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Effects of low-dose oxygen ions and protons on cardiac function and structure in male C57BL/6J mice

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Abstract

Purpose: Astronauts traveling beyond low-Earth orbit will be exposed to high linear-energy transfer charged particles. Because there is concern about the adverse effects of space radiation on the cardiovascular system, this study assessed cardiac function and structure and immune cell infiltration in a mouse model of charged-particle irradiation.

Materials and methods: Male C57BL/6J mice were exposed to oxygen ions (¹⁶O, 600 MeV/n at 0.25–0.26 Gy/min to a total dose of 0, 0.05, 0.1, 0.25, or 1 Gy), protons (150 MeV, 0.35–0.55 Gy/min to 0, 0.5, or 1 Gy), or protons (150 MeV, 0.5 Gy) followed by ¹⁶O (600 MeV/n, 0.1 Gy). Separate groups of mice received ¹³⁷Cs γ -rays (1 Gy/min to 0, 0.5, 1, or 3 Gy) as a reference. Cardiac function and blood velocity were measured with ultrasonography at 3, 5, 7, and 9 months after irradiation. At 2 weeks, 3 months, and 9 months, cardiac tissue was collected to assess apoptosis, tissue remodeling, and markers of immune cells.

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Disclosure statement

The authors report no conflicts of interest.

Results: Ejection fraction and fractional shortening decreased at 3 and 7 months after ^{16}O . These parameters did not change in mice exposed to γ -rays, protons, or protons followed by ^{16}O . Each of the radiation exposures caused only small increases in cleaved caspase-3 and numbers of apoptotic nuclei. Changes in the levels of α -smooth muscle cell actin and a 75-kDa peptide of collagen type III in the left ventricle suggested tissue remodeling, but there was no significant change in total collagen deposition at 2 weeks, 3 months, and 9 months. Increases in protein amounts of cluster of differentiation (CD)2, CD68, and CD45 as measured with immunoblots at 2 weeks, 3 months, and 9 months after exposure to protons or ^{16}O alone suggested immune cell infiltration. For type III collagen, CD2 and CD68, the efficacy in inducing protein abundance of CD2, CD68, and CD45 was $^{16}\text{O} > \text{protons} > \gamma\text{-rays} > \text{protons followed by } ^{16}\text{O}$.

Conclusions: Low-dose, high-energy charged-particle irradiation caused mild changes in cardiac function and tissue remodeling in the mouse.

Keywords

space radiation; mouse model; cardiovascular system; degenerative tissue effects; immune cell infiltration

1. Introduction

Astronauts traveling beyond low-Earth orbit will be exposed to low-dose-rate space radiation [1]. Under these conditions, the nucleus of every cell in the body will likely encounter a proton or secondary electron every few days and an ion with a high atomic number and high energy (HZE) once a month [2]. Although the incidence of HZE-particle passage may seem low compared to protons or secondary electrons, the high ionization potential of HZE particles makes them significant contributors to biological damage [2].

Exposure of the heart to ionizing radiation at doses of several Gy during cancer therapy can have adverse cardiovascular effects such as pericarditis, fibrosis, coronary heart disease, and accelerated atherosclerosis [3,4]. In addition, studies of atomic bomb survivors [5] and other human populations exposed to ionizing radiation [6,7] have provided evidence for the development of cardiovascular disease following (whole body) exposure to radiation doses that are manifold lower than those used for radiotherapy. These study outcomes have raised concerns about the potential risk of degenerative cardiovascular disease during or after exposure to charged-particle irradiation during deep-space travel [8,9].

There are limited opportunities to obtain data on the biological effects of charged particles from human subjects, leaving us almost exclusively dependent on animal models. As such, some studies have begun to characterize the cardiovascular effects of whole-body exposure to high-energy charged particles in rodent models. Yan et al. demonstrated changes in cardiac function as measured by echocardiography and hemodynamics in male C57BL/6 mice at time points from 1 to 10 months after irradiation with 0.15 Gy iron ions (^{56}Fe , 1 GeV/n) [10]. In a subsequent study, changes in the expression of genes involved in cell death, the oxidative stress response, and inflammation were identified in cardiomyocytes up to 1 month after 0.15 Gy ^{56}Fe (1 GeV/n) [11]. In a mouse model, radiation-induced changes in cardiac function were different when ^{56}Fe (1 GeV/n) was administered before 3 fractions

of protons (1 GeV) or administered after 3 fractions of protons [12]. Therefore, biological outcomes in the heart may depend on the order in which charged particles are delivered. Tungjai et al. demonstrated an increase in cardiac cleaved poly (adenosine diphosphate-ribose) polymerase, a marker of apoptosis, and indices of inflammation, such as increased activated NF- κ B [nuclear factor kappa-light-chain-enhancer of activated B cells], interleukin-1 β and interleukin-6, and tumor necrosis factor- α , in male CBA/CaJ mice up to 6 months after exposure to silicon ions (^{28}Si , 300 MeV/n, 0.01 – 0.5 Gy) [13]. Soucy et al., with ^{56}Fe (1 GeV/n) irradiation (0.5 and 1 Gy) in male Wistar rats, demonstrated increased aortic stiffness using pulsed-wave Doppler [14], and Ghosh et al. showed reduced endothelium-dependent and -independent vasodilation in the gastrocnemius muscle feed artery in ^{56}Fe (600 MeV/n, 1 Gy)-exposed male C57BL/6 mice [15].

The HZE component of space radiation includes the nuclei of any element with an atomic number greater than 2, with C, O, Ne, Mg, Si, and Fe being among the most abundant [16, 17]. The ionization density of these nuclei increases as the atomic number increases [16]. However, while the ionization density and resulting cellular damage by Fe are high, astronauts within a spacecraft will be exposed primarily to smaller fragmented products of heavy ions that interact with the spacecraft hull [17]. Hence, behind a shield of 5 g/cm² aluminum, the expected average density of a common spacecraft wall, protons are thought to contribute to about 30% of the dose equivalent to the heart of an astronaut, and particles with an atomic number between 11 and 20 are expected to contribute to about 15% of the heart dose equivalent [18]. Therefore, we designed a study in a C57BL/6 mouse model, widely used to study the effects of radiation on the cardiovascular system [10,12,15,19], to assess effects of low doses of protons and oxygen ions (^{16}O) on cardiac function and structure. Doses of protons and ^{16}O were selected based on the abundance of protons versus heavy ions in space. Additional cohorts of mice were exposed to γ -rays as a reference radiation. Relative biological effectiveness (RBE) values for proton or heavy ion-induced cardiovascular effects have not yet been determined. However, based on prior studies in protons compared to heavy ions [10,19], we expected the cardiovascular toxicity levels to be ^{16}O >protons> γ -rays. Therefore, we exposed mice to γ -rays at doses up to 3 times the highest dose of protons and ^{16}O . The overall purpose of the study was to characterize the short- and long-term effects of protons and ^{16}O at space-relevant doses on cardiac function, apoptosis, markers of immune cells, and extracellular remodeling in the heart and make comparisons with the effects of γ -rays.

2. Material and Methods

2.1 Animal housing and radiation

Male C57BL/6J mice (1–2 months old) were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed 5 per cage at the University of Arkansas for Medical Sciences (UAMS) until they were 6 months old. Mice were fed a low-soy diet (2020 $\times$, Harlan Laboratories, Indianapolis, IN, USA) because soy contains radioprotective compounds, including genistein [20]. They had access to chow and water *ad libitum* and were housed on a 12:12 hour light:dark cycle.

At 6 months old, mice were transported to Brookhaven National Laboratory (BNL; Upton, NY) by overnight air transportation (World Courier, London, UK). At BNL, mice were housed on a 12:12 hour light:dark cycle and fed the 2020× diet (Harlan Laboratories) *ad libitum*. After a 1-week acclimation period at BNL, mice were exposed to whole-body proton and/or ^{16}O irradiation at the NASA Space Radiation Laboratory (NSRL) [21]. Unanesthetized mice were placed in individualized clear and well-ventilated Lucite cubes (3 in $\times$ 1½ in $\times$ 1½ in) and positioned 4 to 5 at a time in the NSRL beam line.

There were 4 cohorts of mice that received radiation at NSRL. In cohort 1, mice received a single dose of 0.1 Gy, 0.25 Gy, or 1.0 Gy ^{16}O (600 MeV/n) at a dose rate of 0.25–0.26 Gy/min during NSRL run 15A. In cohort 2, mice were exposed to a single dose of 0.5 Gy or 1 Gy protons (150 MeV) at a dose rate of 0.35–0.55 Gy/min during NSRL run 15C. In cohort 3, mice were exposed to a single dose of 0.05 Gy ^{16}O (600 MeV/n) at a dose rate of 0.04–0.05 Gy/min during NSRL run 16B. In cohort 4, mice were exposed to a single dose of protons (150 MeV, 0.5 Gy, 0.17–0.19 Gy/min) followed by ^{16}O an hour later (600 MeV/n, 0.1 Gy, 0.18–0.33 Gy/min) during NSRL run 16B. Radiation dosimetry was performed by the NSRL physics team. For each NSRL run, age-matched sham-irradiated mice were also transported to NSRL and placed in identical clear Lucite cubes, but they were not exposed to ^{16}O or protons. One or two days after irradiation or sham treatment, mice were returned to UAMS by overnight air transportation. After returning to UAMS from BNL, all mice were given 2020× chow containing 150 ppm fenbendazole for 8 weeks as a routine quarantine procedure.

At 6 months old, a fourth cohort of mice was exposed to γ -rays using a cesium-137 (^{137}Cs) source (Mark 1 Model 68A, J L Shepherd & Associates, San Fernando, CA) at UAMS as a reference, at a dose rate of 1 Gy/min and to total doses of 0.5 Gy, 1 Gy, or 3 Gy. For this purpose, unanesthetized mice were placed in a clear, well-ventilated pie-shaped Plexiglas holder with individual compartments (5 mice per holder) and exposed in the ^{137}Cs source irradiator on a turntable (6 rpm) for even radiation exposure. Radiation dosimetry was performed with DOSE-MAP® gafchromic films (Ashland Specialty Ingredients, Wayne, NJ) and with an ion chamber calibrated for cesium (Exradin A20, Standard Imaging, Middleton, WI). For sham irradiation, age-matched animals were placed in the pie-shaped holder for 5 minutes but not exposed to radiation.

A total of 35 mice (40 in the combined proton and ^{16}O group) were allocated to each radiation dose. Of these mice, 10 were followed for up to 2 weeks after irradiation or sham-treatment, 10 were followed up to 3 months, and 15 (20 in the consecutive proton and ^{16}O group) were followed up to 9 months. Supplemental File, Table S1 lists the number of animals allocated to each radiation dose and time point. All animal procedures within this study were approved by the Institutional Animal Care and Use Committees at UAMS (protocol numbers 3523 and 3754) and BNL (protocol number 477).

2.2 High-resolution ultrasonography

Mice underwent ultrasonography in random order and performed by one individual (M.B.). Because anesthesia is known to alter cardiac physiology [22], we developed a procedure in which mice were under anesthesia for the shortest possible time during ultrasonography.

Hence, the day before ultrasonography, mice were anesthetized with 2% isoflurane, and hair was removed from the thorax and abdomen with depilatory cream. On the day of ultrasonography, mice were anesthetized with 1.5% isoflurane and placed supine on a heated platform that monitors respiration rate and generates an electrocardiogram. The mice were scanned immediately with a Vevo® 2100 high-resolution pre-clinical small animal imaging system (VisualSonics, Toronto, ON) with a MS400 (18–38 MHz) transducer. Echocardiograms were obtained in the short axis M-mode at the mid-left ventricular level. These echocardiograms were similar to those obtained by Yan et al. [10], albeit by different ultrasound equipment and a higher-frequency probe (18–38 MHz vs. 15 MHz as in [10]). Pulsed-wave Doppler was used to determine mitral valve E and A velocities in a four-chamber view of the heart. Pulsed-wave Doppler was also used to determine abdominal aortic blood flow as an indicator of both cardiac and vascular function. For this purpose, the probe was placed in the transverse axis, immediately anterior to the renal artery branch point.

One individual (P.S.) analyzed all of the ultrasonography output files, in random order. She was aware of the animal identification numbers, but these numbers did not identify the experimental groups. All ultrasound analyses were spot checked by M.B. on a weekly basis. In addition, when there was doubt about an individual ultrasound recording or results obtained, P.S. and M.B. would re-analyze the recordings together to make sure there was agreement. The Vevo® 2100 software version 1.6.0 cardiac package was used to obtain echocardiographic parameters, such as stroke volume (SV), ejection fraction (EF), fractional shortening (FS) and cardiac output (CO) from 3 M-mode scans per animal. The vascular package was used to assess mitral valve E and A velocities and blood flow parameters from 3 consecutive wave patterns per pulsed-wave Doppler scan, in up to 3 scans per animal. Blood velocity measures were obtained from velocity time integral (VTI) traces and from separate velocity measurements in each scan. All ultrasonography measures are listed in Supplemental File, Table S2.

2.3 Tissue collection

Mice were anesthetized with 3% isoflurane and weighed. A modified infusion set (27G with shortened tubing) was used to inject a single dose of heparin (30–40 U/kg) into the abdominal vena cava. Then, the heart was collected, rinsed, weighed, and cut into 2 longitudinal halves. One half of the heart was fixed in 5% formalin for 24 hours and embedded in paraffin for histology and immunohistochemistry. The other half was divided into atria, right ventricle, and 4 samples of left ventricle; all tissue samples were snap-frozen in liquid nitrogen and stored at –80°C until preparation for immunoblotting. Within 10 minutes after collecting the heart, the abdominal aorta was collected and fixed in 5% formalin for histology. Lastly, skin and muscle were removed from the tibia bone, and calipers were used to measure tibia length as an indicator of body size.

2.4 Histology

For histopathological analysis, 5-µm longitudinal sections of heart were stained with hematoxylin and eosin (H&E). For each experimental group, 1 section of 5 randomly selected hearts were shipped to a veterinary pathologist for analysis (Stanley D. Kosanke,

DVM, PhD; Heartland Veterinary Pathology Services, Edmond, OK). Sections were assessed and scored for inflammation, myofiber necrosis, interstitial fibrosis, microvascular damage, and epicardial fibrosis.

To quantify tissue collagen deposition, 5- μ m longitudinal sections of heart and 5- μ m cross sections of aorta were rehydrated and incubated with Sirius Red supplemented with Fast Green. Sections were scanned with a ScanScope CS2 slide scanner and analyzed with ImageScope 12 software (Leica Biosystems, Wetzlar, Germany). The relative tissue area of collagens was calculated as the red-stained area expressed as a percentage of the total tissue area of each section.

To quantify mast cells, sections were incubated in 0.5% Toluidine Blue in 0.5 N HCl for 3 days at room temperature, followed by 0.7 N HCl for 10 minutes. Eosin was used as a counterstain.

Apoptotic nuclei were identified with the CardioTACS™ Kit (Trevigen, Gaithersburg, MD), which is based on DNA end-labeling with terminal deoxynucleotidyl transferase, following the manufacturer's instructions.

Both Toluidine Blue and CardioTACS-stained sections were examined with an Axioskop transmitted-light microscope (Carl Zeiss, Oberkochen, Germany). Mast cells and apoptotic nuclei were counted by one individual (M.C.) who was aware of the animal identification numbers but not the treatment groups. Numbers of mast cells and apoptotic nuclei were divided by the total tissue area of each section as determined with ImageScope 12 software (Leica Biosystems).

2.5 Immunohistochemistry

Immunohistochemistry was used to identify cluster of differentiation (CD)45-positive cells. Sections were rehydrated, and antigen retrieval was performed by incubating the sections in Target Retrieval buffer (DAKO, Glostrup, Denmark) at 120°C in a decloaking chamber (Biocare Medical, Pacheco, CA) for 20 min, cooling them for 45 mins in the retrieval buffer, and rinsing in distilled water. Immediately after, sections were incubated in 1% H₂O₂ in methanol to block endogenous peroxidase, followed by 10% normal serum in 3% dry powdered milk and 0.2% bovine serum albumin to block non-specific antibody binding. Sections were incubated overnight with rat anti-CD45 (1:100 dilution, clone 30-F11, Santa Cruz Biotechnology, Santa Cruz CA), followed by biotinylated goat anti-rat IgG (1:400, Bio-Rad, Hercules, CA) and an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 45 minutes. Bound antibodies were visualized with 0.5 mg/mL 3,3'-diaminobenzidine (Sigma-Aldrich) and counterstained with hematoxylin. Stained sections were examined with an Axioskop transmitted-light microscope (Carl Zeiss), and CD45-positive cells were counted in 10 optical areas per section. Cells were counted by one individual (M.C.) who was aware of the animal identification numbers but not the treatment groups. The number of CD45-positive cells was divided by the total area of the 10 views.

2.6 Immunoblot analysis

To investigate protein expression indicative of cardiac remodeling, immune cell infiltration, or apoptosis, immunoblot analysis was performed on 6 frozen left ventricle tissue samples randomly selected from each radiation group, radiation dose, and time point. A Potter-Elvehjem mechanical compact stirrer (BDC2002, Caframo LabSolutions, Georgian Bluffs, ON, Canada) was used to homogenize the tissue in a 1% Triton-X100 radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (1:100) and phosphatase inhibitors (1:100; Sigma-Aldrich, St. Louis, MO). Protein concentration was determined with a bicinchoninic acid protein assay (Bio-Rad), and 25 µg protein was added to a 2× Laemmli buffer containing β-mercaptoethanol (5%). Gel electrophoresis was performed, and proteins were transferred to a polyvinylidene difluoride membrane.

Membranes were first incubated in tris-buffered saline (TBS) containing 0.05% Tween-20 and 5% non-fat dry milk to reduce non-specific antibody binding. They were then incubated overnight at 4°C with rat anti-α-smooth muscle actin (α-SMA, 1:4,000 dilution, Abcam, Cambridge, MA) or one of the following antibodies (Santa Cruz Biotechnology): rabbit anti-collagen type III (1:1,000), rabbit anti-mast cell tryptase (1:20,000), rabbit anti-cleaved and full-length caspase-3 (1:3,000), rabbit anti-CD2 (1:3,000), rabbit anti-CD68 (1:1,000), rat anti-CD45 (1:40,000), or mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:20,000) as a loading control; all were prepared in TBS containing 0.1% Tween-20 and 5% non-fat dry milk. After incubating with horseradish peroxidase-conjugated secondary antibodies: goat anti-rabbit IgG (Cell Signaling Technology, Danvers, MA; 1:10,000), goat anti-rat IgG (Santa Cruz Biotechnology; 1:30,000 or 1:40,000) or goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA; 1:20,000), membranes were covered in enhanced chemiluminescence (ECL) Plus Western Blotting Detection Reagent (GE Healthcare Life Sciences, Chicago, IL) and placed on CL-Xposure Film (Thermo Scientific, Waltham, MA). Films were developed and imaged with an AlphaImager® gel documentation system (ProteinSimple, San Jose, CA). Densitometry was performed with ImageJ software. All proteins were normalized to GAPDH, and then calculated relative to 0 Gy at each time point.

2.7 Statistics

Ultrasonography measured 20 parameters as listed in Table S2. Each measure was first fitted with a repeated-measures analysis of covariance comprised of radiation (an among-animal factor), time (a within-animal factor), their interaction as factors, and time-specific heart rate as a (linear) covariate. Heart rate during ultrasonography was included as a covariate because we and others have found that echocardiography outcomes may depend on heart rate [22]. Regarding the repeated measures, the best (lowest) Bayesian Information Criterion was used to choose the within-animal covariance from among these structures: exchangeable, autoregressive, Toeplitz, or general. From the residuals of the initial analysis, normal assumptions were evaluated using visual inspection of Q-Q plots, and homogeneous variance assumptions were evaluated with Levene's test for equal variances. Normal assumptions were reasonable for all outcomes. For all outcomes of immunoblotting, except CD45, standard deviations were larger at some post-radiation time points than at other time points. Hence, for those immunoblotting results homogeneous variances could not be

assumed, and the final analyses included adjustments to the covariance structure to accommodate heterogeneous variances when evidence indicated a need. The denominator degrees of freedom were estimated with Kenward-Roger's method [23]. In each analysis, the radiation groups were compared to the sham group within the same cohort and for the same time point. These analyses were conducted for each cohort separately.

All other analyses provided a total of 16 measures (Table S3). Each of these measures was fitted with an analysis of variance (ANOVA) with radiation, time, and the radiation $\times$ time interaction as (among-animal) factors. Using residuals from initial ANOVAs, normal and homogeneous variance assumptions were evaluated. Normal assumptions were reasonable for all outcomes. For those outcomes exhibiting evidence of heterogeneous variance, final analyses accounted for these variance differences. In each analysis, the radiation groups were compared to the sham group within the same time point. These analyses were conducted for each cohort separately.

All tests were conducted at a .05 significance level. These analyses were conducted with the MIXED procedure in SAS/STAT software, version 9.4 (SAS System for Windows, SAS Institute, Inc.). The purpose of the study was to identify which of all individual measures in this study (listed in Tables S2 and S3) may be of interest for future investigation in this field of research. Therefore, we chose not to make adjustments for multiple comparisons, so not to affect the statistical outcome for an individual parameter. Instead, positive false-discovery rates (pFDR) are calculated and presented in section 2.9.

2.8 Power analyses

Comparisons of interest were between sham and each irradiated group within a time point (e.g., 2 weeks, 3 months, 9 months). Because these comparisons are within a time point, repeated measures analyses had no bearing on power, but the longitudinal designs did reduce the number of animals used. We computed effect sizes detectable with a power between 0.8 and 0.85 using 0.05 significance level *t*-tests conducted within the analyses described above. For the earliest time point (35 mice/radiation group), the detectable effect size was 0.70 SD, where SD is the within-group standard deviation. For the middle time point (25 mice/radiation group) and last time point (15 mice/radiation group) detectable effect sizes were 0.80 SD and 1.0 SD, respectively.

2.9 Raw data and estimation of false discovery rate

Raw data are available in the NASA Ames Life Sciences Data Archive at <https://lsda.jsc.nasa.gov/>.

Positive false-discovery rates, the expected proportion of false discoveries given at least 1 significant hypothesis test, were estimated with the method presented in Storey's 3rd algorithm [24]. There were two sets of analyses: one for 20 longitudinal outcomes, and another for 16 static outcomes (i.e., outcomes which were measured only once per mouse). When analyzing one outcome, cohorts were analyzed independently. Within each of these cohort-specific analyses, we compared each radiation dose to its sham control within each time point. Because the purpose of the study was to identify which of these individual measures may be of interest for future research, we did not make adjustments for multiple

comparisons. Altogether, we performed 770 hypothesis tests for the longitudinal outcomes, of which 85 radiation-to-sham comparisons had $p < .05$. The estimated pFDR was 0.32 (and was no greater than 0.43 with 95% confidence). For the static outcomes, we performed 407 hypothesis tests, with 130 radiation-to-sham comparisons having $p < .05$. The estimated pFDR was 0.07 (and was no greater than 0.09 with 95% confidence).

3. Results

3.1 Animal characteristics

We first summarized animal survival and bodyweight after each of the radiation types. Of the 460 mice used in this study, 8 mice had to be euthanized before the pre-set endpoint because of wounds due to fighting (males) or alopecia (females).

All mice followed up to 3 and 9 months gained weight (Figure 1). However, mice exposed to protons at a dose of 1 Gy showed a significantly reduced bodyweight compared to sham controls at 9-month follow-up (Figure 1A). In contrast, mice exposed to 0.25 Gy ^{16}O experienced a small but significant decrease in bodyweight compared to sham controls at 3 months, followed by a small increase at 9 months (Figure 1B). Apparently, during the first half of the study, mice exposed to 0.25 Gy of ^{16}O were lacking in weight gain compared to sham controls, and then compensated for that lack of weight gain during the second half of the study. No significant changes were seen in the group exposed to protons followed by ^{16}O (consecutive exposure) (Figure 1C). Lastly, γ -rays at doses of 1 and 3 Gy caused small but significant decreases in bodyweight compared to sham controls (Figure 1D).

3.2 Heart weight

At sacrifice, the dry heart weight was measured and divided by tibia length to correct for body size. Changes in heart weight and heart weight divided by tibia length were small, and there was no clear trend with time post-irradiation or radiation dose (Figures S1 and S2). Therefore, the heart weight data provided no obvious indications of ventricular remodeling.

3.3 In vivo cardiac dimensions and function

The Supplemental Materials lists values for left ventricular inner diameter (LVID) and cardiac volume (Table S4), left ventricular anterior wall (LVAW) and left ventricular posterior wall (LVPW) thickness (Table S5), and the parameters EF, FS, SV, and CO (Table S6). To characterize diastolic function, we measured mitral valve E and A velocities from cardiac four-chamber views (Table S7). Four-chamber views were not obtained from mice exposed to ^{16}O at doses from 0.1–1 Gy, and results are therefore not provided. Lastly, we used pulsed-wave Doppler to obtain 3 measures of blood flow in the abdominal aorta (Table S8). All measures showed a relationship with heart rate and were therefore analyzed by adjusting for a linear effect of heart rate, as described in 2.7 Statistics. All comparisons described below were between irradiated animals and time-matched sham controls.

^{16}O (600 MeV/n):

Exposure to ^{16}O caused a decrease in LVID in diastole at doses of 0.1 and 0.25 Gy compared to sham controls at 3 months, and this translated in a decrease in cardiac volume

in diastole (Table S4). Cardiac volume at end of systole was decreased at 9 months after 0.25 Gy. A dose of 0.05 Gy caused an increase in LVAW thickness in diastole and LVPW thickness in both diastole and systole at 2 months (Table S5). Increases in LVPW thickness in diastole were also seen at 9 months after 0.1 Gy and 0.25 Gy. A dose of 1 Gy, on the other hand, caused a decrease in LVPW thickness at 3 months (Table S5). EF and FS were reduced at 3 months after 1 Gy and at 7 months after 0.1 Gy (Table S6). SV and CO were also reduced, as measured at 3 months after 0.25 Gy and 1 Gy (Table S6), suggesting a small decrease in systolic function in these animals. Mitral valve E and A velocities were not altered in mice exposed to ^{16}O at a dose of 0.05 Gy (Table S7) and were not measured after doses of 0.1–1 Gy. Lastly, abdominal aorta peak velocity was reduced at 9 months after 0.1 Gy and 1 Gy, and VTI was also reduced at 9 months after 0.1 Gy ^{16}O (Table S8).

Protons (150 MeV):

Protons at doses of 0.5 and 1 Gy caused an increase in LVID in diastole at 5 and 7 months and an increase in LVID in systole at 9 months (Table S4). In addition, LVAW thickness in systole was reduced at 9 months after 0.5 and 1 Gy (Table S5). Protons caused no significant changes in EF or FS (Table S6). Five out of 15 sham-irradiated mice had a low cardiac volume when measured at 5 months (less than 24 mm³ in systole and less than 54 mm³ in diastole). These same 5 mice remained at a low systolic and diastolic volume at 7 and 9 months, which affected the average values of cardiac volume, stroke volume, and cardiac output at these time points (Tables S4 and S6). Therefore, the clinical significance of the differences between sham radiation and proton exposed animals at the time points of 5–9 months is uncertain. Protons caused an increase in mitral valve E velocity at 5 months and increases in mitral valve E and A velocities at 7 months, but no change in the ratio of E/A (Table S7). Lastly, increases were observed in abdominal aorta peak velocity at 7 months after 0.5 Gy protons and at all time points after 1 Gy (Table S8).

Protons (150 MeV, 0.5 Gy) followed after an hour by ^{16}O (600 MeV/n, 0.1 Gy):

A decrease in LVAW thickness at diastole at 9 months was the only change in ultrasonography parameters that was observed in the consecutive exposure group compared to time-matched sham controls (Table S5).

γ -rays:

Exposure to γ -rays caused a decrease in LVID in systole at 9 months after 3 Gy (Table S4), an increase in LVAW thickness in systole at 9 months after 1 Gy (Table S5), a decrease in LVPW thickness in diastole at 5 months after 1 Gy (Table S5), but no changes in EF, FS, SV or CO (Table S6). In addition, γ -rays caused an increase in mitral valve A velocity at 7 months after 0.5 Gy. This was followed by a decrease in mitral valve A velocity at 9 months, which lead to an increase in the mitral valve E/A ratio at this time point. (Table S7). Hence, there is a small indication of diastolic dysfunction in this dose group. An increase in mitral valve A velocity was also seen at 9 months after 3 Gy γ -rays, but there was no change in E/A (Table S7). When abdominal aorta blood flow was examined, γ -rays were shown to cause an increase in peak velocity at 5 and 7 months after 1 Gy (Table S8).

3.4 Cardiac apoptosis

To determine whether radiation induced apoptosis, we performed immunoblots for cleaved and full-length caspase-3 in left ventricle tissue. Figure 2 shows results of ^{16}O exposed mice. Figure S3 in the Supplemental Materials shows results of all other radiation types. ^{16}O (0.1 and 0.25 Gy) caused an increase in cleaved caspase-3 only at 3 months (Figure 2B) and an increase in caspase-3 at 3 months and 9 months (Figure 2C). Protons (0.5 and 1 Gy) also increased the amount of cleaved caspase-3 at 3 months, but caused no changes in full-length caspase-3 (Figure S3). The consecutive-exposure condition only increased cleaved caspase-3 at the 2-week time point (Figure S3). In contrast, exposure to γ -rays (0.5 – 3 Gy) increased levels of cleaved caspase-3 at 9 months (Figure S3). Apparently, at the dose ranges investigated, ionizing radiation caused caspase-3 activation in the heart for only a short period of time, and the peak of caspase-3 activation seemed to depend on radiation type.

Because we noticed the largest changes in cleaved caspase-3 at 3 and 9 months after ^{16}O exposure and at 3 months after protons, we selected these time points to assess numbers of apoptotic nuclei, using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-based assay (CardioTACS). Increases were seen in the number of apoptotic nuclei 9 months after ^{16}O (0.25 Gy) (Figure 3A) and 3 months after proton irradiation (1 Gy) (Figure 3B). Altogether, the results of caspase-3 and TUNEL assays provided only small indications of cardiac apoptosis in response to radiation.

3.5 Cardiac pathology, collagen content, and markers of cardiac extracellular remodeling

Remodeling of the cardiac extracellular matrix may be associated with changes in collagen content and the number of myofibroblasts, mainly responsible for the production of collagens. First, we examined type III collagen protein in the left ventricle. While mouse type III collagen is approximately 300 kDa in size, the anti-collagen III antibody detected a protein band of approximately 75 kDa (Figure 4A). We previously detected this band in samples of the mouse left ventricle and determined it to be specific for type III collagen [19]. We believe that it is a truncated peptide of type III collagen that may indicate remodeling of existing collagens in the heart. Increases in this 75-kDa peptide of type III collagen were seen at all time points after exposure to protons, ^{16}O , and γ -rays. The highest dose of each type of radiation gave the most consistent up-regulation (Figure 4B,C,E). Surprisingly, no changes were seen when protons were followed by ^{16}O (Figure 4D).

An increase in the number of myofibroblasts in radiation fibrosis in the heart correlates with an increase in α -SMA [25]. However, the protein levels of α -SMA in the left ventricle decreased at 9 months after proton irradiation (1 Gy) (Figure S4). While ^{16}O irradiation decreased α -SMA at 2 weeks, dose-dependent increases were observed at 3 months and at 9 months. Lastly, α -SMA increased at 9 months after γ -ray irradiation (1 and 3 Gy) (Figure S4).

Histopathological analysis of H&E-stained sections of the heart revealed no indications of inflammation, myofiber necrosis, interstitial fibrosis, microvascular damage, or epicardial fibrosis (data not shown). Quantification of Sirius Red + Fast Green stained sections of the heart revealed small but significant decreases in the tissue area occupied by collagens at 9

months after ^{16}O (0.05 and 0.25 Gy) and at 9 months after consecutive exposure (protons followed by ^{16}O) (Figure S5). Increases in collagen deposition were not seen in any of the radiation groups.

Sirius Red + Fast Green was also used to assess collagen deposition in the wall of the abdominal aorta at 9 months after exposure to ^{16}O , protons followed by ^{16}O , or γ -rays. We observed a small but significant decrease in the consecutive exposure group and an increase after γ -rays (1 and 3 Gy) (Figure S6).

Because we previously observed a close correlation between mast cell number and radiation-induced pathological changes, such as degeneration and fibrosis, after localized cardiac x-ray exposure [26], we assessed cardiac mast cells as an additional indicator of active tissue remodeling. First, we quantified mast cells with histological staining. At the 2-week time point, the number of mast cells decreased after proton irradiation (0.5 Gy), increased after ^{16}O (0.1 Gy), and decreased after γ -rays (3 Gy). At the 3-month time point, only proton irradiation (0.5 Gy) increased mast-cell number (Figure S7). Histological staining did not detect any mast cells in the abdominal aorta wall (data not shown).

Lastly, we determined the levels of mast cell tryptase in the left ventricle as an indication of mast cell activity. In contrast to the lack of a dose-dependent increase in mast-cell number in ^{16}O (0.1–1 Gy) exposed mouse hearts, there was a dose-dependent increase in mast cell tryptase (Figure 5A and C). Similarly, in contrast to the lack of a dose-dependent increase in mast-cell number in γ -ray (0.5–3 Gy) exposed mouse hearts, there was a dose-dependent increase in mast cell tryptase (Figure 5E). These results suggest that existing mast cells in the left ventricle of ^{16}O and γ -ray exposed mice may have displayed increased cellular activity. While ^{16}O at doses of 0.25 and 1 Gy increased mast cell tryptase at all time points, the lowest dose of ^{16}O (0.05 Gy) caused a decrease at 3 and 9 months (Figure 5D). Proton irradiation increased mast cell tryptase at 2 weeks after exposure (0.5 and 1 Gy), at 9 months (1 Gy), but not at 3 months (Figure 5B).

Altogether, while results of histopathology and histology indicate that there was no significant radiation fibrosis, changes in the levels of a truncated peptide of type III collagen and mast cell tryptase in the left ventricle suggest that there may have been some remodeling of the existing extracellular matrix in the heart.

3.6 Markers of immune cells in the heart

To determine whether radiation altered immune cell abundance or function in the heart, we used immunoblotting to assess left ventricular protein levels of T-cell marker CD2 and monocyte/macrophage marker CD68, to assess cells of both a lymphoid and a myeloid lineage with known roles in cardiac remodeling. Increases in CD2 levels were observed at 9 months after 1 Gy protons (Figure 6B), 3 months after 0.1 Gy ^{16}O , 3 and 9 months after 0.25 Gy ^{16}O , at all time points after 1 Gy ^{16}O (Figure 6C), and 9 months after consecutive exposure (protons followed by ^{16}O) (Figure 6D). Lastly, while γ -rays caused a decrease in CD2 at 2 weeks (3 Gy), increases were observed at 3 months (0.5 and 3 Gy) and 9 months (1 and 3 Gy) (Figure 6E).

CD68 is a cell-surface marker of monocytes and macrophages, and we observed an increase in the amount of CD68 in the left ventricle at all time points after exposure to 1 Gy of protons (Figure 7B), 2 weeks after 0.1 and 0.25 Gy ^{16}O , and 3 months and 9 months after 0.25 and 1 Gy ^{16}O (Figure 7C). No changes were seen in the consecutive exposure group (Figure 7D). In mice exposed to γ -rays, left ventricle CD68 increased at 2 weeks and 9 months (1 and 3 Gy), but not at 3 months (Figure 7E).

Finally, we investigated the effect of radiation on leukocytes in the heart by assaying CD45, the leukocyte common antigen. Results of ^{16}O and γ -rays are presented in Figure 8. Results of the other radiation exposures are shown in Figure S8. Immunoblotting revealed a decrease in the amount of CD45 in the left ventricle 3 months after proton irradiation (1 Gy) (Figure S8A) and 3 months after consecutive exposure (Figure S8B). For ^{16}O , increases in CD45 were evident at 9 months after a dose of 0.05 Gy (Figure S8B), and at 3 months after 1 Gy and 9 months after 0.1 Gy and 1 Gy (Figure 8D). Exposure to γ -rays increased CD45 at all time points after a dose of 1 Gy and at 3 months after 0.5 Gy and 3 Gy (Figure 8F). Protons (1 Gy) caused a decrease in left ventricular CD45 compared to time-matched sham controls at 3 months (Figure S8A). Lastly, exposure to protons followed by ^{16}O caused a decrease in CD45 as measured at 3 months (Figure S8B). Altogether, there was no clear dose-dependent relationship between CD45 abundance and specific types of radiation.

We then performed immunohistochemistry to quantify CD45-positive cells in selected experimental groups (9 months after ^{16}O [0.1, 0.25 and 1 Gy] and γ -rays [1 and 3 Gy]). Consistent with immunoblotting results, the number of CD45-positive cells tended to increase at 9 months after 0.1 and 1 Gy ^{16}O , but the increase was not statistically significant (Figure 8E). Similarly, there was only a trend towards increased numbers of CD45-positive cells after γ -ray exposures (Figure 8G).

4. Discussion

The purpose of this study was to identify measures of cardiac function, cardiac apoptosis, extracellular matrix remodeling, and immune cell infiltration that may be altered by high-energy charged-particle radiation in a mouse model. For this purpose, we exposed male C57BL/6J mice to various doses of protons, ^{16}O , protons followed by ^{16}O (consecutive exposure), or γ -rays (as a reference); mice were then followed for up to 9 months after irradiation. Some of these same mice were used to assess short- and long-term effects of ^{16}O on the hematopoietic system and the brain, and these results have been published previously [27–33]. At 9-months follow-up, mice exposed to 1 Gy of protons and 1 and 3 Gy of γ -rays had lost some bodyweight compared to sham controls. This may have been due to intestinal radiation injury or a change in metabolism. However, radiation-induced changes in bodyweight were too small to potentially affect cardiac function or remodeling.

In the current study, exposure to ^{16}O significantly decreased the EF and FS at 7 months, as well as decreased the cardiac volume in systole and decreased the mean and peak velocities in the abdominal aorta at 9 months. Altogether, these results suggest that ^{16}O reduced systolic function in these mice. Exposure to protons or γ -rays did not cause changes in ultrasonography parameters indicative of systolic function, suggesting that heavy ions may

be more effective in altering systolic function in the mouse heart. However, in contrast to the results of ^{16}O in our study, in a recent study no indications of changes in systolic function were found in male C57BL/6 mice at 9 months after exposure to ^{16}O (600 MeV/n) or ^{56}Fe (1 GeV/n) [34]. Similarly, exposure of male C57BL/6N mice to three fractions of protons (1 GeV, 0.17 Gy each) or three fractions of protons (1 GeV, 0.17 Gy each) followed by a single dose of 0.15 Gy ^{56}Fe (1 GeV/n) did not lead to changes in cardiac function [12]. On the other hand, Yan et al. identified a small increase in EF in male C57BL/6 mice from 1 to 10 months after a single dose of 0.15 Gy ^{56}Fe (1 GeV/n) [10], suggestive of the induction of compensatory mechanisms in the ^{56}Fe exposed mouse heart. In the same study by Yan et al., the EF decreased in mice that underwent an experimental myocardial infarction 3 or 10 months after exposure to ^{56}Fe , indicating that radiation reduced their ability to recover from a second insult [10]. Lastly, similar to charged particle radiation, male C57BL/6 mice also show small changes in cardiac function as measured with single photon emission computed tomography (SPECT)/CT after local doses of X-rays up to 8 Gy [35]. Since the effects of low-dose radiation on the mouse heart seem to be small, differences in the methods used to measure cardiac function, such as echocardiography, *in vivo* pressure volume loops, or SPECT/CT, and experimental factors such as the use of anesthesia, may affect the study outcome. If small changes are observed in cardiac function parameters, care must be taken to determine their clinical relevance.

In contrast to ^{16}O , protons increased the SV at 5 and 7 months after exposure and increased the mean and peak blood velocities at 5 to 9 months, suggesting a small increase in systolic function. Protons did not alter the ratio of mitral valve E to A velocity. Therefore, we saw no indications of diastolic dysfunction in these animals. Interestingly, Yan et al. also observed that male C57BL/6 mice exposed to 0.5 Gy of protons (1 GeV) had improved cardiac function when experimental myocardial infarction was induced 3 or 10 months after irradiation [10]. Thus, in contrast to ^{16}O , exposure to protons may induce molecular responses in the heart that help combat the effects of aging and injury.

In line with findings described above and with results obtained by Yan et al. [10], fewer of our measures of cardiac function and structure were altered when protons were followed by ^{16}O than to single exposures of either radiation type. It appears that a low dose of protons may induce repair mechanisms in the heart that protect the heart from subsequent exposures to low-dose heavy ions, even when there is only one hour between the two radiation exposures. A similar observation was made in a previous study, in which irradiation of male C57BL/6 mice with protons (150 MeV) at a dose of 0.1 Gy 1 day before ^{56}Fe (600 MeV/n, 0.5 Gy) prevented changes in cardiac protein expression in response to ^{56}Fe [19]. Moreover, when male C57BL/6 mice were exposed to ^{56}Fe (1 GeV/n, 0.15 Gy) and protons (1 GeV, 0.17 Gy per fraction) in various orders, *in vivo* cardiac function depended on the order in which the two types of particles were delivered [12]. Along the same lines, exposing human fibroblasts to 0.2 Gy of protons (1 GeV) in culture induced adaptive and bystander effects that protected the cells against a subsequent exposure to 0.5 Gy of ^{56}Fe (1 GeV) after 6 to 24 hours; this effect was not observed when the time between the two exposures was increased to 72 hours [36]. Altogether, these *in vitro* and *in vivo* studies indicate that low-dose proton exposures can affect the biological response to heavy ions. This phenomenon should be

taken into account when fractionated radiation exposures are tested in cell culture and animal studies.

Cleaved caspase-3 is the activated, pro-apoptotic form of caspase-3. We observed increases in the amount of cleaved and full-length caspase-3 in the left ventricle after exposure to protons and ^{16}O , extending previous findings that cleaved poly(ADP-ribose) polymerase (PARP), a caspase-3 substrate during radiation fibrosis [37], increased at time points from 1 week to 6 months after exposure to ^{28}Si (300 MeV/n) [13]. However, while in the current study increases were seen in left ventricular protein levels of cleaved caspase-3, these changes were small and their clinical significance is uncertain. Moreover, the number of TUNEL-positive nuclei in the left and right ventricle was also small and did not always coincide with increases in cleaved caspase-3. Altogether, the results suggest that radiation did not cause significant apoptosis in the heart. Indeed, recent studies showed that, while mechanisms are not yet fully understood, several cell types can survive exposure to ionizing radiation despite a robust level of caspase-3 activation [38,39]. Hence, further studies are required to determine the extent, timing, and role of apoptosis in the heart after exposure to heavy ions.

We did not observe an increase in total collagen deposition in the heart or aorta, indicating that radiation fibrosis did not occur. On the other hand, Yan et al. previously reported a significant increase in collagen deposition in the heart, also measured by histological staining, in male C57BL/6 mice 10 months after exposure to ^{56}Fe (1 GeV/n, 0.15 Gy) or protons (1 GeV, 0.5 Gy) [10]. This difference in outcomes may be due to radiation type or energy. However, we did observe an increase in the abundance of a 75-kDa cleavage product of type III collagen, one of the most abundant collagens in the heart, in the left ventricle of irradiated animals. We have previously observed this band in the mouse heart, verified that it was specific for collagen type III, and showed that its density increased after 0.5 Gy of ^{56}Fe (600 MeV/n) [19]. In the current study, mast cell tryptase and α -SMA protein levels also increased at 3 and 9 months after ^{16}O . Altogether, our results suggest that while there was no clear radiation fibrosis, there may have been some remodeling of the existing extracellular matrix in the heart in response to radiation.

Previous studies showed an increase in the number of CD68-positive cells in the hearts of mice at 2 and 4 weeks after exposure to ^{56}Fe (0.15 Gy, 1 GeV/n), indicating the recruitment of macrophages [10,12]. While we do not know which immune cells play a damaging or reparative role in the heart after irradiation, an increase in cardiac inflammatory cytokines in response to ^{28}Si (300 MeV/n) suggests that heavy ions are capable of inducing inflammation in the heart [13]. Thus, we assayed the common leukocyte marker CD45, as well as CD2 and CD68, markers of T-cells and monocytes/macrophages, respectively, in the left ventricle after irradiation. Single exposures to protons or ^{16}O increased CD2 and CD68 protein levels at all time points and increased CD45 at 3 and 9 months after irradiation. On the other hand, we did not observe significant changes in the number of CD45-positive cells as assayed with immunohistochemistry. It is possible that changes seen in immune cell markers with immunoblotting are due to a change in expression of these markers by existing immune cells in the heart. In addition, we did not measure the numbers of CD2, CD68, or CD45-positive cells in the blood. Therefore, we do not know whether changes in these immune cell markers

paralleled similar changes in the circulation. Also, we did not examine the status of neutrophils or other immune cell populations in heart or blood. Future studies should quantify immune cells in both blood and heart using sensitive methods such as flow cytometry, to determine the extent of immune cell infiltration in the heart in response to low-dose radiation, as well as examine inflammation in the irradiated heart.

This study included γ -rays at doses of 0.5 to 3 Gy as reference radiation. In interpreting the results, one should keep in mind that the animals exposed to protons and ^{16}O were in the radiation beam stationary and were exposed laterally, while animals in the γ -ray groups were exposed laterally, but on a turntable. A dose dependency in response to γ -rays was seen only in the levels of CD2, CD68, and mast cell tryptase in the left ventricle. In comparing radiation doses at the 9-month time point, the fold changes in mast cell tryptase were all similar after exposure to 1 Gy of protons, 0.25 Gy of ^{16}O , and 1 Gy of γ -rays (Figure 4). CD2 were all similar after exposure to 1 Gy of protons, 1 Gy ^{16}O , and 3 Gy γ -rays. In addition, fold changes in CD2 at doses of 0.1 and 0.25 Gy ^{16}O were similar to those for 0.5 and 1 Gy γ -rays, respectively (Figure 5). Also at 9 months, the fold changes in CD68 abundance relative to sham controls were similar in response to 1 Gy of protons, 0.1 Gy of ^{16}O , and 0.5 Gy of γ -rays. In addition, 1 Gy of ^{16}O and 1 Gy of γ -rays induced similar fold changes in CD68 (Figure 6). Therefore, overall, protons were 1 to 3 times more effective than γ -rays at increasing the levels of these immune cell markers, while ^{16}O was 3 to 5 times more effective than γ -rays.

Due to practical considerations in animal housing and radiation, our study had some limitations. First, this study focused on male mice only. In the future, we will consider sex as a biological variable in the cardiovascular response to radiation by including female animals. Second, it is difficult to model radiation dose and dose rate in ground-based experiments. In deep space, tissue dose rate from GCR is estimated at 0.3–0.6 mGy/day (1–1.8 mSv/day dose-equivalent) [40]. Therefore, a 2-year mission may result in tissue dose of no more than 0.2–0.4 Gy from a mixture of ions. A dose of 1 Gy ^{16}O administered at a high dose rate may not be relevant for radiation exposures in deep space. Research is under way to start addressing the biological effects of mixed ion fields and chronic exposure to low dose-rate ionizing radiation [41–43]. Third, because this study involved a large number of mice, mice had to be divided into 4 cohorts. Since each cohort has its own sham-irradiated animals, the sham animals are different for different radiation type. Therefore, care must be taken in comparing relative differences between sham controls and irradiated animals between different radiation types. Lastly, after returning to UAMS from BNL, all mice were administered fenbendazole-containing chow for 8 weeks as a quarantine procedure. While fenbendazole does not cause significant changes in tissue pathology [44], there are conflicting reports about its effects on the immune response in mice [45–46]. Hence, we cannot exclude the possibility that fenbendazole had some effect on the parameters in our study, potentially by modifying the immune response.

In conclusion, our data provide some indications of mild changes in cardiac function, remodeling, and immune cell infiltration after low doses of protons and ^{16}O in the male mouse heart, and the nature of the changes depended on the type of radiation. Moreover, the cardiac response was very different when mice were exposed to ^{16}O one hour after protons.

Future studies should investigate the effects of mixed ion fields and protracted radiation exposures. Lastly, the cardiac response in females also needs to be investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	Analysis of variance
BNL	Brookhaven National Laboratory
CD	Cluster of differentiation
CI	Confidence interval
CO	Cardiac output
E/A	Ratio of mitral valve E and A velocities
EF	Ejection fraction
FS	Fractional shortening
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H&E	Hematoxylin and eosin
HZE	High atomic number and energy
LVAW	Left ventricular anterior wall
LVID	Left ventricular inner diameter
LVPW	Left ventricular posterior wall
MVA	Mitral valve A velocity
MVE	Mitral valve E velocity
NSRL	NASA Space Radiation Laboratory
PARP	Poly(ADP-ribose) polymerase
pFDR	Positive false discovery rate
α-SMA	α-Smooth muscle cell actin

SPECT/CT	Single photon emission computed tomography
SV	Stroke volume
TBS	Tris-buffered saline
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UAMS	University of Arkansas for Medical Sciences
VTI	Velocity time integral

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Figure 1. Bodyweight at time of sacrifice.

Bodyweight was measured after exposure to protons (A), ^{16}O (0–1 Gy) (B), ^{16}O (0 and 0.05 Gy) and protons (0.5 Gy) followed by ^{16}O (0.1 Gy) (C), and after γ -rays (D). Graphs indicate average bodyweight and standard error of the mean (SEM); $n = 9$ –15/group.

* $p < 0.05$ compared to time-matched sham controls.

Figure 2. Effects of radiation on cleaved and full-length caspase-3 after exposure to ^{16}O .

(A) Representative image of immunoblot for cleaved and full-length caspase-3 at 9 months after ^{16}O irradiation. (B) Left ventricle protein levels of cleaved caspase-3 after exposure to ^{16}O (0.1 – 1 Gy). (C) Left ventricle protein levels of full length caspase-3 after exposure to ^{16}O (0.1 – 1 Gy). Left ventricular protein levels were normalized to GAPDH and then calculated relative to time-matched sham-irradiated controls (arbitrary units). Graphs indicate average values and SEM. * $p < 0.05$ compared to time-matched sham controls. $n = 6$ /group.

Figure 3. Effects of radiation on apoptosis after exposure to protons or ^{16}O .

Number of apoptotic nuclei per tissue area after exposure to ^{16}O (A) or protons (B). $n = 9-15/\text{group}$. Graphs indicate average values and SEM. * $p < 0.05$ compared to time-matched sham controls.

Figure 4. Immunoblot analysis of type III collagen in left ventricle.

(A) Representative image of an immunoblot indicating a 75-kDa band recognized by the anti-collagen III antibody, in samples at 3 months after exposure to ^{16}O . (B) – (E) Left ventricle protein levels of the type III collagen 75-kDa peptide. Left ventricular peptide levels were normalized to GAPDH and then calculated relative to time-matched sham-irradiated controls (arbitrary units). Graphs indicate average values and SEM. $n = 5\text{--}6/\text{group}$. * $p < 0.05$ compared to time-matched sham controls.

Figure 5. Effects of radiation on mast cell tryptase in the heart.

(A) Representative image of immunoblot for mast cell tryptase at 9 months after ^{16}O exposure. (B) – (E) Mast-cell tryptase abundance in the left ventricle. Left ventricular protein levels were normalized to GAPDH and then calculated relative to time-matched sham-irradiated controls (arbitrary units). Graphs indicate average values and SEM. $n = 5\text{--}6/\text{group}$. * $p < 0.05$ compared to time-matched sham controls.

Figure 6. CD2 protein levels in the left ventricle.

(A) Representative image of immunoblot for CD2 at 3 months after ^{16}O exposure. (B) CD2 abundance in the left ventricle. Left ventricular protein levels were normalized to GAPDH and then calculated relative to time-matched sham-irradiated controls (arbitrary units). Graphs indicate average values and SEM. $n = 5-6/\text{group}$. * $p < 0.05$ compared to time-matched sham controls.

Figure 7. CD68 protein levels in the left ventricle.

(A) Representative image of immunoblot for CD68 at 9 months after ^{16}O exposure. (B) CD68 abundance in the left ventricle. Left ventricular protein levels were normalized to GAPDH and then calculated relative to time-matched sham-irradiated controls (arbitrary units). Graphs indicate average values and SEM. $n = 5-6/\text{group}$. * $p < 0.05$ compared to time-matched sham controls.

Figure 8. Effects of radiation on CD45-positive cells in the heart after ^{16}O and γ -rays.

(A) Representative immunoblot of CD45 at 3 months after ^{16}O . Please note that the 0 Gy samples had been loaded on the gel as the second group from the left, to the right of the 0.1 Gy samples. (B) Representative image of CD45 immunohistochemistry, 3 months after sham-irradiation, 40 $\times$ magnification; Scale bar: 60 μm . (C) Representative image of CD45 immunohistochemistry, 3 months after ^{16}O (0.25 Gy), 40 $\times$ magnification; Scale bar: 60 μm . (D) Left ventricle protein levels of CD45 after ^{16}O . (E) Number of CD45-positive cells per tissue area at 9 months after ^{16}O . (F) Left ventricle protein levels of CD45 after γ -rays. (G) Number of CD45-positive cells per tissue area at 9 months after γ -rays. In immunoblotting, left ventricular protein levels were normalized to GAPDH and then calculated relative to

time-matched sham-irradiated controls (arbitrary units). All graphs indicate average values and SEM. $n = 6/\text{group}$. * $p < 0.05$ compared to time-matched sham controls.

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