RAT MODELS OF PARTIAL-BODY IRRADIATION WITH BONE MARROW-SPARING (LEG-OUT PBI) DESIGNED FOR FDA-APPROVAL OF COUNTERMEASURES FOR MITIGATION OF ACUTE AND DELAYED INJURIES BY RADIATION

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Abstract

Objective: The goal of this study was to develop rat models of partial body irradiation with bone-marrow sparing (leg-out PBI), to test medical countermeasures (MCM) of both acute radiation syndrome (ARS) and delayed effects of acute radiation exposure (DEARE) under the FDA animal rule.

Methods: The leg-out PBI models were developed in female and male WAG/RijCmcr rats at doses of 12.5–14.5 Gy. Rats received supportive care consisting of fluids and antibiotics. Gastrointestinal ARS (GI-ARS) was assessed by lethality to day 7 and diarrhea scoring to day 10. Differential blood counts were analyzed between days 1–42 for the natural history of hematopoietic ARS (H-ARS). Lethality and breathing intervals (BI) were measured between days 28–110 to assess delayed injury to the lung (L-DEARE). Kidney injury (K-DEARE) was evaluated by measuring elevation of blood urea nitrogen (BUN) between days 90–180.

Results: The $LD_{50/30}$, including both lethality from GI-ARS and H-ARS, for female and male rats is 14.0 Gy and 13.5 Gy respectively, while the $LD_{50/7}$ for only GI-ARS is 14.3 Gy and 13.6 Gy respectively. The all-cause mortality, including ARS and L-DEARE, through 120 days ($LD_{50/120}$) is 13.5 Gy and 12.9 Gy respectively. Secondary end points confirmed occurrence of 4 distinct sequelae representing GI, hematopoietic, lung and kidney toxicities after leg-out PBI.

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Conclusion: Adult rat models of leg-out PBI developed showed the acute and long-term sequelae of radiation damage that has been reported in human radiation exposure case studies. Sex-specific differences were observed in the DRR between females and males. These rat models are among the most useful for the development and approval of countermeasures for mitigation of radiation injuries under the FDA animal rule.

Keywords

rat; partial body radiation irradiation; biological indicators; health effects

INTRODUCTION

The aim of the National Institute of Allergy and Infectious Diseases (NIAID) Radiation and Nuclear Countermeasures Program is to develop medical countermeasures (MCM) to treat the acute and delayed effects of radiation that may occur from a nuclear attack or terrorist event (DiCarlo et al. 2008, DiCarlo et al. 2011). Victims of high-dose radiation exposure are likely to experience a range of symptoms to multiple organ systems which manifest at different time points following exposure. In the early days to weeks following exposure, lethality may occur due to gastrointestinal (GI-ARS) or hematopoietic acute radiation syndrome (H-ARS) or combined injury to both systems.

In the months-years following exposure, patients are susceptible to delayed effects of acute radiation exposure (DEARE) to multiple organ systems including the lungs (L-DEARE) and kidneys (K-DEARE). For these reasons, it is critical to develop mitigators for multiple-organ injury (MOI) that successfully rescue victims from injuries due to a radiological or nuclear attack.

It is not ethical to expose human subjects to high-dose radiation; therefore, approval of MCM for ARS and DEARE are conducted using the FDA 'Animal Rule' and guidance document (FDA 2002, FDA 2015) that requires established and validated radiation animal models that resemble radiation exposure scenarios consequent to a nuclear attack. In a mass casualty scenario, radiation exposures will likely be heterogeneous, nonuniform and total- or partial-body. The threshold radiation dose for lethal bone marrow injury is below that of the delayed effects of radiation, therefore, to allow dose- and time-dependent survival and assessment of DEARE, a minimal volume of bone marrow must be spared. A partial body irradiation (PBI) rat model with bone-marrow (BM) shielding of only one limb has the potential to define the dose- and time-dependent link between the multiple organ injuries of the ARS and DEARE, providing a more definitive approach to assess MCM efficacy consequent to a terrorist incident.

Several laboratories have established animal models of PBI/BM-sparing or extended uniform total body irradiation (TBI) to assess DEARE (Booth et al. 2015, Unthank et al. 2015, Fish et al. 2016, Chua et al. 2019, Medhora et al. 2019, Fish et al. 2020, Miller et al. 2020, Patterson et al. 2020). Established models and dose response relationships (DRR) are described for non-human primates (NHP), where multiple ARS and DEARE sequelae manifest after PBI with BM-sparing of ~5% (MacVittie et al. 2012, MacVittie et al. 2014, MacVittie et al. 2015) or ~2.5% (Farese et al. 2019). Other, varied exposure protocols have

been established using PBI with BM-sparing as well as unilateral, nonuniform TBI to mimic the exposure environment and assess MCM efficacy, albeit focused against the acute H-ARS (Chapel et al. 2003, Drouet et al. 2004, Bertho et al. 2005, Herodin et al. 2007, Drouet et al. 2008). Mouse PBI models with BM shielding have also been developed, although these models have been primarily used to characterize GI-ARS, GI-DEARE and lung injury (Johnston et al. 2011, Booth et al. 2012a, Booth et al. 2012b, Booth et al. 2015). Several teams have used the total body irradiation (TBI) mouse models, over the potentially lethal H-ARS dose range, to assess the incidence of organ-specific DEARE in long-term survivors in control and MCM-treated cohorts (Baker et al. 2009, Chua et al. 2012, Chua et al. 2014, Unthank et al. 2015, Chua et al. 2019, Unthank et al. 2019, Miller et al. 2020, Patterson et al. 2020, Wu et al. 2020).

Some of the most sophisticated PBI models to date have been developed using WAG/RijCmcr rats with shielding to part of one hind limb (~7.5% of BM, Taketa et al. 1970) by leg-out PBI (Fish et al. 2016, Fish et al. 2020). The initial model demonstrated that young adult, female, rats (Fish et al. 2016) manifested GI, lung and renal toxicities, but lethal lung injury was relatively rare and only occurred in <15% rats. Therefore, evaluation of L-DEARE MCMs would require a restrictively large number of animals to observe statistical differences in survival during pneumonitis. Moreover, in male rats, only the 50% lethal dose (LD_{50}) for radiation-nephropathy was described with limited data available for lung and GI toxicities (Fish et al. 2020). Though the BM was shielded in these studies, the natural progression of bone marrow damage was not evaluated, leaving BM sequelae poorly characterized in the leg-out PBI rat model. The goal herein was to optimize and fully assess adult female and male rat models of leg-out PBI, that manifested lethal GI, lung and kidney damage, while also assessing hematopoietic injury after irradiation. The goal required the radiation dose, diet and supportive care to be refined based on previous studies (Fish et al. 2016, Moulder et al. 2019, Fish et al. 2020) and as described in the results herein.

MATERIALS & METHODS

Animal care

All animal protocols were approved by Institutional Animal Care and Use Committees (IACUC) at the Medical College of Wisconsin. WAG/RijCmcr rats bred at MCW were weaned to Teklad 8604 (Envigo, Madison WI) rodent diet along with reverse osmosis hyperchlorinated water. The rats were housed (3–5 per cage) in a 14 h/10 h light/dark cycle, at 22° C with humidity maintained between 30–70%. Based upon direction from the IACUC, rats were designated as moribund and euthanized if they met specified veterinarian's criteria as described previously (Medhora et al. 2015). The terminal endpoint for such rats was necropsy.

Irradiation

Leg–out PBI rat models: WAG/RijCmcr female and male rats were unilaterally irradiated, dorsal to ventral, without the use of anesthetics at 11–12 weeks of age. All irradiations were done between 8–10 am. For leg-out PBI, non-anesthetized rats were immobilized in a plastic jig and irradiated using a XRAD 320KV orthovoltage x-ray system

(Precision X-Ray, North Branford, Connecticut). The x-ray system was operated at 320 kVp and 13 mAs with a half value layer of 1.4 mm Cu with a dose-rate of 1.69 Gy min^{-1} for female rats and 1.73 Gy min^{-1} for males. Doses ranged from 13 –14.5 Gy for female rats and 12.5 –14.5 Gy for males. During irradiation, each rat was confined to a chamber which allows irradiation of 2 rats simultaneously. One hind limb of each rat was carefully externalized from the chamber and shielded with a 0.25-inch lead block. The dose to this leg was approximately 0.15 Gy/Gy (1.9–2.2 Gy over the dose range). The dual-chambered jig was placed on a plane perpendicular to the direction of the beam, with distance from source to the midline of rats set at 61.0 cm for females and 60.8 cm for males. Collimator jaws and dosimetry were used as previously described (Medhora et al. 2014). The irradiation field at midline was large enough to cover both chambers with adequate (at least 2 cm) margins.

Model refinement

Diet and supportive care: WAG/RijCmcr rats were weaned to Teklad 8604 (Envigo, Madison WI) rodent diet. Two weeks prior to irradiation rats were switched to a moderate antioxidant diet (Teklad 2018 Global 18% protein rodent diet, Envigo, Madison WI); diet details are listed in Table 1. The Teklad 2018 global diet has been used at other institutions for rodent radiation studies (Patterson et al. 2021, Booth et al. 2012a, Booth et al. 2012b, Booth et al. 2015) and is a better reflection of the human diet than Teklad 8604/8904 used previously (Fish et al. 2016, Moulder et al. 2019, Fish et al. 2020).

Supportive care was enhanced from a previous publication (Fish et al. 2016) of a similar model to include bolus hydration by daily subcutaneous injection of saline 40 ml kg^{-1}day^{-1} from days 2–10 versus days 3–7. This allowed for pelleted stool formation to return to normal. The antibiotic enrofloxacin 10 mg kg^{-1}day^{-1} was delivered from days 2–14 instead of days 2–28, as neutrophil count was back to nearly normal levels by day 15 after leg-out PBI. Powdered food was provided from days 35 to 70 after irradiation to accommodate tooth loss that resolved by day 70.

Dose response relationship (DRR) for female rats: 139 female rats were given leg-out PBI at doses of 13 Gy (n= 32), 13.5 Gy (n=45), 14 Gy (n=46), 14.25 Gy (n=6) or 14.5 Gy (n=10). An additional 5 rats served as non-irradiated age-matched controls. All rats received supportive care. Rats were followed for morbidity through 150 days. GI-ARS (0–7 days after irradiation), H-ARS (8–30 days after irradiation), L-DEARE (31–120 days after irradiation) and K-DEARE (121–150 days after irradiation) were monitored and confirmed by necropsy. This model was used to determine the DRR, the LD for the different sequelae after leg-out PBI in adult female rats. Female rats were followed for 150 days after PBI. Body weights were recorded weekly. The probit plots estimate the LD for 10, 30, 50, 70 and 90% morbidity with upper and lower 95% confidence intervals (CI).

DRR for male rats: 114 male rats were given leg-out PBI at doses of 12.5 Gy (n=24), 13 Gy (n= 32), 13.5 Gy (n=35), 14 Gy (n=13), or 14.5 Gy (n=10). An additional 8 rats were non-irradiated, age-matched controls. Supportive care was the same as that administered to the female rats. This male model was used to determine the DRR and LD for the different sequelae after leg-out PBI in adult male rats. Since radiation nephropathy manifested later

in males, the study duration and observation was extended to 180 days. Body weights were recorded weekly. The probit plots estimate the LD for 10, 30, 50, 70 and 90% rats that were moribund with upper and lower 95% CI.

Secondary endpoints for GI- and H-ARS

The $LD_{50/120}$ for female and male rats represented the best models to study injuries to multiple organs in each sex, since they elaborated H-ARS as well as lethal GI-ARS, L-DEARE and K-DEARE. Secondary end points were measured in these models to confirm the cause of death in the K-M studies.

To test the progression of GI-ARS at the $LD_{50/120}$ for the respective sex, 25 female and 32 male rats received 13.5 or 13 Gy leg-out PBI respectively. The rats were evaluated from days 2–10 for GI-ARS by stool scoring: 0=pelleted stool, 1=soft stool and 2=diarrhea. Rats received a score of 2.5 if they were moribund and therefore euthanized, to account for attrition. H-ARS was assessed by complete blood cell counts (CBC). Blood (0.5–1 ml) was harvested from the jugular vein into EDTA-treated tubes, by a trained technician. Samples were obtained at days 1, 3, 5, 7, 9, 12, 15, 22, 30 and 42 after irradiation. CBC were run on a Heska Elements HT5 blood analyzer. The natural history at 13.5 Gy in females and 13 Gy in males was studied because these were the best doses to test MCM.

Secondary endpoints for L-DEARE and K-DEARE

A subset of rats was assessed from the DRR study to measure functional damage in the lung and kidney. Minimally invasive measurements of the breathing interval (BI) or blood urea nitrogen (BUN) were conducted to monitor pulmonary and renal functions respectively.

Breathing interval measurements in rats: Breathing rates for each rat were measured every two weeks from 28–110 days after exposure as described previously (Medhora et al. 2012a, Medhora et al. 2012b, Medhora et al. 2014). Rats were restrained in a plastic restrainer for 5 minutes for 2 consecutive training days to allow the rats to become acclimated to the apparatus. On the third day, the restrainer was placed in a transparent EMKA plethysmograph (Scireq Scientific Respiratory Equipment Inc., Montreal, QC, Canada) which measured the frequency of pressure changes. Recordings for a maximum of 10 minutes per animal were used. The mean breathing rate for each rat was then calculated from four steady regions of the recording lasting greater than 15 seconds each. The inverse of the breathing rates was calculated to derive the BI or time/breath in seconds. Since higher breathing rates and lower BI indicated more severe lung damage, the BI were set to 0 for animals that died during pneumonitis, to account for attrition (Medhora et al. 2012a, Medhora et al. 2012b).

Measurement of blood urea nitrogen (BUN): Previous published work has shown that rising BUN levels are superior to histopathology for assessing radiation nephropathy (Moulder et al. 2011). To measure BUN, rats were anesthetized with 3–5% isoflurane for blood draws via the jugular vein conducted by an experienced technician. The BUN was assayed from serum as described previously (Cohen 1994, Medhora et al. 2014) using a urease-nitroprusside colorimetric assay. BUN values were expressed as mg/dl of serum and

medians with 95% CI were used for statistical analysis. Irradiated rats with BUN>120mg dl^{-1} were euthanized and given a value of 120 mg dl^{-1} to account for attrition, since such rats were previously confirmed to have severe and irreversible renal damage (Moulder et al. 1993a, Moulder et al. 1993b).

Lung histology: A subset of 13.5 Gy leg-out PBI irradiated female and 13 Gy leg-out PBI male rats that were moribund between 42–70 days (n=6 rats) were randomly selected for histological evaluation of the lungs. These rats typically presented with lung injury at necropsy. The left lung was harvested, fixed and embedded in paraffin. Whole mount lung sections (4 μm thick) were stained with hematoxylin and eosin (H&E) and also stained with tryptase antibody (IMGENEX catalogue #IMG-80250, 1:150) (Gao et al. 2013) and five randomly selected fields (20x) of each lung were evaluated. The foamy macrophages, vascular, and alveolar wall thickness were scored as described previously (Medhora et al. 2014, 2015); injury was measured on a 5-point scale and a mean composite lung injury score was calculated. Mast cells were assessed by positive tryptase staining in five randomly selected fields (20x) of each lung.

Scores from irradiated moribund rats were compared to those from non-irradiated animals (controls). Since no non-irradiated control rats were moribund in this study, lungs from these rats sacrificed at the termination of the study at 150 or 180 days for female and male respectively, were used for comparison (controls). Scoring for all rats (moribund or non-irradiated controls) was performed by operators masked to the treatment groups. Data is presented as means and 95% CI.

Kidney histology: At morbidity or termination of the study (140–150 days post 13 Gy leg-out PBI for female and 150–180 day for male rats), kidneys were harvested, cut into half and immediately fixed in 10% buffered formalin and processed for paraffin embedding. Kidney sections were stained with Masson's trichrome stain, and the glomerular fibrosis was scored blinded in coded samples by two investigators (FG and JN) (Medhora et al. 2019). Glomerular fibrosis scoring was as follows: the percent blue staining within each glomerulus was assessed; 0–25% blue staining was given a score of 1, 25–50% blue staining a score of 2, 50–75% blue staining a score of 3 and 75–100% blue staining a score of 4. The number of glomeruli scored in each of the percentage groups was multiplied by the score. The scores were then aggregated to get a composite histologic score. A total of 50 glomeruli were scored for each rat. Higher scores indicated more severe renal fibrosis.

Statistical analyses

Dose-response curves were fit by probit analysis and used to determine 50% lethal doses (LD$_{50}$). The significance of dose-response trends was assessed by the Mantel extension test; this is a non-parametric test of monotonic trends that does not (unlike probit analysis) assume an exact shape for the dose response. Morbidity data are shown by Kaplan Meier plots and tested for differences between groups by Peto-Peto Wilcoxon tests. Breathing intervals are shown as means. BUN values are medians with 95% confidence intervals. Statistical differences between multiple groups for BUN were calculated by the one-way ANOVA and Dunnett's multiple comparison tests. For histology scoring, t-test was used for

two-group comparisons for scores of lung injury score and mast cell count between morbid rats versus non-irradiated controls of the same sex.

RESULTS

DRR for female rats

Female WAG/RijCmcr rats were irradiated with a single dose ranging from 13.0–14.5 Gy leg-out PBI as described and provided supportive care. The Kaplan-Meier curves (solid lines in figure 1) show the DRR for organ-specific lethal sequelae in irradiated female rats from days 1–160.

ARS: At the 14.5 Gy dose all animals were moribund during ARS (green line). At doses of 14.25 Gy (orange line) and below (blue, red and black lines) survival through GI- and then H-ARS were observed, survival increased in a dose-dependent manner. The rat surviving at 14.25 Gy was euthanized at 32 days, as the percent survival was too low to study DEARE at this dose.

L-DEARE: Survivors from the GI- and H-ARS, at doses 14 Gy and below, developed dose- and time-dependent L-DEARE between days 60 to 120. At 14 Gy leg-out PBI, ~90% of rats that survived ARS were moribund by 120 days from L-DEARE. The median (95% CI) survival time for this group was 65 (12–75) days. At 13.5 Gy, ~50% of rats surviving ARS were moribund by 120 days, median survival time 142 days (76–150) and ~22% at 13.0 Gy, median survival time 150 (144–150) days. Pleural effusions were observed in most moribund rats upon necropsy between days 60–120 confirming radiation pneumonitis. Pleural fluid was measured, and volume recorded at necropsy.

K-DEARE: The dose-dependent renal phase of morbidity followed L-DEARE between 135–160 days when rats exhibited typical signs of radiation-nephropathy (Moulder et al. 2011) with increased levels of blood urea nitrogen (BUN). It should be noted that all female rats at these doses of leg-out PBI will succumb to radiation-nephropathy given enough time beyond 150 days (Medhora et al. 2019).

Body weight (BW) loss: Superimposed over the Kaplan-Meier curves (figure 1) is the natural history of the average percent change in BW for each group. Body weight decreased in a dose- and time-dependent manner. Interestingly, the start in decrease in BW preceded organ-specific sequelae observed in the Kaplan-Meier plots after leg-out PBI. During ARS, the BW decreased by ~20–25 % at doses from 13–14 Gy. The survivors of ARS then gained weight up to before the onset of L-DEARE, when the percent BW decreased again from 12% to 20%. The rats that survived L-DEARE (13–13.5 Gy leg-out PBI groups) increased in BW until before they started to progress to renal failure, after which the BW dropped precipitously and did not recover.

DRR for male rats

Male WAG/RijCmcr rats were irradiated with single doses ranging from 12.5–14.5 Gy leg-out PBI and given supportive care as described. The Kaplan-Meier curves (figure 2) show the DRR for the sequelae induced by radiation.

ARS: At the 14.5 Gy dose all animals were moribund during ARS (green line). At doses of 14 Gy (blue line) greater than 75% of male rats were moribund to GI, with 1 surviving through BM. This rat was terminated at day 42 after irradiation as the percent survival was too low to study DEARE at this dose. At doses below 14 Gy (red, orange, black and purple lines) significant survival through GI- and H-ARS were observed, and survival increased in a dose-dependent manner.

L-DEARE: At doses below 14 Gy the male rats that survived ARS proceeded to develop L-DEARE between 60 to 120 days. At doses of 13.25 and 13.5 Gy leg-out PBI the median survival time was 71 (6–86) and 75 (6–85) days respectively. Rats (~90%) surviving ARS were moribund from pneumonitis by 120 days from L-DEARE. At 13.0 Gy ~55% were moribund by 120 days with a median survival time of 90 (76–150) days while only ~10% became moribund at 12.5 Gy, with a median survival time 176 (171–180) days.

K-DEARE: Male rats succumbed to radiation nephropathy, though the latency period was extended compared to female rats. Eventually all male rats at these doses of leg-out PBI are expected to succumb to radiation-nephropathy.

Body weight (BW) loss: Superimposed over the Kaplan-Meier curves (figure 2) is the natural history of the average percent change in BW for male rats. The decrease in percent BW was more severe with increasing doses of PBI and similar to females coincided with the start of organ-specific sequelae. During ARS, the BW decreased by ~20–25 % for PBI doses from 12.5 – 13.5 Gy. The survivors of ARS then gained weight up to before the onset of L-DEARE when the percent BW decreased from ~6 to 15%. The rats that survived L-DEARE increased in BW until they progressed to renal failure.

Probit analysis in Female and Male rats

GI- and H-ARS: The mortality vs dose probit curves for male and female rats through 30 days (ARS) are shown in figure 3a. The $LD_{50/30}$ for female rats is 14.00 (13.90–14.13) Gy after leg-out PBI. Male rats are slightly more sensitive with a $LD_{50/30}$ of 13.53 Gy (13.39–13.73) Gy leg-out PBI. The $LD_{50/30}$ included rats moribund to both GI-ARS and H-ARS. The $LD_{50/7}$ for GI-ARS is 14.29 (14.2–14.6) and 13.56 (13.4–13.8) for female and male rats, respectively (not shown in figure 3).

All-cause mortality: All-cause mortality through 120 days (figure 3b) includes rats moribund for GI-ARS, H-ARS and L-DEARE. The $LD_{50/120}$ is 13.45 (13.31–13.57) and 12.86 (12.73–12.96) for female and male rats, respectively. Comparing these probit curves for female and male rats the dose modifying factor (DMF) is 1.05 (1.04–1.06). These data show that male rats are more sensitive to radiation induced multiple organ injury (MOI) compared to female rats through 120 day after irradiation. To calculate the LD_{50} for

L-DEARE only, rats moribund between days 31 and 120 were evaluated. The $LD_{50/31-120}$, the time-interval that corresponds to lethality due to radiation pneumonitis, is 13.53 (13.4–13.7) and 12.96 (12.8–13.1) for female and male rats, respectively (not shown in figure 3). The LD_{50} values for the different sequelae are listed in Table 2.

Secondary endpoints, ARS

The DRR provided an optimal dose ($LD_{50/120}$) of leg-out PBI, to test MCM efficacy against the DEARE. A respective dose of 13.5 Gy leg-out PBI for female rats and 13 Gy leg-out PBI for male rats which represented four sequelae was chosen to measure secondary endpoints and to confirm organ-specific natural history of injury.

GI-ARS, GI scoring: GI scoring, as described in the Methods, was conducted from days 2–10. Both female and male rats reached maximum GI damage (highest scores) on day 5 after irradiation (figure 4). Recovery to normal pelleted stool was seen by day 9 in survivors.

H-ARS, Complete Blood Counts: Hematopoietic injury was assessed from complete blood counts (CBC) at multiple time points (see Methods) from 1–42 days after leg-out PBI. Neutrophil counts (figure 5a) reached a nadir by day 7 and recovered by day 22 after irradiation in both female and male rats, while the platelet counts (figure 5b) reached a nadir by day 14 and recovered by day 22. The white blood cell (WBC), red blood cells (RBC), lymphocyte and monocyte count also decreased and recovered by 42 days after irradiation (supplemental figures 1–2). Similarly, the hemoglobin and hematocrit fell and recovered (supplemental figures 3). The mean corpuscular volume (MCV) increased with time after irradiation while the mean platelet volume (MPV) did not change (supplemental figures 4). These results elaborate hematopoietic injury during H-ARS in the rat model of leg-out PBI.

Secondary endpoints DEARE

L-DEARE, breathing interval (BI): A subset of rats from the DRR study had their breathing rates assessed from day 28 after irradiation to the start of K-DEARE to determine changes in lung function. The average BI (inverse of the breathing rate) is superimposed on the Kaplan-Meier survival curve for both female and male rats (figure 6). There was a decrease in BI first measured at 49 and 35 days in female and male rats, respectively. A decrease in BI is an indicator of radiation pneumonitis.

L-DEARE, pleural effusions: Sixteen of forty female (16/40) rats receiving 13.5 Gy leg-out PBI and surviving the ARS were moribund between 60 and 104 days after irradiation with an average time to morbidity of 76 (70–82) days. Pleural effusions of >1 ml was observed upon necropsy in 14 of 15 (14/15) female rats, 1 rat could not be evaluated.

L-DEARE, absence of lethal radiation nephropathy: BUN was assessed in these female rats euthanized for pneumonitis to confirm rats did not succumb to renal failure. The average (95% CI) BUN of these rats was 36.3 (30–42) mg/dl confirming the absence of lethal radiation nephropathy (>120 mg/dl). Sixteen of 26 (16/26) male rats receiving 13.0 Gy leg-out PBI and surviving ARS were moribund between 65 and 103 days after irradiation, with an average time to morbidity of 85 (79–91) days. In males, pleural effusions of >3

ml were observed upon necropsy in all rats moribund at this time. The average BUN of these rats was 29.0 (25–33) mg/dl, once again demonstrating an absence of severe radiation nephropathy. Endpoints such as the breathing interval, pleural effusions at necropsy and/or histology have been used to confirm morbidity from lung injury during this period (Medhora et al. 2019).

K-DEARE, BUN: The progression of radiation nephropathy is shown by the BUN at 3–4 times after leg-out PBI (figure 7a and 7b). The increase in BUN is a marker of renal dysfunction (Moulder et al. 2011). All surviving female and male rats, after pneumonitis will develop renal failure (BUN>120 mg/dL) at the radiation doses used here (Medhora et al. 2019). Male rats have a longer latency period and succumb to lethal renal injury at 180 days as compared to 150 days for female rats.

L-DEARE, histology, lung: The results of histology scoring of lung sections, from female and male irradiated rats, during pneumonitis (days 60–100 after PBI) are shown in figure 8a. Mast cells (tryptase positive cells; brown stain) were increased in both female (euthanized at 69 days after 13.5 Gy leg-out PBI) and male (euthanized at 89 days after 13.0 Gy leg-out PBI) irradiated rats compared to non-irradiated-sex-matched control rats (euthanized at 150 and 180 days respectively). The quantification (figure 8b) shows the increase of mast cell with the mean and 95% CI, p<0.05. Lung injury scoring was also assessed. Increases in blood vessel thickness (see black arrows), alveolar-wall thickness (green arrows) and an increase in the numbers of foamy macrophages (not shown in these tryptase stained sections) were scored in the lungs. The composite score for these injuries reached statistical significance for both 13.5 Gy PBI female and 13.0 Gy PBI male rats when compared to non-irradiated, sex-matched control rats p<0.05 (figure 8c). Typically, during radiation pneumonitis in WAG/RijCmcr rats (Szabo et al. 2010) there is an increase in tryptase positive mast cells and in lung injury scores.

K-DEARE, histology, kidney: The results of histological scoring of Masson's trichrome stained kidney sections from female and male rats at 140–180 days are shown in figure 9. Irradiated rats (lower panel) demonstrated marked kidney injury with the presence of microscopic cysts, interstitial fibrosis, tubular proteinaceous casts and glomerular injury in the cortical and medullary regions (Medhora et al. 2019). A composite score for fibrosis (blue staining in the glomeruli) reached statistical significance in both female and male rats as compared to non-irradiated, sex matched controls p<0.05 (figure 9b).

DISCUSSION

The FDA Animal Rule, animal models:

The goal of this study was to develop animal models of concomitant ARS and DEARE to test efficacy of countermeasures for approval by the FDA animal rule. Specifically, there is a need to develop and test mitigators of radiation-induced pneumonitis in animals undergoing concomitant injury to multiple organs. Human lungs are sensitive to radiation. With supportive care and treatment for the H-ARS, it is likely that victims of radiological incidents will survive the to proceed to develop multiple organ injury (MOI). This DEARE

characterized by radiation-induced pneumonitis and nephropathy (Moulder 2014a, Moulder et al. 2014b). There are no mitigators approved by the FDA to treat DEARE, making it imperative to validated animal models that develop radiation-induced MOI, in order to test mitigators of ARS and DEARE.

Recent interaction, presentations and discussions with the FDA, Division of Imaging and Radiation Medicine (DIRM) have initiated renewed emphasis on the use of a PBI/BM-sparing model to assess MCM efficacy against acute radiation-induced lung injury as opposed to the whole thorax lung irradiation (WTLI) model. Furthermore, the primary clinically relevant endpoint for FDA approval, remains focused on a significant decrease in all-cause mortality. In this regard, the statistical design and analysis of the study protocols to determine MCM efficacy to mitigate lethal radiation induced lung injury (RILI) using the PBI/BM-sparing models in the rat, mouse and NHP must account for mortality consequent to GI- and H-ARS, as well as lethal DEARE. This is a potential dilemma for each model that is certainly species and likely sex-dependent relative to the inherent dose- and time-dependent ARS and DEARE mortality. The database herein defined those parameters for the WAG/RijCmcr rat strain. All-cause mortality due to PBI with BM-sparing in the "leg-out" model remains a significant consideration, relative to pivotal study design for FDA approval under the animal rule.

The initial WAG/RijCmcr rat models used young adult (11–12 weeks old), juvenile (5–6 weeks old) female and male rats (Fish et al. 2020); these studies described herein are young adult, 11–12 weeks old rats. Furthermore, in previous studies only a small percentage of female and male rats (~15%) exposed to 13 Gy leg-out PBI developed lethal radiation pneumonitis (Fish et al. 2016) indicating large numbers of animals would be needed to test significant mitigation of lung damage. Using doses higher than 13 Gy would increase early GI toxicity (Fish et al. 2020), leaving fewer rats at risk for radiation pneumonitis. For these and other reasons, the existing rat models needed to be refined.

Model refinement, sex-dependent ARS and DEARE, LD values:

It is well known that antioxidants can alter the radiation sensitivity in animals (Brown et al. 2010, Kennedy et al. 2011, Johnke et al. 2014, Singh et al. 2017). WAG/RijCmcr rats on diets depleted of isoflavones demonstrated increased sensitivity to radiation (Moulder et al. 2019). Therefore, a moderate antioxidant diet, Teklad 2018, that contained an isoflavone content of 150–250 mg/kg was selected to replace Teklad 8604 (containing 350–550 mg isoflavones/kg) used in previous studies (Fish et al. 2016, Fish et al. 2020). A rigorous dose-response relationship (DRR) for survival was conducted in the current study, in male and female rats with Teklad 2018, as shown in figures 1 and 2.

The probit analyses in figure 3a show the $LD_{50/30}$ to be slightly lower in male (13.5 Gy) than in female rats (14 Gy). This radio-sensitivity of male rats is also evident for mortality due to L-DEARE. The DRR for the rat model estimates the $LD_{50/120}$ for males to be 12.86 Gy while the $LD_{50/120}$ for females is 13.45 Gy. The multi-organ sequelae characteristic of the ARS and DEARE may be species-, strain- and sex-dependent. Mortality may also be model dependent, as the evolution of the DEARE occurs in survivors of the ARS. These variables have significant effects on model development. Interestingly, NHP studies report

no difference in radiosensitivity between male and female animals though the group sizes are smaller than studies with rats and the diet is different and they have more genetic variability. Recently however, a sex difference in the NHP TBI model ($LD_{50/60}$) has been reported (Beach et al. 2021). Differences in response to lung injury between males and females was absent in C57L/J mice (Jackson et al. 2014, Jackson et al. 2017) though males had a longer latency period prior to pneumonitis as compared to female mice (Dabjan et al. 2016).

MCM efficacy, all-cause mortality:

At 13.5 Gy, female rats on Teklad 2018 diet, demonstrated ~10% GI-lethality from ARS. From the >90% rats that survived, ~50% succumbed to L-DEARE (figure 1), making the 13.5 Gy dose better suited for MCM testing in female rats than 13 Gy described with Teklad 8604 diet. In male rats, 13 Gy leg-out PBI yielded ~60% lethality during radiation pneumonitis with the Teklad 2018 diet, though ARS lethality was relatively high, between 10–20% (figure 2). With 12.5 Gy, GI injury was below 10%, but lung lethality was very low (<5%) rendering this dose unsuitable for MCM testing for L-DEARE in male rats. Taken together, the DRR studies suggested that the most suitable models for testing MCM to mitigate L-DEARE are 13.5 Gy and 13 Gy for young adult female and male rats respectively and using the Teklad 2018 diet. These doses were chosen to further characterize the models.

The PBI/BM-sparing models allow for the analysis of time- and dose-dependent morbidity and mortality of ARS and DEARE, encompassing GI-, H-ARS, and L-DEARE. The time segments for the GI- and H-ARS mortality (d1–30) for rat are distinct and separated by long latency periods, approximately 50 days for the rat (figure 1 and 2), prior to the incidence of morbidity and mortality attributable to delayed lung injury. Interestingly, K-DEARE is delayed in male rats as compared to the females allowing males to survive longer after radiation (figures 1 and 2). This difference could be attributed to hormones, since the latency in juvenile male WAG/RijCmcr rats is comparable to that of juvenile females (Medhora et al. 2019).

There is an observed sex-dependent difference in mortality in WAG/RijCmcr rat strain obtained after statistical analysis using all-cause mortality at respective $LD_{50/90}$, and $LD_{50/120}$ for female and male rats with 80% power to detect p<0.05. In fact, the difference in the LD_{50} for L-DEARE between females and males will impact MCM study design parameters. Thus, separate efficacy studies with different radiation doses for each sex should be proposed for rats.

Clinical biomarkers:

Interestingly, by superimposing the body weight over the survival plots in figures 1 and 2, it becomes evident that changes in body weight is a simple biomarker to predict the onset and severity of GI, lung and renal injuries after leg-out PBI. At doses of 13, 13.5 and 14 Gy in female rats, and 12.5, 13 and 13.5 Gy in male rats, there was an initial drop in body weight starting from day 2, with the nadir being lower for the higher doses of radiation in both females and males. It is not clear if this nadir also reflects hematopoietic injury since the 2 sequelae, GI and bone marrow toxicities, appear within a few days of each

other. Around 42 and 85 days the body weights begin to fall again just before the onset of lethal radiation pneumonitis and nephropathy respectively, with the steepest declines in body weight coinciding with the highest doses of radiation.

Though the primary end point of this study was to assess the time course of survival probability via the Kaplan-Meier analysis, measurements of secondary endpoints are essential to define the natural history and morbidity inherent in each concomitant, organ sequelae. These data confirm and establish the duration of acute, prolonged and delayed radiation effects to better inform MCM efficacy and FDA considerations. Therefore, secondary endpoints for GI, bone marrow, lung and renal toxicities were measured for the optimal 13.5 Gy female and 13 Gy male leg-out PBI models with the Teklad 2018 diet. Between 5–9 days, GI injury as determined by scoring diarrhea, occurring in both female and male rats (figure 4). Though ~7.5% of the bone marrow was spared during radiation, depletion of neutrophils and platelets reached a nadir at ~7 and ~14 days respectively, demonstrating H-ARS toxicity. These and other hematopoietic injuries were not severe enough to cause significant lethality. Decrease in breathing intervals was recorded from 28 days after leg-out PBI. The breathing intervals continued to drop, coincident with lung morbidity. It is known that breathing rate and intervals recover with time after radiation (Zhang et al. 2008, Kma et al. 2012, Medhora et al. 2014, Fish et al. 2016), though breathing intervals during radiation nephropathy remain to be measured in a leg-out PBI model. Renal injury increased exponentially in irradiated female and male rats after 13.5 and 13 Gy leg-out PBI respectively (figures 7a and 7b). These doses of irradiation are well above the threshold for renal damage (Moulder et al. 2011, Fish et al. 2020) so that recovery of kidney function is not possible, and all the rats that survive L-DEARE eventually succumb to radiation nephropathy. It is important to note that renal injury can be effectively mitigated by angiotensin converting enzyme (ACE) inhibitors in the high antioxidant diet model (Moulder et al. 2011, Medhora et al. 2012, Fish et al. 2016). The ACE inhibitor lisinopril is effective in mitigating DEARE in this new model with the moderate antioxidant diet (T-2018, unpublished results).

There are a number of advantages of the WAG/RijCmcr leg-out PBI rat model, most importantly, the occurrence of at least 4 distinct dose-, time- and sex-dependent sequelae. In similar NHP and mouse studies, experiments were terminated at a fixed time usually 180 days, (Booth et al. 2015, Cui et al. 2016, Cohen et al. 2017, MacVittie et al. 2020) and often all the animals were euthanized from severe pneumonitis indicating the dose of radiation may be considerably higher than the threshold for L-DEARE. Secondly, the irradiated WAG/RijCmcr rats developed pleural effusion which are observed in non-human primates (Garofalo et al. 2014, MacVittie et al. 2017, MacVittie et al. 2020) and humans (Zhao et al. 2017) but not in certain strains of mice (Jackson et al. 2010, Jackson et al. 2011). In addition, G-CSF and GM-CSF, which have already been approved for H-ARS by the FDA, can be tested in the PBI/BM-sparing rat models to determine compatibility with new drugs being tested for ARS or different DEARE sequelae. For example, the mitigator lisinopril, an ACE inhibitor, has been shown to attenuate radiation pneumonitis and nephropathy in the WAG/RijCmcr leg-out PBI rat model as poly-pharmacy approach with G-CSF (Fish et al. 2016).

There are also limitations of the WAG/RijCmcr leg-out PBI rat model. For example, the renal injury is more pronounced than reported in comparable NHP (Cohen et al. 2019) and mouse models (Unthank et al. 2015, Unthank et al. 2019). Humans experience radiation nephropathy (Lawton et al. 1991, Cohen et al. 1993), though the severity is not known, since the doses of radiation used in the clinic are restricted to ensure non-lethal toxicities to normal tissues. It is possible that shielding one kidney may increase the lifespan of the rats after leg-out PBI, revealing other DEARE-related injuries in different organs such as heart and brain. Limitations of the current study also include the absence of detailed histological examination of intestinal crypts to accurately record GI toxicity. Measurement of GI scoring was not optimal, as observed by the large error bars in figure 4. Also, long term GI and BM injuries were not measured as reported for mice (Booth et al. 2012a, Booth et al. 2012b, Booth et al. 2015). Future studies are underway with the same models to record injuries to the heart and brain and to determine biomarkers using metabolomics and lipidomic profiles.

In summary, a robust and versatile animal model for testing MCM for acute and delayed radiation injuries is described herein for female and male WAG/RijCmcr rats. The PBI/BM-sparing rat models allow for the analysis of time- and dose-dependent morbidity and mortality of ARS and DEARE, encompassing GI-, H-ARS, L-DEARE and K-DEARE. The time segments for the GI- and H-ARS mortality (d1–30) for rat are distinct and separated by long latency periods, approximately 50d, prior to the incidence of morbidity and mortality attributable to delayed lung injury. Survivors of L-DEARE, after second latency period develop radiation nephropathy (K-DEARE). These rodent models are currently among the best developed to contribute to MCM discovery by the criteria of the FDA Animal Rule.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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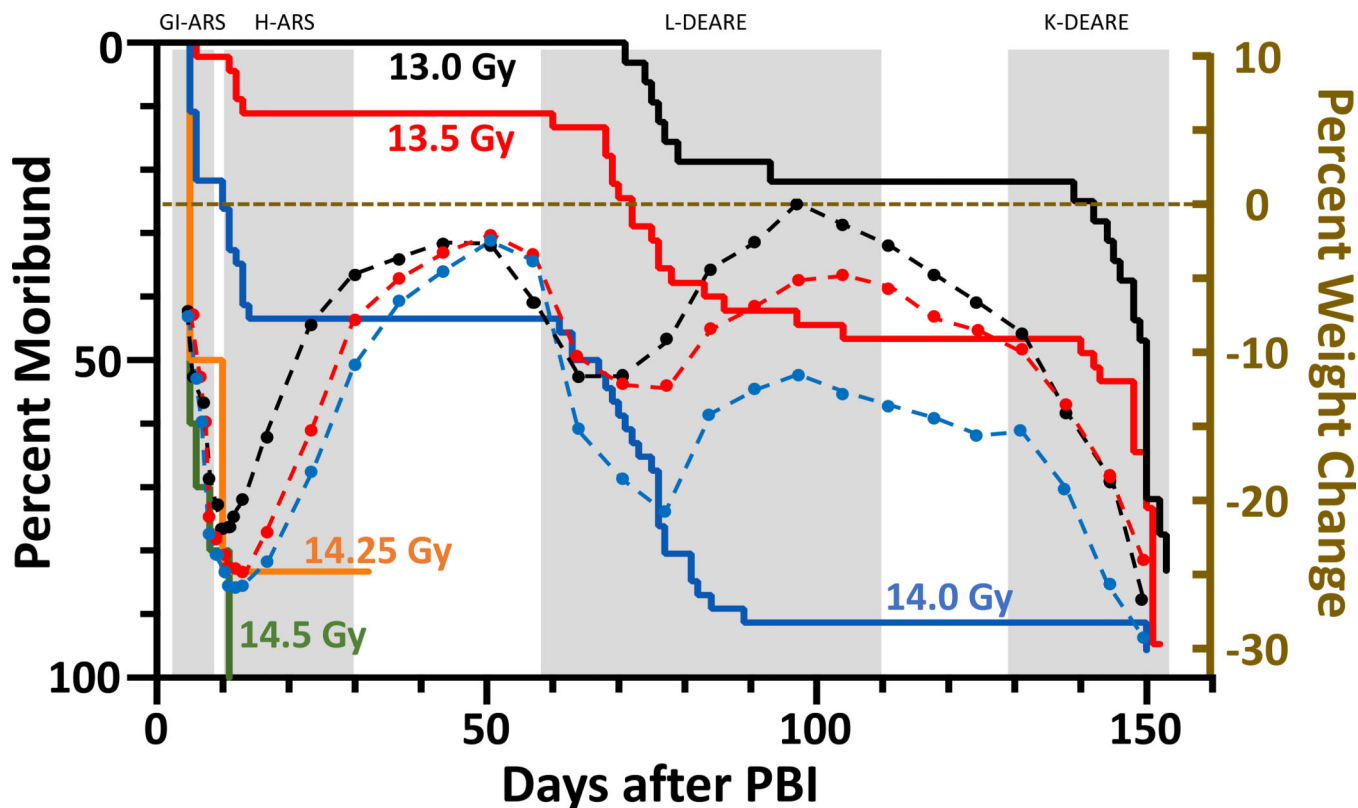
Figure 1.
Kaplan-Meier (KM) survival plots demonstrating dose- and time-dependent lethal sequelae observed after leg-out PBI in female rats. Female rats (11–12-week-old) received PBI at doses ranging from 13.0 to 14.5 Gy. Doses above 14 Gy (green and orange lines) result in > 80% morbidity during ARS. Survivors of the ARS (13 Gy (black line), 13.5 Gy (red line) and 14 Gy (blue line)) showed a dose-dependent survival during pneumonitis but were moribund from radiation nephropathy. At the doses delivered, all rats will develop radiation nephropathy after 120 days. To correlate body weight with organ specific sequelae, superimposed over the KM plot are the time courses (dashed lines) of average percent change in body weight from irradiation for 13.0 (black dots), 13.5 (red dots) and 14 Gy (blue dots). The body weight decreases prior to organ specific lethal sequelae.
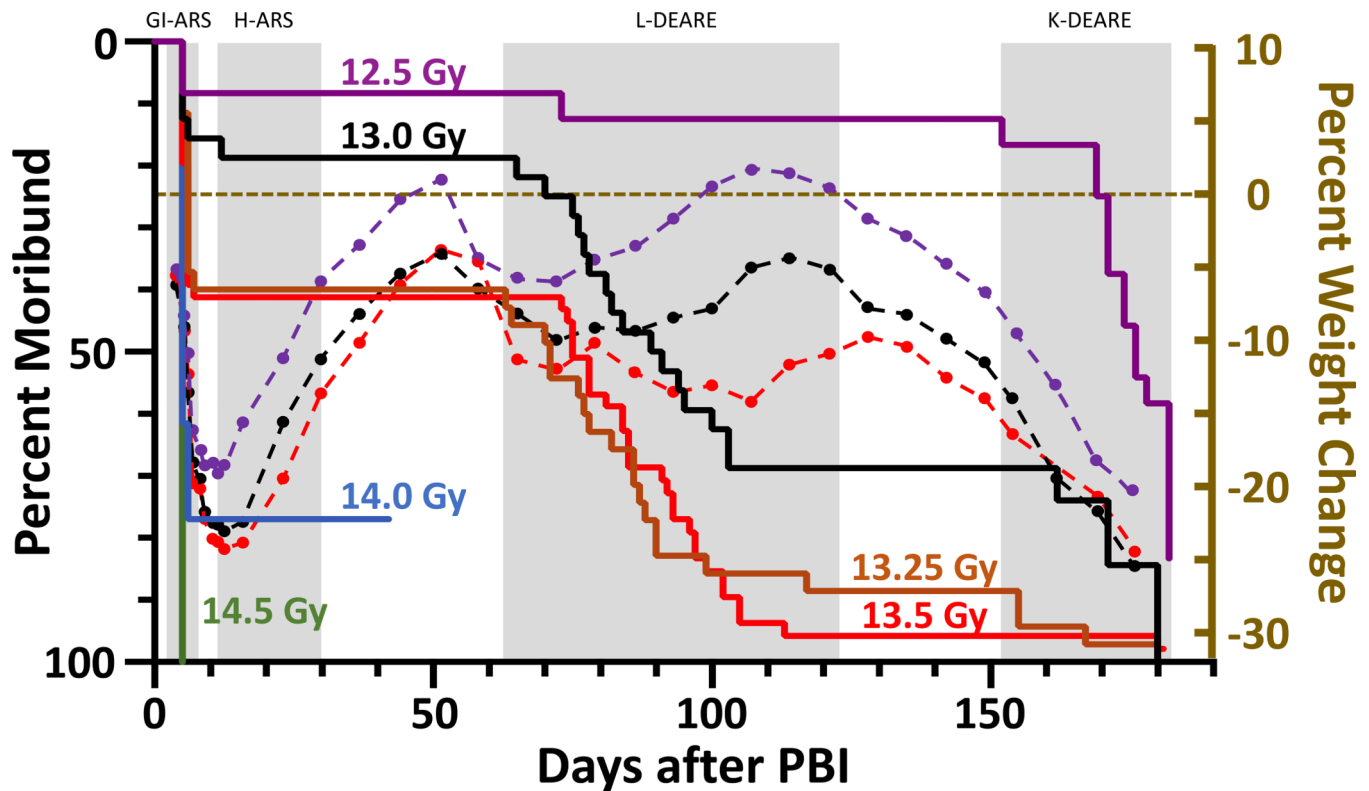
Figure 2.
Kaplan-Meier (KM) survival plot demonstrating dose- and time-dependent lethal sequelae observed after leg-out PBI in male rats. Male rats (11–12-week-old) received PBI at doses ranging from 12.5 to 14.5 Gy. Doses 14 Gy and above (blue and green lines) result in > 70% morbidity during ARS. Rats receiving doses at or below 13.5 Gy survived ARS in a dose-dependent manner. Survivor of ARS (12.5 Gy (purple line), 13 Gy (black line), 13.25 Gy (orange line) and 13.5 Gy (red line)) showed a dose-dependent survival during pneumonitis but succumbed to radiation nephropathy. To correlate body weight with organ specific sequela, superimposed over the KM plot are the time courses (dashed lines) of average change in body weight from irradiation for 12.5 (purple dots), 13.0 (black dots) and 13.5 Gy (red dots). The body weight decreases prior to organ specific lethal sequelae.
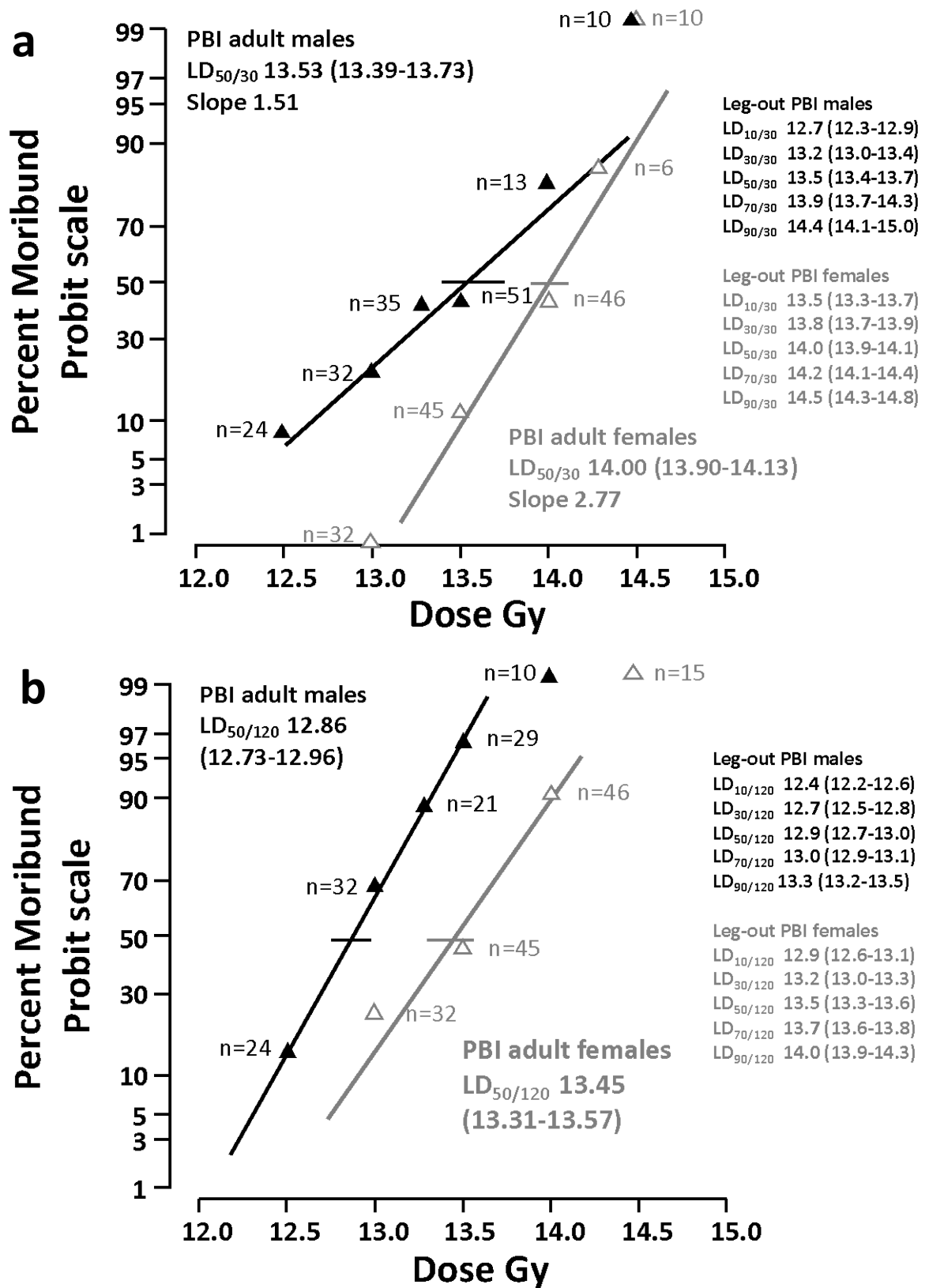
a

n=10 ▲△ n=10

PBI adult males
$LD_{50/30}$ **13.53 (13.39-13.73)**
Slope 1.51

Leg-out PBI males
$LD_{10/30}$ **12.7 (12.3-12.9)**
$LD_{30/30}$ **13.2 (13.0-13.4)**
$LD_{50/30}$ **13.5 (13.4-13.7)**
$LD_{70/30}$ **13.9 (13.7-14.3)**
$LD_{90/30}$ **14.4 (14.1-15.0)**

Leg-out PBI females
$LD_{10/30}$ **13.5 (13.3-13.7)**
$LD_{30/30}$ **13.8 (13.7-13.9)**
$LD_{50/30}$ **14.0 (13.9-14.1)**
$LD_{70/30}$ **14.2 (14.1-14.4)**
$LD_{90/30}$ **14.5 (14.3-14.8)**

PBI adult females
$LD_{50/30}$ **14.00 (13.90-14.13)**
Slope 2.77

n=13, n=6, n=35, n=51, n=46, n=32, n=45, n=24, n=32

b

n=10 ▲ △ n=15

PBI adult males
$LD_{50/120}$ **12.86**
(12.73-12.96)

Leg-out PBI males
$LD_{10/120}$ **12.4 (12.2-12.6)**
$LD_{30/120}$ **12.7 (12.5-12.8)**
$LD_{50/120}$ **12.9 (12.7-13.0)**
$LD_{70/120}$ **13.0 (12.9-13.1)**
$LD_{90/120}$ **13.3 (13.2-13.5)**

Leg-out PBI females
$LD_{10/120}$ **12.9 (12.6-13.1)**
$LD_{30/120}$ **13.2 (13.0-13.3)**
$LD_{50/120}$ **13.5 (13.3-13.6)**
$LD_{70/120}$ **13.7 (13.6-13.8)**
$LD_{90/120}$ **14.0 (13.9-14.3)**

PBI adult females
$LD_{50/120}$ **13.45**
(13.31-13.57)

n=29, n=21, n=46, n=32, n=45, n=32, n=24

Figure 3a.

Probit Analyses to determine LD_{50}. The left panel shows the dose response relationship (DRR) for female and male WAG/RijCmcr rats for percent moribund rats on a Probit scale, versus leg-out PBI dose (Gy) assessed at 30 days (ARS). The $LD_{50/30}$ for all-cause mortality through ARS, including GI-ARS and H-ARS for female rats is 14.0 Gy (13.90–14.13) and for male rats is 13.53 Gy (13.39–13.73).

3b. The right panel shows the DRR for female and male WAG/RijCmcr rats assessed at 120 days, including GI-ARS, H-ARS and L-DEARE (see Table 2).
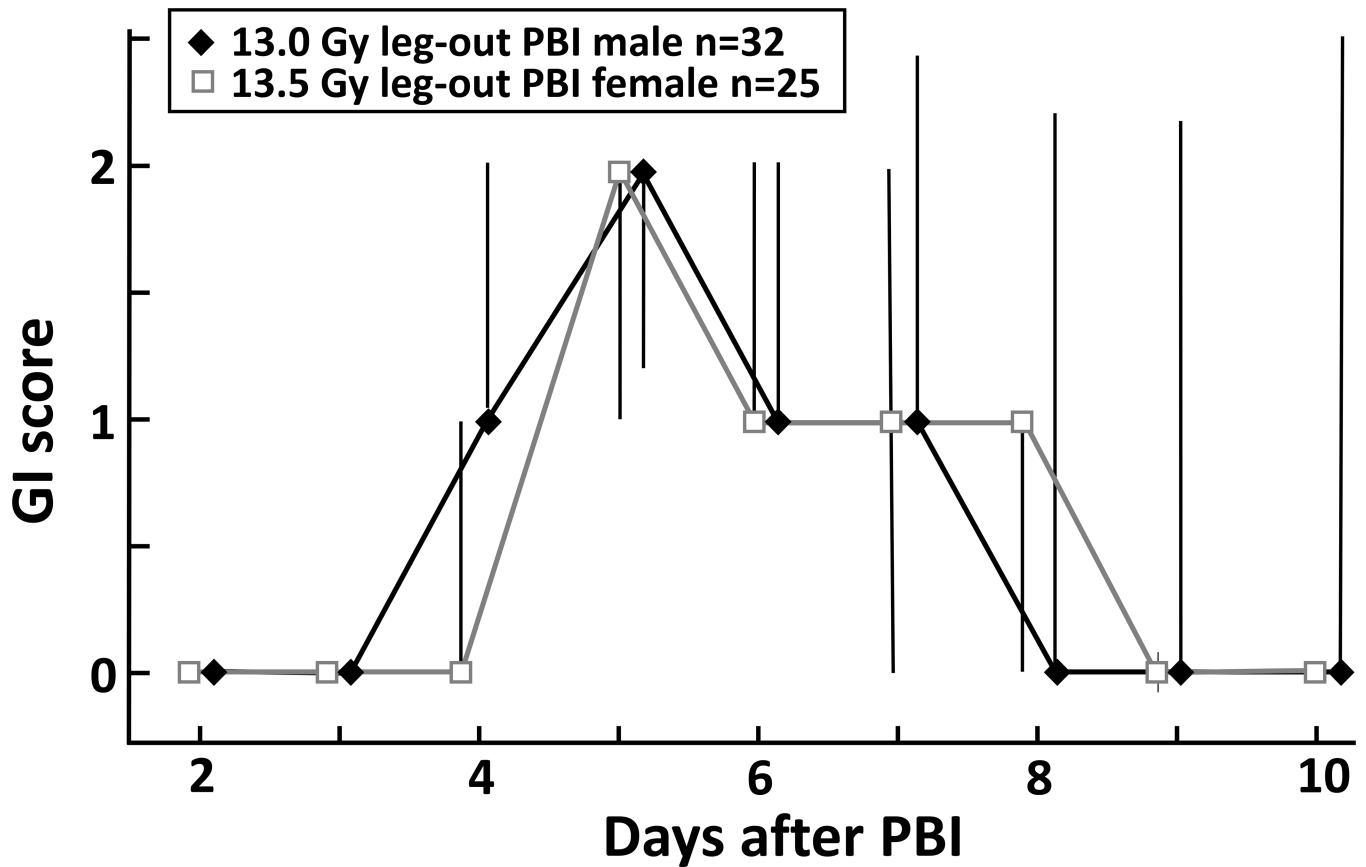
Figure 4.
To evaluate GI-ARS, the $LD_{50/120}$ was selected as this dose best represents models suitable to test mitigators for L- and K-DEARE. GI injury was scored with a modified scoring system. In both female and male rats, the GI injury scores peak at day 5 after irradiation and return to normal by day 10. Data presented as medians and 20–80 percentiles.
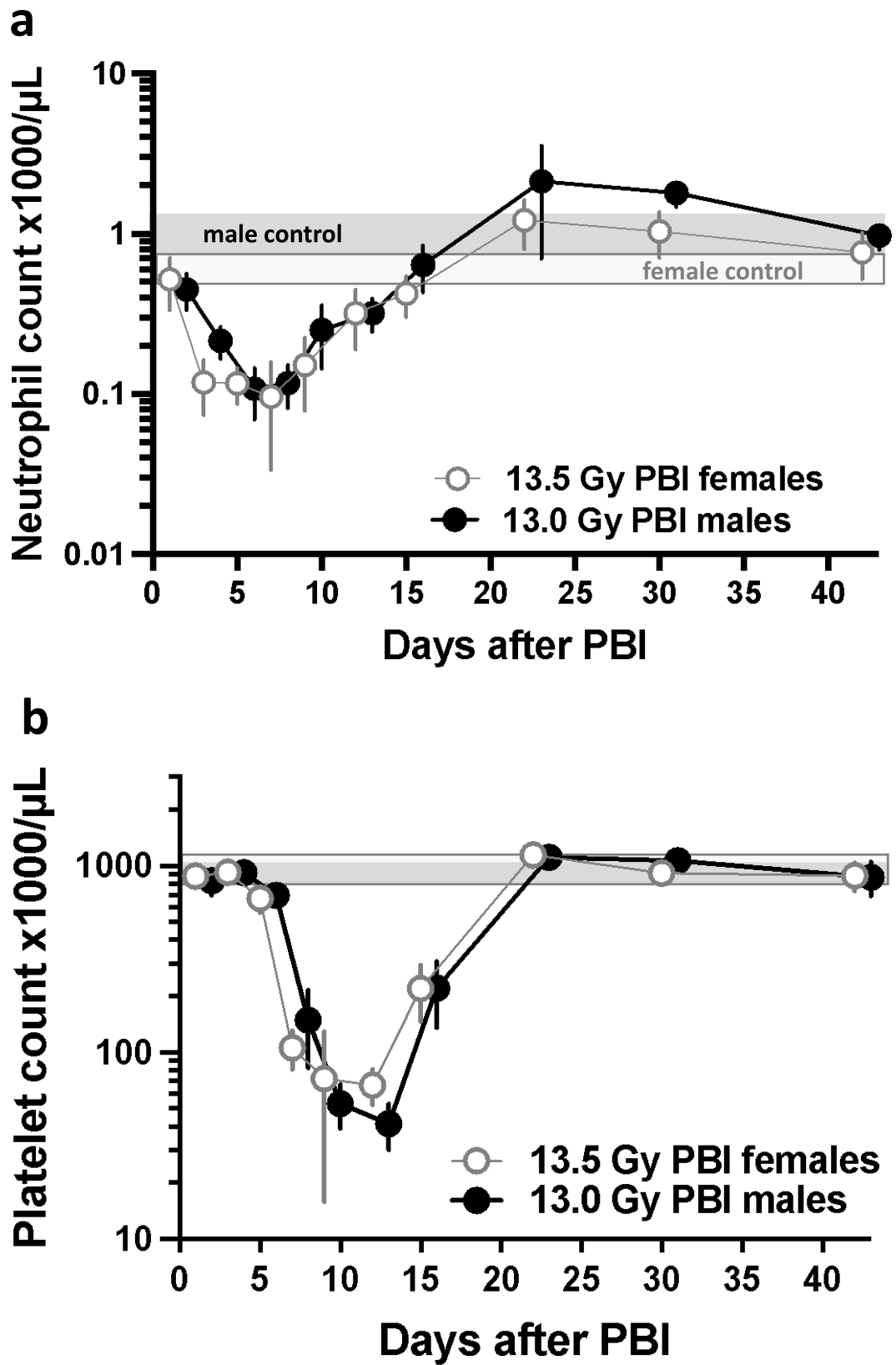
Figure 5a.

Time course of neutrophil counts during H-ARS at the $LD_{50/120}$. Complete blood counts were assessed from day 1 to 42 after irradiation. The neutrophil count for both female (open gray circles) and male (black circles) rats reached a nadir of ~100 neutrophils/μl, between 5–7 days. The neutrophil counts recover to >1000 neutrophils/μl by day 22 after irradiation. Non-irradiated males have a slightly higher neutrophil concentration (solid grey bar) compared to non-irradiated females (white/gray bar). Data are presented as means and 95% CI for non-irradiated female (white/gray bar) and male (solid grey bar) rats.

5b. Time course of platelet counts during H-ARS at the $LD_{50/120}$. There was a drop in platelet counts and recovery during H-ARS at the $LD_{50/120}$. The platelet nadir occurred at 14 days after irradiation with a count of ~35 and 70 platelets $\times 10^3$/μl for male and females, respectively. The platelet counts recover to >1000 $\times 10^3$ platelets/μl by day 22 after irradiation. The bars represent the 95% confidence limits for non-irradiated female (white/gray bar) and male (solid grey bar) rats. Data are presented as means and 95% CI.
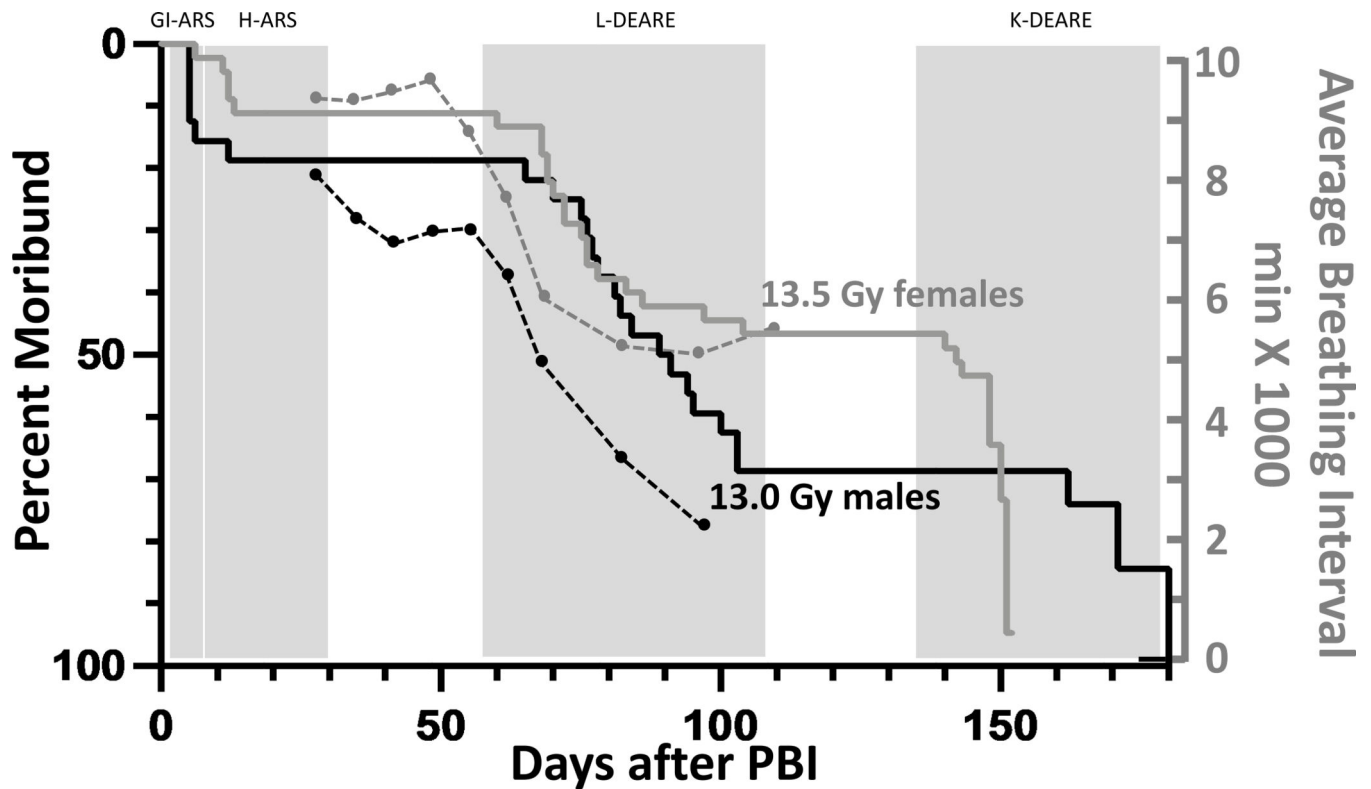
Figure 6.
Kaplan-Meier (KM) survival plot demonstrating the LD$_{50/120}$ for all cause mortality for female (gray line) and male (black line) rats after leg-out PBI. To correlate breathing intervals with the onset of lethal lung-DEARE, the KM plot are superimposed with the breathing intervals showing the time course of lethal lung injury (axis on the right). The average breathing intervals were recorded from 28–100 days for males (black dots) and 28–110 days for females (gray dots) in a subset of rats in the DRR study. Note a fall in the breathing interval that precedes lethal radiation pneumonitis. Data are presented as average BI (minutes x1000).
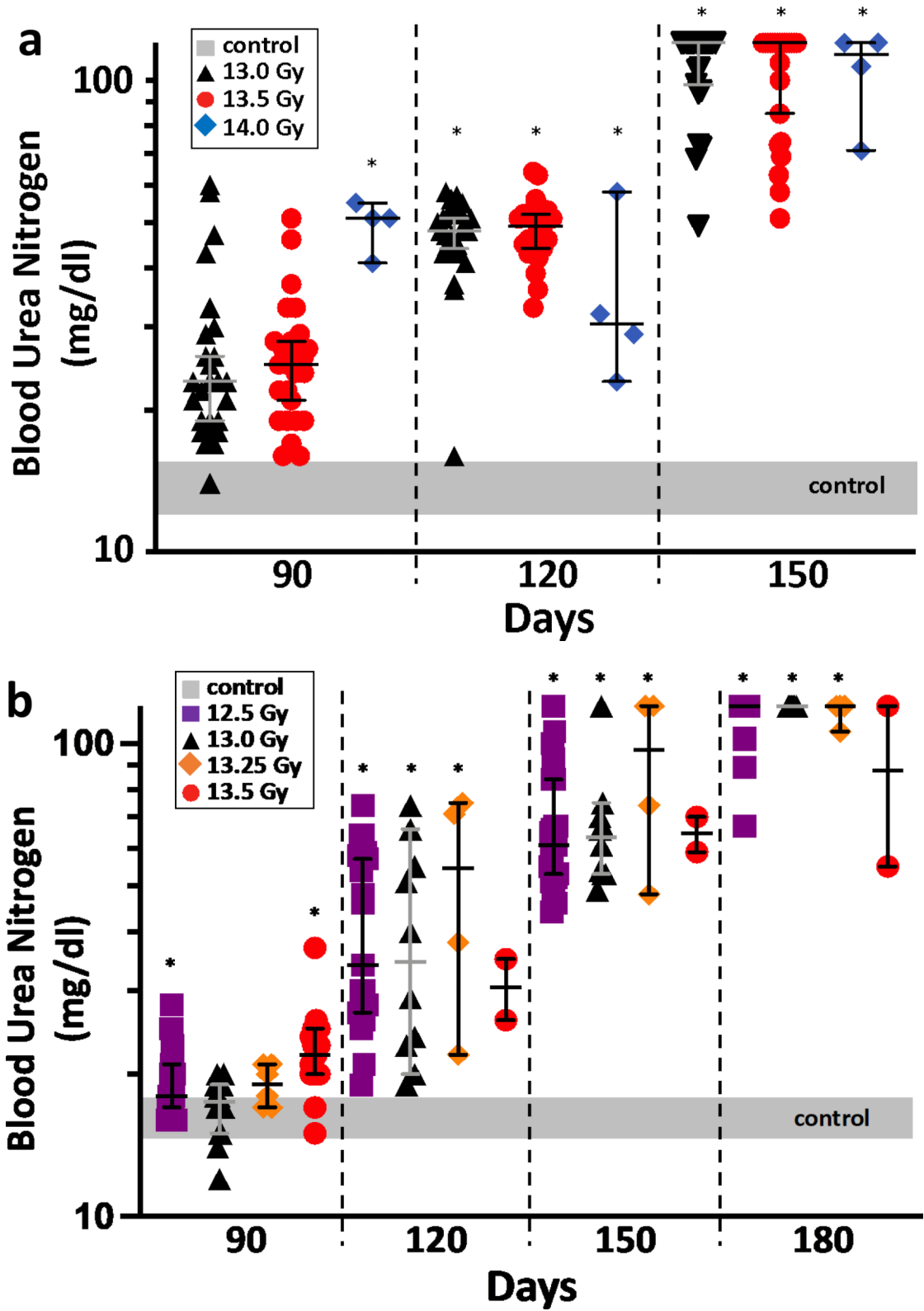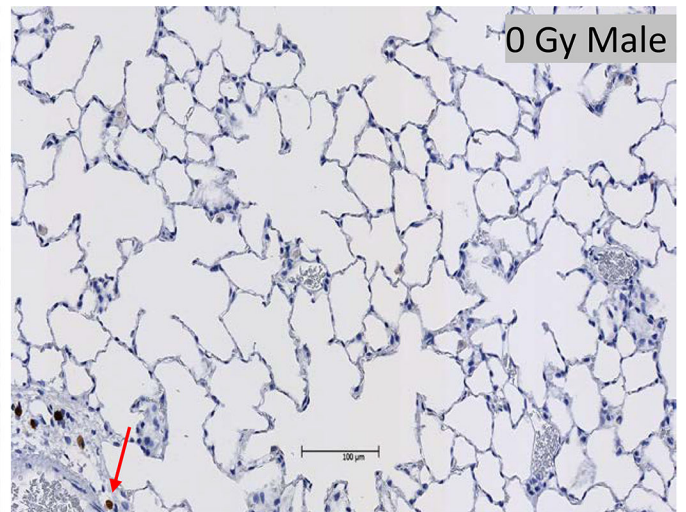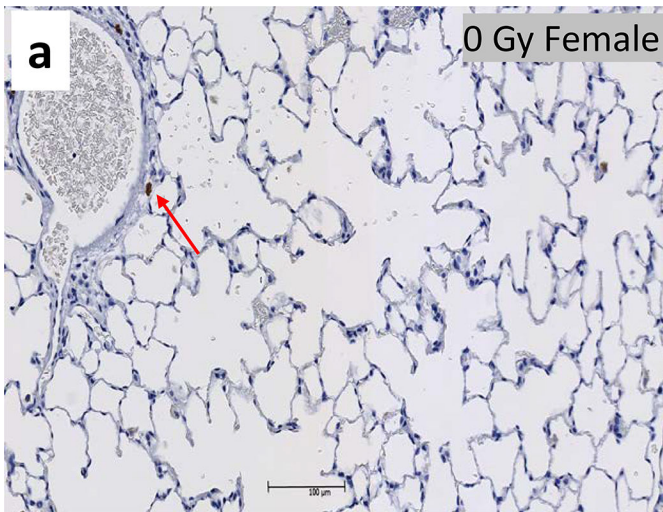
Figure 7a.

Measurement of K-DEARE. Progression of renal injury after leg-out PBI in female WAG/RijCmcr rats is shown in the left panel. Female rats from the DRR study, at doses of 13 Gy (black triangles), 13.5 Gy (red circles) and 14 Gy (blue diamonds) leg-out PBI, had increased BUN level from day 120 as compared to non-irradiated control rats (grey bar). Irradiated female rats at the doses presented, progress to renal failure (defined as a BUN>120 mg/dl) by 150 days after leg-out PBI. Data presented as medians and 95% CI. The asterisk (*) represents p<0.05 versus nonirradiated sex-matched rats.

7b. Progression of renal injury after leg-out PBI in male WAG/RijCmcr rats. Male rats at doses of 12.5 Gy (purple squares), 13 Gy (black triangles), 13.25 Gy (orange diamonds) and 13.5 Gy (red circles) leg-out PBI, had increased BUN levels from day 120 on, compared non-irradiated control rats (grey bar). Note that male rats have a longer latency period for renal injury than female rats, so they were followed to 180 days. Irradiated male rats at the doses presented, progress to renal failure by 180 day after leg-out PBI. Data presented as medians and 95% CI. The asterisk (*) represents p<0.05 versus nonirradiated sex-matched rats.
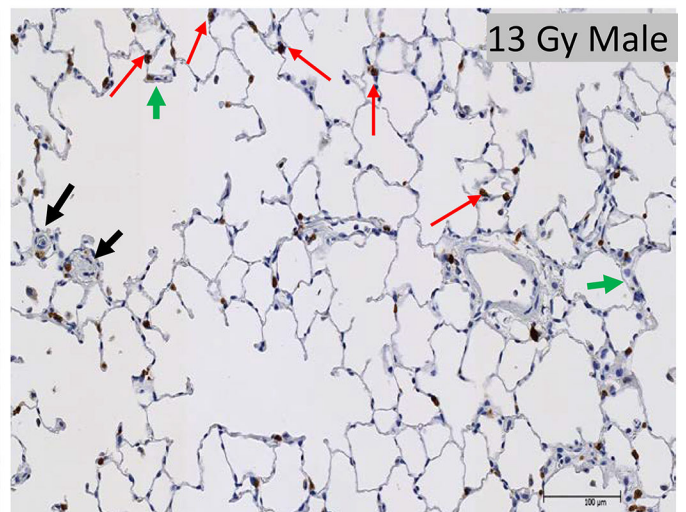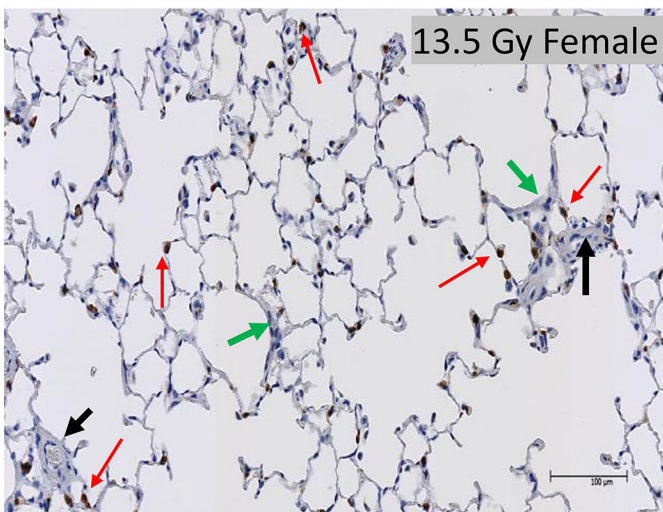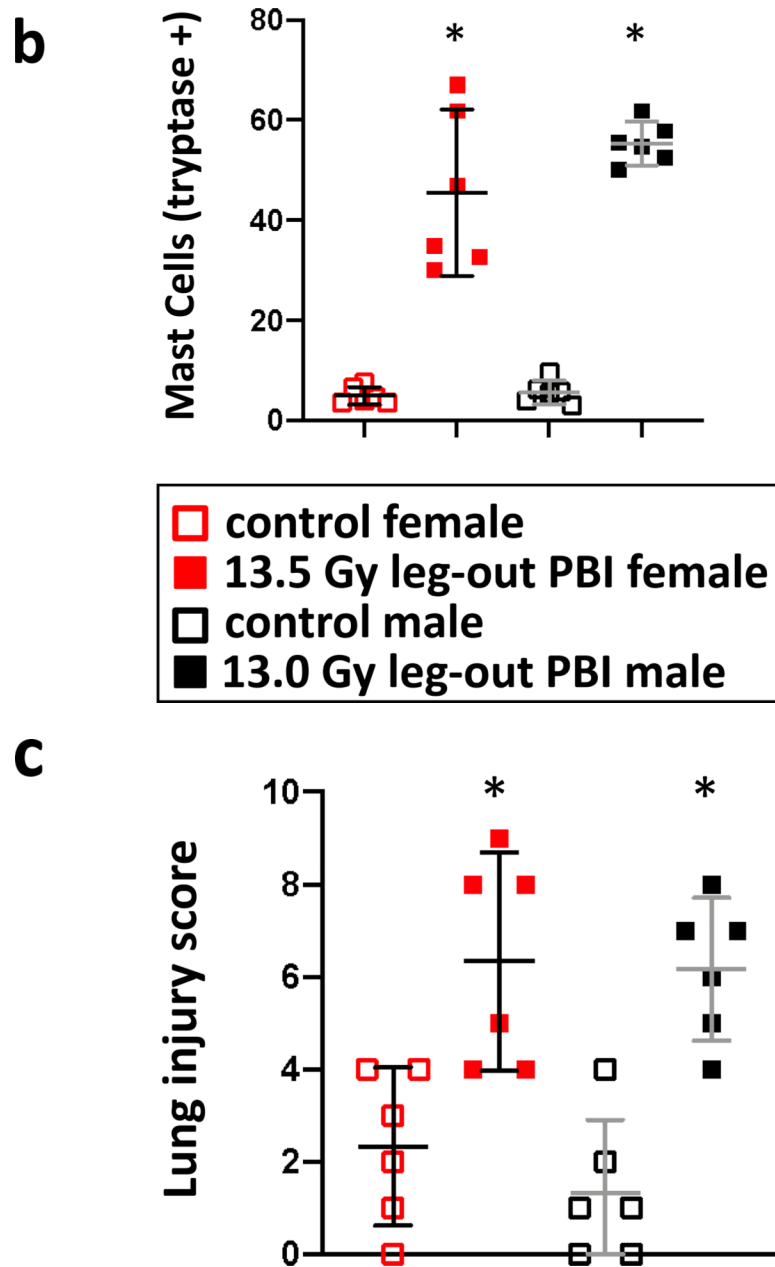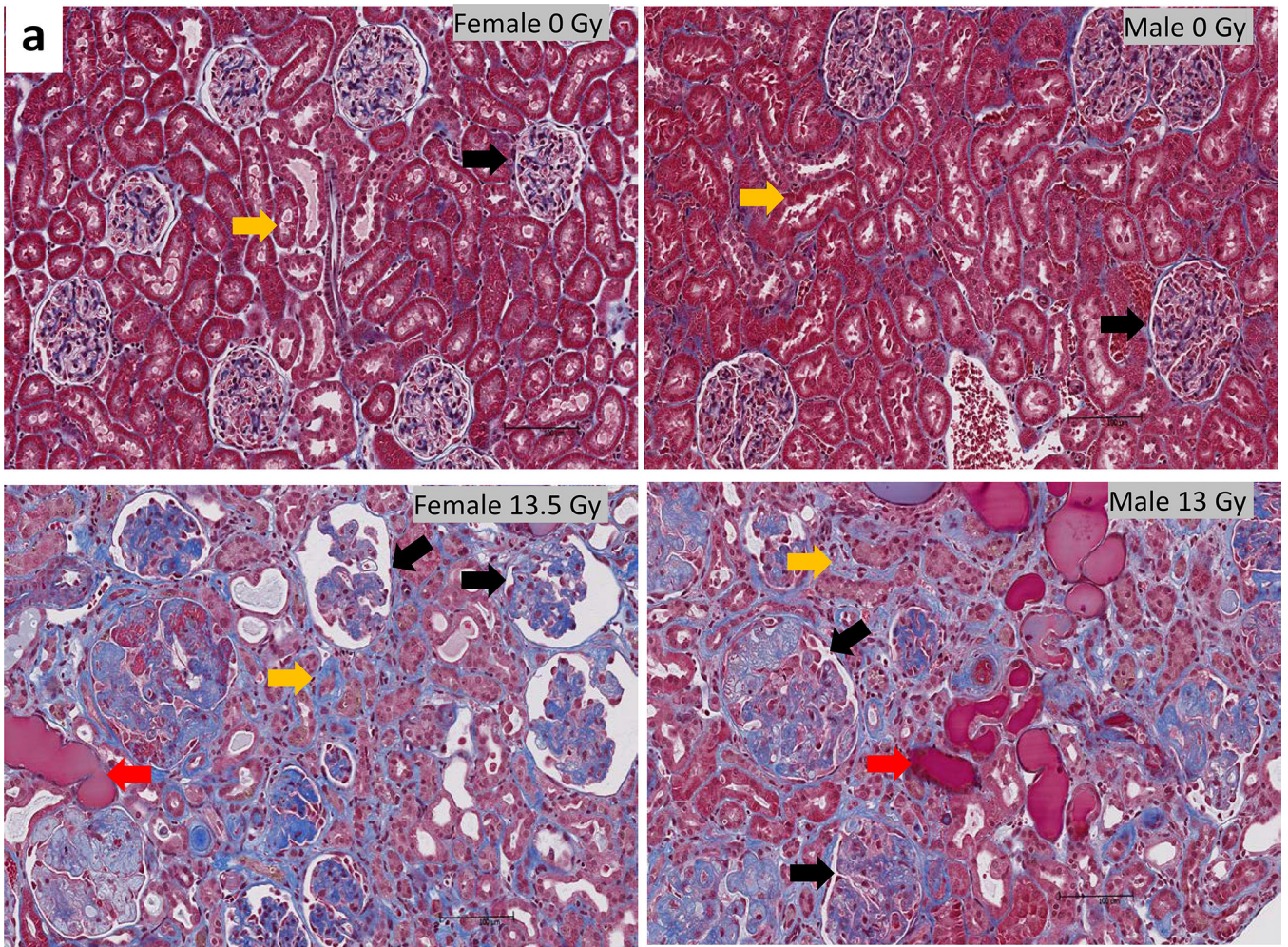
Figure 8a.

Histology of lung injury during pneumonitis. Irradiated female and male rats that were moribund between days 60–100 were accessed for radiation induced lung injury (RILI). Whole mount lung sections (4 μm thick) were stained with anti-tryptase antibody for mast cells (stained brown, red arrows).

8b. Graphical representation of lung mast cell counts from female and male rats. Lungs from both female and male irradiated rats had increased numbers of mast cells compared to their sex-matched control rats. Nonirradiated control rat lungs were harvested at termination (150-day females and 180-day males) as no rats in these groups were moribund during pneumonitis. Data is presented as means and 95% CI. The asterisk (*) represents $p<0.05$ versus nonirradiated sex-matched rats.

8c. Lung injury scores. Lung injury was scored as described in Materials and Methods. Individual scores for foamy macrophages, vascular wall thickness and alveolar wall thickness were determined on a 5-point scale as described (Medhora et al. 2014, 2015). A calculated mean composite lung injury score was derived and is shown. Data is presented as means and 95% CI. The asterisk (*) represents p<0.05 versus nonirradiated sex-matched rats.
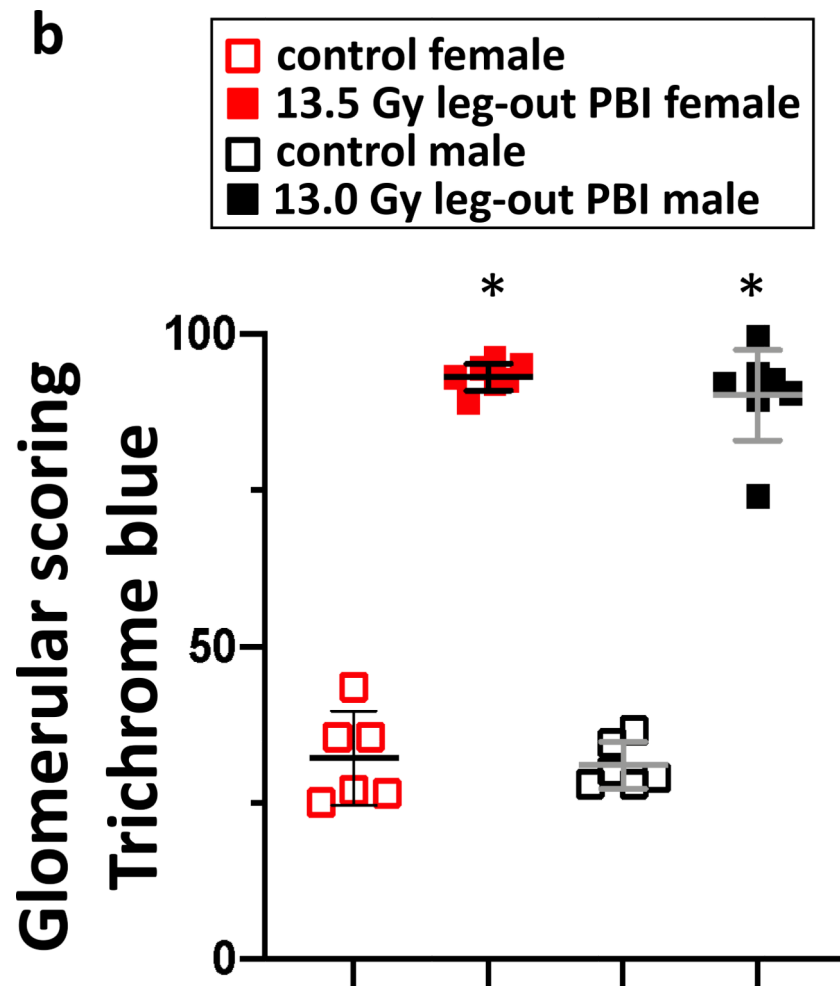
Figure 9a.
Histology of kidney injury and fibrosis during radiation nephropathy. Representative photomicrographs of kidney cortex from control female (upper left panel) and male (upper right panel) rats. Irradiated females at 150 days after 13.5 Gy leg-out PBI (lower left panel) and males at 180 days after 13 Gy leg-out PBI male (lower right panel) rats are shown. Arrows show normal (upper panel) or damaged (lower panel) glomeruli (black), tubules (yellow) and tubular casts (red).

9b. Graphical representation of the results from composite fibrosis scoring of kidneys from female and male rats are shown. Average scores for non-irradiated female and male rats are represented by the open squares, red and black respectively. Irradiated female and male rats are represented by solid red and black squares respectively. Irradiation dramatically increased glomerular fibrosis compared to nonirradiated sex-matched rats. Data presented as means and 95% CI. The asterisk (*) represents $p<0.05$ versus nonirradiated sex-matched rats.

TABLE 1:

DIET COMPARISON

Nutrient	T.8904 Teklad Rodent Diet	2018 Teklad global 18% protein Diet
Protein (%)	24.3	18.6
Carbohydrate (%)	40.2	44.2
Fat (%)	4.7	6.2
Energy density (kcal/g)	3.0	3.1
Sodium (%)	0.3	0.2
Phosphorus (%)[a]	0.7	0.4
Selenium (mg/kg)	0.34	0.23
Isoflavones (mg/kg)	350–550	150–250

[a] Available (non-phytate)

TABLE 2:

LD$_{50}$ Values

Survival Days (d) after PBI	Sequela	Gender	LD$_{50}$ (95% CI) Gy
0–7d	GI-ARS	Female	14.3 (14.2–14.6)
0–7d	GI-ARS	Male	13.6 (13.4–13.8)
8–30d	H-ARS	Female	14.2 (14.0–14.5)
8–30d	H-ARS	Male	NA[#]
0–30d	All-cause ARS	Female	14.0 (13.9–14.1)
0–30d	All-cause ARS	Male	13.5 (13.4–13.7)
30–120d	L-DEARE	Female	13.5 (13.4–13.7)
30–120d	L-DEARE	Male	13.0 (12.8–13.1)
0–120d	All-cause through L-DEARE	Female	13.5 (13.3–13.6)
0–120d	All-cause through L-DEARE	Male	12.9 (12.7–13.0)
120+d	K-DEARE	Female	NA[*]
120+d	K-DEARE	Male	NA[*]

[#] Did not have sufficient morbidity to calculate the LD50.

[*] Doses used in this study are above the LD50 for radiation nephropathy.