

Dental pulp stem cells ameliorate D-galactose-induced cardiac ageing in rats

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45 Abstract

46 **Background.** Ageing is a key risk factor for cardiovascular disease and is linked to several
47 alterations in cardiac structure and function, including left ventricular hypertrophy and increased
48 cardiomyocyte volume, as well as a decline in the number of cardiomyocytes and ventricular
49 dysfunction, emphasizing the pathological impacts of cardiomyocyte ageing. Dental pulp stem
50 cells (DPSCs) are promising as a cellular therapeutic source due to their minimally invasive
51 surgical approach and remarkable proliferative ability.

52 **Aim.** This study is the first to investigate the outcomes of the systemic transplantation of DPSCs
53 in a D-galactose (D-gal)-induced rat model of cardiac ageing.

54 **Methods.** Thirty 9-week-old Sprague–Dawley male rats were randomly assigned into three groups:
55 control, ageing (D-gal), and transplanted groups (D-gal + DPSCs). D-gal (300 mg/kg/day) was
56 administered intraperitoneally daily for 8 weeks. The rats in the transplantation group were
57 intravenously injected with DPSCs at a dose of 1×10^6 once every 2 weeks.

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Sprague–Dawley rats

58 **Results.** The transplanted cells migrated to the heart, differentiated into cardiomyocytes,
59 improved cardiac function, upregulated Sirt1 expression, exerted antioxidative effects,
60 modulated connexin-43 expression, attenuated cardiac histopathological alterations, and had
61 anti-senescent and anti-apoptotic effects.

62 **Conclusion.** Our results reveal the beneficial effects of DPSC transplantation in a cardiac ageing
63 rat model, suggesting their potential as a viable cell therapy for ageing hearts.

64
65 **Keywords:** dental pulp stem cell; cardiac ageing; rat model; D-galactose
66

67 Introduction

68 Ageing is a progressive, multifaceted process that is linked to decreased physiological
69 performance and an elevated risk of mortality (Guo et al., 2022). Although cardiac ageing is a
70 natural process, it is linked to various pathological factors that may result in age-related disorders
71 (Redgrave et al., 2023). Extensive research has demonstrated that ageing is linked to changes in
72 cardiac morphology and function, such as an increase in left ventricular hypertrophy and
73 cardiomyocyte volume as well as a decrease in cardiomyocyte number and ventricular
74 dysfunction, emphasizing the pathological impacts of cardiomyocyte senescence (Dai et al.,
75 2012; Shimizu et al., 2019).

76 A substantial body of evidence indicates that cardiovascular diseases and mechanisms involved
77 in cellular senescence are closely related (Pagan et al., 2022). The ageing heart is accompanied
78 by mitochondrial malfunction, and the consequent generation of reactive oxygen species
79 (ROS) may lead to age-related cardiac dysfunction (Shimizu et al., 2019). This generation of
80 ROS in cardiac tissue triggers the upregulated expression of ageing markers p53, p21, and p16

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85 (Shimizu et al., 2019). These markers are cellular regulatory factors involved in the cell cycle
86 and are remarkably upregulated in senescent cells. P53, a key player in the DNA damage
87 response (DDR) pathway, can induce either temporary or permanent cellcycle arrest (cellular
88 senescence). When presented with stimuli triggering DDR, cell-growth arrest and senescence are
89 mediated via the murine double minute 2 (MDM2)-P53-P21 and P16-retinoblastoma protein
90 (pRb) pathways. Administration of D-galactose results in the elevated expression of P53, P21,
91 and P16 (Bei et al., 2018, Wand et al., 2022). Sirt1, which is a member of a group of NAD-
92 dependent deacetylases collectively referred to as sirtuins, is involved in various molecular
93 pathways and is recognized as a pivotal protein in the control of ageing and metabolism. Several
94 reports have demonstrated that Sirt1 has a role in regulating cardiac myocyte growth and survival
95 under stress (Alcendor et al., 2007; Cencioni et al., 2015; Lu et al., 2014; Ministrini et al., 2021).
96 Furthermore, an imbalance in apoptotic control may be a major factor in ageing. Mitochondria
97 generate apoptogenic substances, such as cytochrome c, which regulate cellular apoptosis. The
98 ratio of pro- and anti-apoptotic Bcl-2 family members, or pro- and anti-apoptotic proteins, is
99 critical in the intrinsic apoptosis process (Phaneuf et al., 2002, No et. al., 2020).
100 Mesenchymal stem cells (MSCs) have exhibited encouraging therapeutic prospects for numerous
101 disorders and organ repairs. Adult stem cells have been successfully harvested from various
102 organs and can differentiate into various cell phenotypes. Their implantation presents a limited
103 risk of tumorigenesis and poses almost no ethical issues (Brown et al., 2018). MSCs have been
104 harvested from bone, adipose, lung, umbilical cord, and dental pulp tissue (Berebichez-Fridman
105 et al., 2019). Growing experimental and clinical evidence supports that MSCs constitute a
106 promising therapeutic approach for the remedy of cardiac dysfunction. They mediate the
107 production of a plethora of growth factors, replace lost cells, and create a niche that promotes
108 endogenous cardiac repair (Bahrami et al., 2023; Barrere-Lemaire et al., 2023; Bui et al., 2023;
109 Gu et al., 2023). Limited studies have investigated the potential beneficial effects of MSC
110 injection in animal models of ageing hearts. In rodent ageing models, bone marrow- and adipose-
111 derived MSCs have proven their efficacy in improving cardiac function and downregulating
112 ageing-linked cardiac damage, which is associated with a downregulation in the expression of
113 apoptosis and senescence markers. However, in these studies, homing, survival, and potential
114 differentiation of MSCs into cardiomyocytes have not been examined (Zhang et al., 2015; Chang
115 et al., 2021).
116 Considering the long-term clinical benefits, autologous MSC transplantation is superior to
117 allogeneic cell transplantation. However, the outcomes of autologous cell therapy remarkably
118 deteriorate with age due to their depletion and limited self-renewal capacity. Recent research has
119 highlighted the impact of ageing and illnesses on both human and rodent tissues, demonstrating
120 phenotypic and functional alterations in endogenous MSCs derived from the bone marrow,
121 adipose tissue, and heart (Li et al., 2013, Gnani et al., 2019, Hong et al., 2020, Guo et. al., 2023).
122 Conversely, recent studies have shown that dental pulp stem cells (DPSCs) exhibit resistance to
123 senescence effects and possess enhanced differentiation potential in vitro and regeneration
124 capabilities in vivo compared to bone marrow mesenchymal stem cells (BMMSCs) (Ma et al.,

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152 2019). Furthermore, DPSCs isolated from elderly individuals demonstrate active metabolism;
153 their derived miRNAs and exosomes represent a rich source of nanovesicles for the treatment of
154 age-related disorders, indicating the healthy condition of these cells and emphasizing their
155 suitability for autologous applications (Jezzi et al., 2019). Such suitability, which is attributed to
156 the resistance of these cells to ageing process, indicates that they are a promising option for the
157 treatment of age-related diseases.

158 A previous study provided the first proof-of-principle that the DPSC secretome protects against
159 D-gal-induced ageing of multiple organs (Kumar et al., 2022). The D-galactose-induced ageing
160 model is well-established and widely recognized. D- galactose treatment can efficiently induce
161 cardiac ageing as indicated by elevated levels of several cardiac ageing markers, including
162 oxidative stress, decreased expression of antioxidants such as superoxide dismutase (SOD),
163 upregulated levels of p53, and enhanced cardiac apoptosis (Bo-Htay et., 2018; Wang et al.,
164 2022). However, the efficacy of the systemic administration of DPSCs in ameliorating age-
165 associated cardiac function and structural deterioration has not yet been evaluated. Several
166 studies have reported the capability of DPSCs to differentiate into cardiomyocytes in vitro (Xin
167 et al., 2013; Sung et al., 2016); however, to the best of our knowledge, such trans-differentiation
168 abilities have not yet been evaluated in ageing hearts. In the current study, we aimed to assess,
169 for the first time, the possible efficacy of an intravenous injection of DPSCs in a D-gal-induced
170 rat model of cardiac ageing to evaluate their potential as a preventive therapy for age-associated
171 cardiovascular diseases.

172

173 Materials & Methods

174 2.1. Animals

175 Thirty male Sprague–Dawley rats (8 weeks old, 180–200 g) were purchased from the Theodor
176 Bilharz Research Institute, Imbaba, Egypt, and kept in the animal house of the Faculty of Medicine,
177 Menoufia University, Egypt as previously described by El-Akabawy et al., 2023. The animals were
178 acclimatized to laboratory conditions for 1 week before the start of the experiment. The rats were
179 kept in standard cages: two were rats kept in each cage, under standard laboratory conditions (22
180 ± 5 °C, 60 ± 5% humidity, and a 12-h/12-h light/dark cycle). Standard laboratory chow and tap
181 water were provided ad libitum. At the end of the experiment, rats were anaesthetized via an
182 intraperitoneal injection of ketamine (90 mg/kg) and xylazine (15 mg/kg) and were decapitated
183 (El-Akabawy et al., 2023). Rats were euthanized in the event of rapid weight loss or impaired
184 ambulation via lethal injection of pentobarbital sodium (200 mg/kg). In this study, no rats were
185 euthanized prior to the planned end of the experiment. All experimental procedures involving
186 animals were approved by the Institutional Review Board of Ajman University, UAE [IRB# M-F-
187 A-11-Oct].

188 2.2. DPSC isolation and culture

190 Dental pulp tissues were obtained from the pulpal cavity of Sprague–Dawley male rat incisors
191 (aged 6–8-week-old) and cultured as previously described (Patel et al., 2009). Briefly, the dental
192 pulp tissues were promptly harvested and enzymatically digested in a solution of 3 mg/mL
193 collagenase type 1 (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C. The cells (1 × 10⁶

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cells) were cultured in T25cm flasks (Falcon). The culture media consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% foetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin. The flasks were incubated at 37 °C in a humidified incubator with 5% CO₂. Media were replaced every three days. The cells were passaged at 80% confluence using 0.05% trypsin–EDTA (Sigma-Aldrich) for 3–5 min. To evaluate cell viability, the cell suspension was mixed with 0.4% Trypan blue (Gibco), and 10 µL of the mixture was loaded in each chamber of a haemocytometer. Counting of the viable and non-viable cells was conducted within 5 min. Cells were used at passage 4.

2.3. Flow cytometry

Cells were resuspended in staining buffer [2% FBS/ phosphate buffered saline (PBS)] and surface-stained with FITC-conjugated mouse anti-rat CD105 (BioLegend, UK), FITCH-conjugated mouse anti-rat CD90 (BD Pharmingen, USA), PE-conjugated rabbit anti-rat CD34 (Abcam, UK), or PE-conjugated rabbit anti-rat CD45 (Abcam, UK) at 4 °C for 30 min. Isotype-matched antibodies were used as controls. Cells were analysed using an EPICS XL flow cytometer (Beckman Coulter) (El-Akabawy et al., 2022).

2.4. Experimental design

Rats were randomly divided into three groups: control, D-galactose (D-gal)-treated, and D-gal + DPSCs-treated (n = 10 in each group). G Power software was used to determine the sample size. Rats (aged 9 weeks) in the D-gal- and D-gal + DPSCs-treated groups were given intraperitoneal injections of d-gal (300 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) daily for 8 weeks. Rats in the D-gal + DPSCs group received intravenous administration into the tail vein of 1 × 10⁶ DPSCs labelled with the membrane-bound fluorescent marker PKH26 (Sigma-Aldrich) once every two weeks (El-Akabawy et al., 2022).

2.5. Measurement of body weight and the heart index

Body weights were measured weekly. At the end of the experiment, the rats were anaesthetized via intraperitoneal injection of ketamine (90 mg/ /kg) and xylazine (15 mg/kg) and were decapitated. Hearts were immediately dissected and weighed. Heart indices were calculated as follows: heart tissue weight (mg)/final body weight (g) (El-Akabawy et al., 2022).

2.6. Transthoracic echocardiography

All transthoracic echocardiography (TTE) measurements were performed using a linear transducer. A linear-array probe was used at a frequency of 10 MHz and attached to a Mindray M7 premium (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., PR China) ultrasound echocardiography Doppler machine. The rats were anaesthetized by intraperitoneal injection of ketamine hydrochloride (25 mg/kg) and xylazine (5 mg/kg). Anaesthesia was followed by hair removal from the anterior part of the chest and thereafter, rats were kept on a specialized warming table to sustain normothermia (Watson et al., 2019)

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267 Ejection fraction (EF) and fractional shortening (FS), left ventricular internal dimension at end-
268 systole (LVIDs), left ventricular end-systolic volume (ESV), left ventricular posterior wall
269 thickness at end-diastole (LVPWd), left ventricular internal dimension at end-diastole (LVIDd),
270 and left ventricular end-diastolic volume (EDV) were assessed following the American Society
271 of Echocardiography guidelines. This assessment was performed in a blind manner by an
272 independent experienced researcher.

273

274 **2.7. Electrocardiogram (ECG) recordings**

275 Three touch electrodes of the PowerLab settings (MLA1214, AD Instruments, New South
276 Wales, Australia) were attached to an animal bioamplifier (FE136 Animal Bio Amp, AD
277 instruments, New South Wales, Australia) and fixed to the animals. Lead placement was
278 conducted according to the lead II configuration for determining heart rate (HR) in small
279 laboratory animals (Stohr, 1998).

280 **ECG recordings were performed as previously described, by Harkin et al., 2022.** A

281 stretch of the ECG readings was obtained to calibrate the second channel for simultaneous beats
282 per minute (BPM) recordings. The 'Ratemeter' (within Chart for Powerlab) was utilized
283 simultaneously to measure the HR on channel 2 from the ECG plot in channel 1. HR was
284 adjusted between 0 and 500 BPM. The ratemeter band was adjusted in such a way that the upper
285 boundary remained positioned lower than the R-wave peak, whereas the lower line was higher
286 than the P- and T-waves and any other noise. This facilitated the monitoring of the ECG waves
287 on channel 1 with simultaneous BPM recorded on channel 2. The ECG was recorded at a
288 sampling speed of 500/s and within a voltage range of 500 mV. A high-pass filter was adjusted
289 to 0.3 Hz, and a low-pass filter (50 Hz) was used.

290 The ECG were digitized and stored using standard PC-based hardware (AD Instruments).
291 PowerLab v.7.3.7 was utilized to illustrate the recording diagrams. The recordings were analysed
292 using LabChart software (AD Instruments). The recorded ECG was used to calculate R-R, QRS,
293 PR, QT, cQT, and ST intervals using the same software. Throughout the experiment, the rats'
294 body temperature was sustained at 38 °C. The time at the start of the recording was set as 0.0
295 minutes. The corrected QT (cQT) was determined using Bazett's formula [$QT_c = QT/RR^{1/2}$]
296 (Goldenberg et al., 2006) installed in the software.

297

298 **2.8. Assessment of oxidative stress and antioxidant indices**

299 Malondialdehyde (MDA) and glutathione (GSH) levels, as well as superoxide dismutase (SOD)
300 activity, were measured in the cardiac tissue using a spectrophotometer. Rat cardiac tissues (100
301 mg) were homogenized in 1 mL of phosphate-buffered saline (PBS; pH 7.0) to assess the MDA
302 level and determine the degree of lipid peroxidation. After mixing the homogenates with 20%
303 trichloroacetic acid (TCA), the mixtures were centrifuged at 5000 rpm for 15 min. A 5%
304 thiobarbituric acid (TBA) solution was added to the supernatants and boiled for 10 min. The
305 absorbance was obtained at 532 nm and a standard curve was used to quantify the MDA levels.
306 The results are presented as nanomoles (nmol) per milligram (mg) of protein.

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Based on the suppression of a nitro blue tetrazolium reduction by O₂ produced by the xanthine/xanthine oxidase system, the superoxide dismutase (SOD) activity was determined, and absorbance was obtained at 550 nm. The findings are represented as units (U) per milligram (mg) of protein. One SOD activity unit was considered as the enzyme concentration required to generate 50% inhibition in 1 mL reaction solution per mg of tissue protein.

To examine GSH concentrations, cardiac tissue homogenates were incubated with a solution of dithiobis nitrobenzoate (DTNB) for 1 h. The absorbance was obtained at 412 nm. A standard curve was used to determine the GSH level. The results are presented in micromoles (mmol) per mg of protein.

2.9. Quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

RNA was isolated from cardiac-tissue homogenates of rats in each group using the RNeasy Purification Reagent (Qiagen, Valencia, CA, USA). RNA purity was assessed using a spectrophotometer, ensuring a 260/280 nm absorption ratio of 1.8–2.0 for all samples. Subsequently, cDNA synthesis was performed employing Superscript II (Gibco Life Technologies, Grand Island, NY, USA). Quantitative PCR (qPCR) was performed on a StepOne™ instrument with software version 3.1 (Applied Biosystems, Foster City, CA, USA). Reaction mixtures contained SYBR Green Master Mix (Applied Biosystems), gene-specific primer pairs (detailed in Table 1), cDNA, and nuclease-free water. Cycling conditions comprised an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Data analysis was conducted using the ABI Prism sequence detection system software, and quantification was performed with the Sequence Detection Software v1.7 (PE Biosystems, Foster City, CA). The comparative cycle threshold method (Livak and Schmittgen, 2001) was used to determine relative expression levels of the target gene, with all values normalized to β -actin mRNA.

2.10. Western blot analysis

Western blot analysis was conducted as previously described by Al-Serwi et al., 2021. Using radioimmunoprecipitation buffer (Sigma-Aldrich), proteins were isolated from the cardiac tissues. The homogenates were centrifuged at 12,000 $\times g$ at 4 °C for 20 min and the protein level was measured in lysate aliquots using a protein assay kit (Bio-Rad, Hercules, CA, USA). Samples were boiled at 95 °C for 5 min, separated (20 μg /lane) using 7% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, and subsequently transferred to nitrocellulose membranes (Bio-Rad). Next, the membranes were blocked for 1 h at room temperature (RT) using 5% bovine serum albumin in Tris-buffered saline (TBS), and then incubated for 12 h at 4 °C with primary antibodies specific for anti-sirt1 (Abcam, Cambridge, UK, cat # ab110304), anti-cleaved caspase-3 (Abcam, Cambridge, UK, cat # ab184787), anti-cytochrome c (Abcam, Cambridge, UK, cat # ab133504), anti-Bax (Abcam, Cambridge, UK, cat # ab32503), and anti-Bcl-2 (Abcam, Cambridge, UK, cat ab194583). After rinsing with TBS, the membranes were

incubated with secondary horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG antibody (1: 3000, Bio-Rad) for 1 h at RT. Proteins were visualized using enhanced chemiluminescence (ECL Plus; Amersham, Arlington Heights, IL, USA) and measured via densitometry using Molecular Analyst Software (Bio-Rad). The relative expression of each protein band was normalized to that of β -actin.

2.11. Histological and immunohistochemical analyses

At the end of the experiment, cardiac tissues were dissected and fixed in 10% formalin and embedded in paraffin wax. For histological assessment, 5- μ m left ventricular sections were de-paraffinized, rehydrated using a graded ethanol series (100%, 90%, and 70%), and stained with haematoxylin and eosin (H&E) or Masson's trichrome.

For immunofluorescence staining, cardiac tissue was fixed at 4 °C for 24 h and cryoprotected in 30% sucrose at 4 °C. Using a cryostat, serial sections (40 μ m) were obtained and kept at -20 °C until use. The sections were incubated in 10% blocking solution at RT for 30 min and then incubated at 4 °C overnight with the following primary antibodies; rabbit Anti-Cardiac Troponin T antibody (1:2000, Abcam, Cat. #ab209813), rabbit Anti-Cardiac Troponin I antibody (1:1000, Abcam, Cat. #ab209809), or rabbit anti-connexin-34 (1:1000, Abcam, Cat. #ab259276). Thereafter, sections were rinsed with PBS and incubated with a secondary antibody (1:500, Alexa-488, Cat. #A-11034, Molecular Probes) for 1 h. After washing with PBS, the sections were mounted in Fluoroshield mounting medium containing DAPI (Abcam, Cat. #ab104139).

2.12. Quantitative histological assessments

For quantitative evaluation, three H&E- and Masson-stained sections per rat were used. By applying ImageJ software (NIH, Bethesda, Maryland, USA), the H&E- and Masson-stained sections were examined to determine the cardiomyocyte area and Masson's-stained area, respectively. A Leica DMLB2/11888111 microscope equipped with a Leica DFC450 camera was used to acquire the images.

Connexin-43 immunofluorescence was measured by randomly capturing five non-overlapping images per slide. A Leica DM5500 B/11888817/12 microscope, fitted with a Leica HI PLAN 10 $\times$ /0.25 objective and a Leica DFC450C camera, was used to capture the images. Connexin-43-stained spots were manually counted using the plugin/cell counting tool (Rangan and Tesch, 2007) in ImageJ software (National Institutes of Health, Bethesda, Maryland, US), and the average per field for each rat was then calculated. This measurement was performed in a blind manner by an independent experienced researcher. For statistical analysis and comparison, ten animals were used per experimental group.

2.13. Statistical analysis

Statistical analysis was performed as previously described by El-Akabawy et al., 2022. The data are presented as the mean $\pm$ SEM. Normal distributions were assessed using the D'Argostino and

Deleted: 5- μ m left ventricular sections

Pearson normality tests, and data were analysed using one- or two-way analysis of variance (ANOVA), followed by a post hoc Bonferroni test. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.03 software (GraphPad Software, San Diego, California, USA). No inclusion/exclusion criteria were applied in this study.

Results

3.1. Characterization of DPSCs

DPSCs derived from the dental pulp tissue of Sprague–Dawley rats were spindle-shaped after 10 days of culture. Flow cytometry was used to characterize the cells at passage 4. The expression of CD90 and CD105 (mesenchymal cell markers), and CD45 and CD34 (hematopoietic lineage markers) were assessed. Over 90% of the cells were identified as CD90+ and CD105+ and less than 10% were identified as CD45+ and CD34+ (Fig. S1). These findings suggest that most of these cells were MSCs.

3.2. DPSC transplantation improves body weight and heart indices

Body weights of the rats in the control, D-gal and transplanted groups did not differ significantly (Fig. 1A). However, the heart index was dramatically increased in aged rats than in control rats, suggesting cardiac hypertrophy. DPSC transplantation dramatically decreased the heart index compared with that of D-gal rats (Fig. 1B), indicating that the transplanted cells improved reduced D-gal-induced hypertrophy.

3.3. Intravenous injection of DPSCs reduces cardiac dysfunction in D-galactose-induced aged rats

Next, we evaluated the effects of DPSC transplantation on D-galactose (D-gal)-induced cardiac ageing in rats using echocardiography. The results revealed widespread LV systolic and diastolic dysfunction, including reduced EF% and FS%, elevated LVIDs, ESV, and LVPWd in D-gal-treated rats. Compared to D-gal-treated rats, DPSC injection improved EF% (Fig. 2B) and FS% (Fig. 2C) and reduced LVPWd (Fig. 2D), LVIDs (Fig. 2E), ESV (Fig. 2F) measurements. We observed an increase in left ventricular internal dimension at end-diastole (LVIDd) (Fig. 2G) and left ventricular end-diastolic volume (EDV) (Fig. 2H) in aged hearts; however, this difference was not statistically significant. D-gal intraperitoneal injection led to significant changes in the ECG results of treated rats in the form of increased QRS duration and cQT interval, but decreased R amplitude and ST height compared to the control group. Rats in the D-gal + DPSCs group exhibited an improved ECG pattern, as indicated by a significantly decreased QRS duration and cQT interval, and an elevated R amplitude and ST height, compared to the aged group (Table 2).

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451
452 **3.4. Effect of DPSC transplantation on the expression of connexin-43**
453 The decreased production of the critical gap junction protein, connexin-43, plays a pivotal role in
454 age-related cardiac dysfunction (Rodriguez-Sinovas et al., 2021). To determine whether DPSC
455 transplantation **restored** connexin-43 expression in the hearts of aged rats, connexin-43
456 immunoreactive areas were evaluated. In D-gal-treated rats, immunoreactive connexin-43 areas
457 were sparse, and their expression in cardiac tissue markedly decreased, whereas in the D-gal +
458 DPSCs group, they were markedly upregulated **(Fig. 3A-D)**.

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460
461 **3.5. DPSC systemic transplantation attenuates cardiac histopathological alterations in D-galactose-induced aged rats**

462 To examine the alterations in the myocardial architecture of different groups, left ventricular
463 cardiac tissue slides were stained with H&E. The left ventricular cardiac tissue of D-gal-induced
464 ageing rats exhibited a distorted myocardial structure, characterized by a disorganized
465 arrangement of cardiomyocytes and expanded intercellular space **(arrows, Fig. A)** when
466 compared to the control rats. In contrast, marked improvement was observed in the ageing rats
467 that received DPSC injection. H&E staining also revealed that the left ventricular
468 cardiomyocyte cross-sectional area was significantly enlarged in D-gal-induced ageing rats,
469 whereas DPSC treatment markedly decreased the cardiomyocyte area in D-gal + DPSC-treated
470 rats **(Fig. B)**. Masson's trichrome staining was used to examine the degree of cardiac fibrosis in
471 the different groups. The collagen-stained area in the interstitial and perivascular areas of the
472 **myocardium** dramatically increased in D-gal-induced ageing rats. However, DPSC treatment
473 significantly reduced collagen accumulation compared to that in the D-gal group
474 **(Fig. 4C and D)**.

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475
476 **3.6. DPSCs migrate to and survive in the cardiac tissue with a few transplanted cells**
477 **differentiated into cardiomyocytes**

478 An immunofluorescence-based examination of the cardiac tissue of D-gal + DPSCs rats was
479 performed to assess the engraftment of injected DPSCs into the heart. **Transplanted DPSCs were**
480 **distinguished from recipient cells via labelling with PKH26, a cell membrane-binding dye with**
481 **red fluorescence**. PKH26-labelled cells were distributed in the cardiac tissues of the D-gal +
482 DPSCs rats **(Fig. 5 B, F, J, and N)**. Interestingly, a few of the transplanted cells differentiated
483 into cardiomyocytes, as indicated by the colocalization of cardiac troponin T (cTnT) **(Fig. 5A-**
484 **H)**, or cardiac troponin I (cTnI) with PKH26-labelled cells **(Fig. 5I-P)**.

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Deleted: **Transplanted DPSCs were distinguished from recipient cells via PKH26 labelling, which is a cell membrane-binding dye with red fluorescence.**

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487 **3.7. DPSCs upregulate Sirt1 expression and exert an antioxidative effect in D-galactose-induced cardiac ageing in rats**

488 We further investigated whether DPSC transplantation has antioxidant effects. Compared with
489 the control rats, we observed that aged rats exhibited higher concentrations of MDA **(Fig. 6A)**.

whereas the SOD (Fig. 6B) and GSH (Fig. 6C) levels were dramatically reduced in aged hearts compared with those in control rats. In D-gal + DPSC-treated rats, MDA concentrations in the cardiac tissue were significantly reduced (Fig. 6A), whereas SOD (Fig. 6B) and GSH (Fig. 6C) levels were higher than those in the aged rats (Fig. 6A-C). Sirt1 demonstrated its ability to delay ageing and induce cardiac antioxidative effects (Alcendor et al., 2007; Cencioni et al., 2015; Lu et al., 2014; Ministrini et al., 2021). Interestingly, DPSC-treated rats exhibited higher Sirt1 expression than D-gal-treated rats (Fig. 7A).

3.8. DPSCs exhibit anti-apoptotic effects in D-galactose-induced cardiac ageing in rats

Cardiac ageing is linked to a marked reduction in Bcl2, an anti-apoptotic marker, and a significant elevation in Bax and cytochrome c, thereby triggering apoptosis (Pollack et al., 2002). Western blot analysis demonstrated that the mitochondria triggered an increase in the pro-apoptotic markers Bax (Fig. 7B), cytochrome c (Fig. 7D), and cleaved caspase-3 (Fig. 7E), whereas the anti-apoptotic marker Bcl-2 (Fig. 7C) was reduced in D-gal-treated rats. However, in D-gal + DPSC-treated rats, the expression of all upregulated apoptotic markers decreased (Fig. 7B, D, and E), while the expression of Bcl-2 was enhanced (Fig. 7C).

3.9. Effects of DPSC administration on senescence-associated markers in D-galactose-induced ageing rats

Senescence-linked β -galactosidase (SA- β -gal) activity is commonly used to recognize cells as senescent. A group of cell cycle regulatory factors, such as P21, and P53, are also considered as senescence markers (Shimizu et al., 2019). The expression of senescence-related markers, such as SA- β -gal (Fig. 8A), p53 (Fig. 8B) and p21 (Fig. 8C), was significantly upregulated in aged rats compared to those in control rats. In D-gal + DPSC-treated rats, DPSCs efficiently reduced the expression of all evaluated ageing markers (Fig. 8A-C).

Discussion

Ageing is characterized by the progressive aggregation of cellular damage, resulting in the gradual dysfunction of several organs. Age-associated alterations in the heart include left ventricular hypertrophy, upregulated collagen deposition, and cardiac dysfunction. Ageing contributes significantly to the progression of age-associated cardiovascular diseases, leading to a notably elevated prevalence of such conditions in elderly populations. Therefore, there is an immediate need to develop more effective therapeutic approaches to manage cardiac insufficiency associated with ageing (Yan et al., 2021; Xie et al., 2023). A substantial body of evidence suggests the potential of MSCs in the treatment of heart disease (Jeong et al., 2018; Fu et al., 2020; Meng et al., 2022; Kalou. et al., 2023). DPSCs exhibit a range of distinct biological characteristics that confer protective effects on injured tissues, including the cardiac tissue (Gandia et al., 2008; Sui et al., 2020; Song et al., 2023). The current study aimed to investigate, for the first time, the potentially favourable outcomes of the systemic injection of DPSCs in D-galactose (D-gal)-induced aged hearts.

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571 Histopathological examination of ageing cardiac tissue revealed cardiomyocyte hypertrophy and
572 enhanced LV fibrosis, consequently reducing LV elasticity and leading to cardiac dysfunction.
573 Ageing hearts exhibit distinctive histological and functional characteristics, such as increased
574 cardiac remodelling and declining cardiac reserve. Compared with normal ageing in rats, D-gal-
575 induced ageing models present cardiac structural and functional ageing alterations (Lazzeroni et
576 al., 2022; Wang et al., 2022). In the current study, we demonstrated that D-gal-induced ageing
577 rats had aberrant cardiac structure and enhanced collagen deposition in the perivascular and
578 interstitial areas of the heart. These histopathological alterations in the cardiac structures were
579 reflected in our echocardiography analysis, which revealed an increase in LVIDd and EDV;
580 however, no significant difference was observed when compared with control rats. In contrast,
581 LVIDs, ESV, and LVPWd were markedly increased in aged rats. Echocardiography also
582 revealed worsening cardiac function, as indicated by decreased FS% and ES%. Interestingly, the
583 observed morphological and functional alterations were dramatically improved in the DPSC
584 transplanted group. Our results are in line with those of previous studies, albeit using other
585 source types of MSCs. It has been documented that the systemic injection of adipose-derived
586 stem cells (ADSCs) significantly ameliorated structural alterations and cardiac performance in
587 D-gal-induced ageing rats (Chang et al., 2021). Significant histopathological improvement was
588 also observed in D-gal-induced ageing rats administered intravenous injections of Wharton's
589 jelly stem cells, as indicated by a reduction in collagen and reversal of ageing-induced structural
590 changes (Hu et al., 2022). Intracardiac injection of BMMSCs was also found to ameliorate
591 natural ageing-associated cardiac hypertrophy and fibrosis and enhance cardiac performance, as
592 reflected by increased EF%, FS%, and reduced LVIDs (Zhang et al., 2015).
593 In this study, the cardiac dysfunction observed in aged rats was combined with significant
594 changes in the ECG patterns. Aged rats exhibited an increased QRS duration and cQT interval,
595 along with a decreased R amplitude and ST height compared to that observed in the control
596 group. A decline in the expression of the vital gap junction protein connexin-43, which is
597 responsible for transmitting signals along the conduction pathway and forming a functional
598 syncytium between myocytes (Rodriguez-Sinovas et al., 2021), was also observed. The impact of
599 disturbed functional syncytium on conducting signals could be exacerbated by the loss of
600 cardiomyocytes, which naturally develops during ageing, along with accompanying reactive
601 fibrosis enhancement (Tracy et al., 2020). In our study, DPSC transplantation reduced fibrosis in
602 aged rats and preserved connexin-43 expression, thereby improving ECG alterations. The
603 therapeutic effects of MSCs on cardiac electrical conductivity have also been documented. Li et
604 al. 2018 observed that BMMSC transplantation increased the density of connexin 43, which
605 improved the dispersion of electrical excitation in rats with myocardial infarction. MSC therapy
606 also resulted in a shorter QRS duration and QTc interval, indicating the capability of MSCs to
607 improve cardiac electrical velocity in a murine double infarction model (Park et al., 2022).
608 Most cardiomyocytes do not undergo active proliferation, and the yield rate of cardiomyocytes in
609 the human heart is less than or equal to 1% per year (Gude et al., 2018). Although cardiac

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615 progenitor cells (CPCs) are considered an interesting source of cell therapy, the aged human
616 heart exhibits a remarkable population of senescent CPCs, which may be responsible for the
617 development of cardiac malfunction instead of promoting regenerative effects (Shimizu and
618 Minamino, 2019). Previous research has shown that MSCs from various sources possess the
619 ability to differentiate into cardiomyocytes (Toma et al., 2002; Guo et al., 2018; Soltani and
620 Mahdavi, 2022; Zhou et al., 2023). Whether MSCs can differentiate into cardiomyocytes remains
621 debatable. While some studies have speculated that the detected regeneration could be a result of
622 donor cell fusion with recipient cardiomyocytes (Alvarez-Dolado et al., 2003), other studies have
623 reported that BMMSCs can give rise to cardiomyocytes under laboratory conditions, even during
624 a lack of cardiomyocytes, thus excluding the likelihood of cell fusion (Pallante et al., 2007).
625 Notably, MSCs derived from various sources are considered safer than genetically engineered
626 MSCs due to their reduced risk of oncogenicity or malformation (Hatzistergos et al., 2011).
627 MSCs of different origins exhibit varying capacities for differentiation into cardiomyocytes. The
628 specific differentiation potential of MSCs from specific sources may lead to more favourable
629 outcomes compared to others (Park et al., 2023). To our knowledge, no previous study has
630 examined the differentiation capacity of DPSCs in a cardiac ageing model. In our study, a
631 considerable number of DPSCs migrated and survived in the cardiac tissue, and some cells
632 colocalized with cTnT and cTnI. Our results are in line with those of several studies reporting the
633 capability of DPSCs to differentiate into cardiomyocytes (Arminan et al., 2009; Xin et al., 2013;
634 Potdar et al., 2015; Sung et al., 2016; Al Madhoun et al., 2021). However, our results suggested
635 that only a small number of DPSCs differentiated into cardiomyocytes. To further investigate the
636 underlying mechanisms by which the transplanted cells improved the structural and functional
637 alterations in D-gal-induced ageing hearts, we sought to evaluate the potential paracrine effects
638 of the injected DPSCs.

639 Emerging evidence consistently supports the notion that oxidative stress plays a significant role
640 in the physiological progression of ageing (Maldonado et al., 2023). When exposed to elevated
641 levels of oxidative stress, p53 displays pro-oxidative activities, intensifying stress levels, and
642 ultimately triggering apoptosis (Liu et al., 2011; Shi et al., 2023). A critical determinant in the
643 ageing process of cardiac tissue is the equilibrium between anti-apoptotic Bcl-2 and pro-
644 apoptotic Bax proteins. Bcl-2 inhibits apoptosis by suppressing cytochrome c release from
645 mitochondria (Pollack et al., 2002). Sirtuins, which are a group of nicotinamide adenine
646 dinucleotide (NAD⁺)-dependent histone deacetylases, are thought to regulate cardiac ageing by
647 influencing mitochondrial stress responses. Sirt1 activation has been suggested to increase the
648 expression of antioxidant enzymes, such as SOD and **catalase, leading to the inhibition of ROS**
649 **generation, which is a key player in oxidative stress** (Alcendor et al., 2007; Cencioni et al.,
650 2015). In our study, ageing hearts demonstrated increased oxidative stress and increased
651 expression of p53, pro-apoptotic BAX, and cytochrome c, and a decrease in the antioxidant
652 markers SOD, Bcl-2, and Sirt1. DPSC transplantation significantly ameliorated these changes.
653 Our results are in line with those of previous studies. Various MSC sources have demonstrated
654 the ability to exhibit antioxidant capacity, primarily through the upregulation of glutathione

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transferase (GST) and the increased activity of SOD. This, in turn, modulates the genes driven by the antioxidant response element (Xia et al., 2021). In studies involving D-gal-stressed H9c2 cells, the culturing of these cells with a conditioned medium derived from MSCs led to significant improvements in cell viability, upregulation of SOD expression, downregulation of oxidative stress levels, and inhibition of p53 activity (Hu et al., 2022). In the context of D-gal-induced ageing in rats, adipose tissue-derived mesenchymal stem cells (ADMSCs) exhibited a downregulation in mitochondria-triggered apoptotic markers, including Bax, cytochrome c, and cleaved caspase-3. Conversely, it has been reported that ADMSCs upregulate Bcl-2 and Sirt1 expression compared to other treatment groups (Chang et al., 2021). Our findings support those of previous studies demonstrating the antioxidant and anti-apoptotic capabilities of DPSCs (Song et al., 2015; Ullah et al., 2018; Hernandez-Monjaraz et al., 2020; El-Kersh et al., 2020; Al-Serwi et al., 2021). Our results demonstrated that antioxidant and anti-apoptotic capabilities of transplanted DPSCs are mediated by downregulation of the expression of p53, pro-apoptotic BAX, and cytochrome c and upregulation of the antioxidant markers SOD, Bcl-2, and Sirt1. In cultured cardiac myocytes, conditioned medium derived from DPSCs significantly inhibits apoptosis under hypoxic/serum deprived conditions. It also reduced the expression of proinflammatory genes induced by lipopolysaccharide. Notably, the anti-apoptotic effect of the conditioned medium of DPSCs was found to be more efficacious than that of conditioned media derived from BMSCs or ADSCs on cardiac myocytes (Yamaguchi. et al., 2015).

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679 Conclusions

680 Our results reveal that the systemic injection of DPSCs ameliorated cardiac structural and
681 functional alterations occurring in a rat model of D-gal-induced cardiac ageing. Our findings
682 support the beneficial effects of intravenously transplanted DPSCs on ageing-related cardiac
683 structural and functional changes. However, to examine whether the transplanted cells could
684 reverse ageing-induced changes, further studies are needed wherein DPSCs should be
685 transplanted after cardiac ageing has been established. In addition, before initiating clinical trials,
686 additional studies are required to promote the differentiation of these cells into cardiomyocytes
687 in vivo. Furthermore, a deeper understanding of the underlying mechanisms responsible for the
688 favourable outcomes of DPSC-secreted factors is crucial.

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690 Conflicts of Interest

691 The authors declare that they have no known competing financial interests or personal
692 relationships that could appear to influence the work reported in this paper.

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694 Authorship contribution statement

695 All authors contributed equally to this study.

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Availability of data and materials

The raw data are available in the Supplemental Files.

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Figure Legends

Figure 1. Body weight (A) and heart index (B) were assessed in control, aged (D-gal), and transplanted (D-gal + DPSCs) rats; $P < 0.001$ vs. control rats; $P < 0.01$ vs. aged rats. Data are expressed as means $\pm$ SEM. N = 10 per group.

Figure 2. DPSCs attenuate cardiac function alterations in D-gal-induced cardiac ageing at 2 weeks post-transplantation after the last DPSC injection. Representative echocardiographic images from rats of control, D-gal, and D-gal + DPSCs groups (A). Ejection fraction (EF)% (B), fraction shortening (FS)% (C), left ventricular posterior wall thickness at end-diastole (LVPWd) (D), left ventricular internal dimension at end systole (LVIDs) (E), left ventricular end-systolic volume (ESV) (F), left ventricular internal dimension at end-diastole (LVIDd) (G), and left ventricular end-diastolic volume (EDV) (H) were measured using echocardiography. $*P < 0.05$,

Deleted: Figure 1. Body weight (A) and heart index (B) were assessed in control, aged (D-gal), and transplanted (D-gal + DPSCs) rats; $P < 0.001$ vs. control rats; $P < 0.01$ vs. aged rats. Data are expressed as means $\pm$ SEM. N = 10 per group.

Deleted: at 2 weeks post-transplantation after the last DPSC injection

Deleted: (A). Ejection fraction (EF)% (B), fraction shortening (FS)% (C), left ventricular posterior wall thickness at end-diastole (LVPWd) (D), left ventricular internal dimension at end systole (LVIDs) (E), left ventricular end-systolic volume (ESV) (F),

1070 ** $P < 0.01$, *** $P < 0.001$, compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with
1071 the D-gal group. Data are expressed as mean $\pm$ SEM. N = 10 per group.

1072
1073 Figure 3. DPSCs upregulated connexin 43 expression in D-gal-induced ageing heart at 2 weeks
1074 post-transplantation of the last DPSC injection. Representative connexin-43 immunofluorescence
1075 images (A-C) and number of connexin-43 spots in ventricular cardiac tissue (D) of control,
1076 ageing (D-gal), and transplanted (D-gal + DPSCs) rats. *** $P < 0.001$ vs. control rats; $^{\#\#}P < 0.01$
1077 vs. aged rats. Data are expressed as mean $\pm$ SEMs. N = 10 per group. Scale bar = 500 μ m.

Deleted: at 2 weeks post-transplantation
of the last DPSC injection

1078
1079 Figure 4. Transplanted DPSCs improved the cardiac histopathological alterations and cardiac
1080 fibrosis in D-gal-induced ageing rats at 2 weeks post-transplantation of the last DPSC injection.
1081 Representative haematoxylin and eosin staining of cross (A) and longitudinal (B) section of the
1082 left ventricle. Representative Masson's trichrome staining and extent of fibrosis (C and D) in the
1083 control, ageing (D-gal) and transplanted (D-gal + DPSCs) groups. *** $P < 0.001$, compared with
1084 the control group; $^{\#}P < 0.001$, compared with the D-gal group. Data are expressed as mean $\pm$
1085 SEM. N = 10 per group. Scale bars = 100 μ m (A) and 500 μ m (C).

Deleted: at 2 weeks post-transplantation
of the last DPSC injection.

Deleted: cross (A) and longitudinal (B)
section of the left ventricle.

1086
1087 Figure 5. Survival and differentiation of transplanted DPSCs into cardiac cells in the transplanted
1088 (D-gal + DPSCs) group at 2 weeks post-transplantation of the last DPSC injection. Some
1089 PKH26-labelled DPSCs coexpressed cardiac troponin T (cTNT) (A-D) or cardiac troponin I
1090 (cTNI) (I-L). The boxed areas in A-D and I-L appear at a higher magnification in E-H and M-
1091 P, respectively. Inserts show a higher magnification of the boxed regions in E-H and M-P.
1092 PKH26-labelled cells (red) B, F, J, N; cTNT-positive cells (green) C, G; cTNI-positive cells
1093 (green) K, O; DAPI-stained nuclei (blue) A, E, I, M. Merged images (D, H, L, P). Scale bars =
1094 100 μ m (A-D and I-L), and 50 μ m (E-H and M-P).

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 μ m (C).

Deleted: transplanted (D-gal + DPSCs)
group at 2 weeks post-transplantation of
the last DPSC injection. Some PKH26-
labelled DPSCs coexpressed

1095
1096 Figure 6. DPSCs induced antioxidative effect on D-gal-induced ageing rats at 2 weeks post-
1097 transplantation of the last DPSC injection. Status of malondialdehyde (MDA, A), superoxide
1098 dismutase (SOD, B), and glutathione (GSH, C) in the hearts of control, aged (D-gal), and
1099 transplanted (D-gal + DPSCs) rats. *** $P < 0.001$ compared with control; $^{\#}P < 0.001$. Data are
1100 expressed as mean $\pm$ SEM. N = 10 per group.

Deleted: at 2 weeks post-transplantation
of the last DPSC injection

1101
1102 Figure 7. DPSCs upregulated Sirt1 expression and exerted anti-apoptotic effects in D-gal-
1103 induced aged hearts at 2 weeks post-transplantation of the last DPSC injection. A) Western
1104 blotting analysis showing the expressions of Sirt1 and apoptotic markers in the hearts of control,
1105 aged (D-gal), and transplanted (D-gal + DPSCs) rats. B) Densitometry analysis of apoptosis-
1106 associated protein levels in different experimental groups. *** $P < 0.001$ compared with control;
1107 $^{\#\#}P < 0.001$. Data are expressed as mean $\pm$ SEM. N = 10 per group.

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of the last DPSC injection

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1126 Figure 8. Protective effects of DPSCs assayed on senescence-associated markers in D-gal-
1127 induced aged heart at 2 weeks post-transplantation of the last DPSC injection. Gene expression
1128 of senescence-associated markers such as senescence-linked β -galactosidase (SA- β -gal, A), p53
1129 (B), and p21(C) in the heart of control, aged (D-gal), and transplanted (D-gal + DPSCs) rats as
1130 measured by RT-qPCR. *** $P < 0.001$ compared with control; $^{***}P < 0.001$. Data are expressed as
1131 mean $\pm$ SEM. N = 10 per group.

1132
1133 Figure S1. Characterization of the dental pulp stem cell (DPSC) population. The cell-surface
1134 phenotype of the DPSCs was assessed by flow cytometry at passage 4, using antibodies against
1135 CD90, CD105, CD45, and CD34. In total, 90.1%, and 94.6% of the cells expressed CD90 and
1136 CD105, respectively, whereas only 3.4% and 8.6 % expressed CD45 and CD34, respectively.

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of the last DPSC injection

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such as senescence-linked β -galactosidase
(SA- β -gal, A), p53 (B), and p21(C)

Deleted: at passage 4

2.5. Measurement of body weight and the heart index

Body weights were measured weekly. At the end of the experiment, the rats were anaesthetized via intraperitoneal injection of ketamine (90 mg/ /kg) and xylazine (15 mg/kg) and were decapitated. Hearts were immediately dissected and weighed. Heart indices were calculated as follows: heart tissue weight (mg)/final body weight (g).

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