more thorough analysis of changes in biological and molecular markers in cardiac and systemic tissues. Additionally, the precise molecular mechanisms driving these remarkable therapeutic responses remain to be fully elucidated and are beyond the scope of this current study. In conclusion, this study demonstrates the utility of PBM therapy as a nonpharmacological, noninvasive, simple, and practical adjunctive therapeutic modality to address cardiovascular diseases and aging.

AUTHOR CONTRIBUTIONS

Sunayana Begum Syed performed the experiments. Ismayil Ahmet designed the experiments, performed the experiments, supervised the experiments, and wrote the manuscript. Praveen R. Arany performed TGF- β 1 analysis and wrote the manuscript. Khalid Chakir performed the experiments. Christopher H. Morrell performed the statistical analysis. Edward G. Lakatta and Praveen R. Arany designed and supervised the experiments. All authors approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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