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DELAYED EFFECTS OF ACUTE RADIATION EXPOSURE (DEARE) IN JUVENILE AND OLD RATS: MITIGATION BY LISINOPRIL

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Abstract

Our goal is to develop lisinopril as a mitigator of delayed effects of acute radiation exposure (DEARE) in the National Institute of Allergy and Infectious Diseases (NIAID) radiation countermeasure program. Published studies demonstrated mitigation of DEARE by lisinopril in adult rats. However, juvenile or old rats beyond their reproductive lifespan have never been tested. Since no preclinical models of DEARE were available in these special populations, appropriate rat models were developed to test lisinopril after irradiation. Juvenile (42 day old, prepubertal), female and male WAG/RijCmc (Wistar) rats were given 13 Gy partial body irradiation (PBI) with only one hind limb shielded. Lethality from lung injury between 39-58 days and radiation-nephropathy between 106-114 days were recorded. All irradiated only juvenile rats were morbid from DEARE by 114 days, while lisinopril (24 mg m⁻² day⁻¹) started 7 days after irradiation and continued, improved survival to 88% (p=0.0015, n 8/group). Old rats (>483 days, reproductively senescent) were irradiated with 13 Gy PBI keeping one leg shielded and additionally shielding the head in some animals. Irradiated old females developed lethal nephropathy and all became morbid by 170 days after irradiation, though no rats displayed lethal radiation pneumonitis. Similar results were observed for irradiated geriatric males though 33% of rats remained alive at 180 days after irradiation. Lisinopril mitigated radiation nephropathy in old rats of both sexes. Finally, comparison of DEARE between irradiated juvenile, adult and old rats showed younger rats were more sensitive to DEARE with earlier manifestation of injuries to some organs.

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INTRODUCTION

Research to identify and develop medical countermeasures for a radiological or nuclear accident or attack became a priority after 9/11 (DiCarlo et al. 2012). In order to develop and stockpile drugs for this indication approval must be obtained from the US Food and Drug Administration (FDA) via the FDA Animal Rule. Under this rule the efficacy of drugs to enhance survival and reduce injury after radiation must be tested in appropriate animal models that mimic the human response. A recent review summarized models, as well as drug candidates that have been tested for this purpose (Singh et al. 2015). Most models described to date manifest **Acute Radiation Syndrome (ARS)** that develops within 30 days after exposure. Depending on the dose of radiation, acute gastrointestinal (GI) injury occurs first, usually after 4-10 days in mice (Booth et al. 2012), rats (Fish et al. 2016), and non-human primates (NHP) (MacVittie et al. 2012). Acute hematopoietic syndrome from 10-30 days after exposure also develops in these species (Plett et al. 2012). Other models of ARS in guinea pig, ferret, dog and minipig have been described (Williams et al. 2010, Singh et al. 2015). If animals survive, a number of **delayed effects of acute radiation exposure (DEARE)** manifest after recovery from ARS. These have been studied in multiple animal species (Williams et al. 2010) as single or multi-organ injuries depending on the number of organs in the field of radiation. The goal is to develop preclinical models that are relevant to a nuclear attack or accident to target multiple organs with the highest single dose of radiation that will manifest the endpoints of interest. However, animals given high doses of radiation will not survive ARS unless GI and bone marrow toxicities are avoided or addressed. Recently, partial body exposures with minimal shielding of bone marrow cells by externalizing portions of one or both hind limbs from the field of radiation (leg-out PBI), have been used to overcome hematopoietic toxicity. Antibiotics and hydration have been used to overcome GI injury at doses 12 Gy (Booth et al. 2012, MacVittie et al. 2012, Fish et al. 2016). Previous knowledge of such preclinical models has been described in adult animals only and there is little information of DEARE with leg-out PBI in juvenile or older animals except for a few reports in mice (Johnston et al. 2013, Misra et al. 2015). Increased sensitivity to infections at delayed times was reported in irradiated neonatal and 14 day old mouse pups, though detailed sequelae that result from exposure to high doses of radiation were not conducted (Johnston et al. 2013).

Previously published studies described a rat model of DEARE affecting multiple organs after a single dose of 13 Gy leg-out PBI (Fish et al. 2016). One hind limb was shielded in adult female rats in this study in combination with hydration (with saline) and antibiotics in the first 30 days after radiation, to survive ARS. Considerable morbidity was observed due to radiation pneumonitis around 40-80 days in these animals. All rats finally developed lethal radiation nephropathy before 160 days after exposure. These DEARE were mitigated by the drug lisinopril, started 7 days after radiation (Fish et al. 2016). Additionally, lisinopril successfully mitigated DEARE when used in the presence of the cytokine G-CSF

(Granulocyte-Colony Stimulating Factor) (Fish et al. 2016). Recently G-CSF has been approved by the FDA to treat acute hematopoietic toxicity (USFDA 2015) but there are no countermeasures in the strategic national stockpile to mitigate or treat DEARE. The present studies address the use of lisinopril as a mitigator of DEARE in special populations using juvenile and old rat models of radiation in order to evaluate DEARE. Female and male rats were irradiated at 42 days to represent a juvenile population. The Wistar strain of rats used under conditions in our facility, reach puberty after 65 days (unpublished). Puberty is described as the age at which reproduction first becomes possible (Sengupta 2013). From this it is estimated that the 42 day old female and male rats are at an equivalent age of 7-9 years in a human.

Older females were irradiated at an average age of 572 days at which time they were reproducibly senescent and were retired from breeding. This would correspond to ~51 years of age in humans. This group of older rats are therefore described as 'old females'.

Over 50% of male rats in our laboratory reached their normal life span by the end of ~ 2 years. Geriatric male rats were irradiated at a mean age of 568 days which would correspond to 80% of their lifespan. This age would be equivalent to ~64 years of age for an 80 year old life expectancy for human males when males in the USA are considered 'geriatric' (~65 years of age). Therefore we have developed juvenile and older/geriatric female and male rat models and tested mitigation of DEARE in each group.

MATERIALS AND METHODS

Animal care

Animal protocols were approved by Institutional Animal Care and Use Committees (IACUC) at the Medical College of Wisconsin. Rats were monitored daily and any deterioration in body condition was recorded. The IACUC criteria for euthanasia for moribund rats were followed under supervision of veterinarians and trained animal handlers.

Age and groups of special populations

Juvenile female and male WAG/RijCMCR rats were 42 days old (41-44 days old and a mean weight 102 grams) at the start of the study. Irradiated rats were randomized into 2 groups, (i) irradiation only group (n=13 females and 8 males) after 13 Gy leg-out partial body irradiation (leg-out PBI) and (ii) irradiation+lisinopril group (n=9 females and 7 males) given 13 Gy leg-out PBI and intervention with the drug lisinopril.

Old female (~ 274 gms) and male rats (~414 gms) were irradiated after they reached 483-651 days of age (mean age was 572 days for females and 568 days for males). Age-matched nonirradiated rats were included with these groups to identify the natural history of rodents in this age group. These reproductively senescent rats were retired breeders from the WAG/RijCmcr colony at MCW. Rats were not irradiated in organized batches as for juvenile animals, but were collected in small groups as they became available and randomized head-to-head into 3 groups; (i) nonirradiated (n=7 females and 9 males); (ii) irradiation only (n=11 females and 15 males) and; (iii) irradiation+lisinopril (n=7 females and 9 males).

One group of adult male rats (n=16) was irradiated between 84-90 days of age with 13 Gy leg-out PBI, for comparison to the young and old male rats.

Irradiations

WAG/RijCmcr female or male rats were irradiated without the use of anesthetics as previously described (Fish et al. 2016). Rats were immobilized in 3 sizes of plastic jigs to fit the different sizes of rats being studied in each experiment. Dosimetry was first performed by a Medical Physicist at midline in the medium sized jig. A Farmer-type ion chamber was used to measure the output in all 3 sizes of jigs at midline, using a wax phantom that filled each jig. Dosimetry was then done in the small and large jigs to calculate comparative dose rates to the medium sized jig. Animals were irradiated using a XRAD 320KV orthovoltage X-ray system as previously described (Fish et al. 2016) with a posterior to anterior beam. Briefly, the x-ray system was operated at 320 kVp and 13 mAs with a half value layer of 1.4 mm Cu. During the irradiation, one hind limb of each rat was carefully externalized from the field and shielded with a 0.25 inch lead block. The dose to this leg was ~2 Gy. In some old female and male rats, the head was also shielded in addition to one hind leg to deliver leg- and head-out partial body irradiation (PBI). For simplicity, all irradiated old rats will be described to receive 'PBI'.

Drug treatments

Irradiated rats were first randomized into two groups: (i) irradiation only (2) irradiation +lisinopril. After irradiation all rats were given Enrofloxacin (10 mg kg⁻¹day⁻¹) from days 1-14 in the drinking water and saline by subcutaneous injection from days 3-7 (40 ml kg⁻¹day⁻¹). The dose of lisinopril was ~24 mg m⁻² day⁻¹ starting from day 7 after irradiation and continuing until study termination. This dose is comparable to that approved for clinical use on a mg m⁻² day⁻¹ basis. Lisinopril was delivered in drinking water at a concentration of 40 mg liter⁻¹ (Fish et al. 2016).

Blood urea nitrogen (BUN) measurements

Previous published work has shown that rising BUN levels are a measure of renal function and correlate with histopathology for assessing radiation nephropathy (Moulder et al. 2011). To measure BUN, rats were anesthetized with 3-5% isoflurane and blood drawn via jugular vein bleeds conducted by a trained technician. The BUN was assayed from serum as described previously (Medhora et al. 2014) using a urease-nitroprusside colorimetric assay. BUN values were expressed as mg dL⁻¹ in serum and medians with 20-80% ranges were used for statistical analysis. Irradiated rats with BUN 120 mg dL⁻¹ were euthanized and given a value of 120 mg dL⁻¹ to account for attrition, since such rats were previously defined to have severe and irreversible renal damage (Moulder et al. 2011).

Necropsy

Morbid or terminal rats were euthanized by overdose with isoflurane. The abdominal and pleural cavities were opened and carefully examined by experienced technicians and all abnormalities were recorded. Major findings that contributed to lethality are listed in Tables 1-3.

Histology

Histology was performed on lungs and kidneys from a subset of randomly selected rats that were examined at termination or when identified as morbid. The left lung was harvested, inflated and fixed with formalin and embedded in paraffin. Whole mount lung sections (4 μm thick) were stained as described with anti-tryptase antibody (IMGEX catalogue #IMG-80250, 1:150, Gao et al. 2013), Masson's trichrome (Kma et al. 2012) or hematoxylin & eosin (H&E). Five randomly selected fields from different areas of trichrome-stained lung sections of old rats were scored. Vessel- and alveolar- wall thickness, as well as, numbers of foamy macrophages were scored as previously described (Medhora et al. 2014, Medhora et al. 2015). A composite score was obtained with equal representation given to each parameter (vessel-wall thickness, alveolar-wall thickness and number of foamy macrophages). The scores from irradiated morbid rats were compared to those from nonirradiated rats (controls). Scoring of rat lungs (morbid or nonirradiated controls) was done at the same time by two investigators (FG and JN) who were masked to the treatment groups. To measure fibrosis, four fields that did not include large vessels or airways were randomly selected from corresponding regions of each lung in a blinded manner. Total blue color (representing collagen) was quantitated by Image J software and normalized with the total red color (representing cells) in the field by one investigator (FG). The average value for blue:red color for each rat was used to compare collagen content in irradiated versus nonirradiated old rats. Histological scores were presented as percent control of nonirradiated rats.

The kidneys were cut into halves and immediately fixed in 10% buffered formalin and processed for paraffin embedding. Kidney sections were stained with Masson's trichrome, and the kidney injury assessed in coded samples by one investigator (AHK) as described earlier (Moulder et al. 1993, Sieber et al. 2009) and as follows: absence of renal cyst (0); presence of microscopic (1+); and macroscopic (2+) cysts. Glomerular sclerosis was assessed by studying 20 random glomeruli per slide as follows: 1-2 sclerosed glomeruli (1+); 3-4 sclerosed glomeruli (2+); or 5 or more sclerosed glomeruli (3+). Interstitial fibrosis was assessed on an increasing scale as none (0); scattered (1+); or diffuse (2+). Glomerular mesangiolysis was assessed as absent (0); variably present (1+); present in most glomeruli (2+); and present in all glomeruli (3+). These scores were then aggregated to get a composite histologic score which was expressed as percent control of nonirradiated rats.

Total and differential blood counts—Blood was harvested from the jugular vein from six juvenile (3 female and 3 male) and 6 older (2 females and 4 geriatric males) rats. Total red and white blood cell counts (RBC and WBC) and differential counts for WBC were conducted in Marshfield Laboratories, WI., a Clinical Laboratory Improvement Amendments (CLIA)-approved veterinary laboratory.

Statistical Analyses

Morbidity data are shown by Kaplan Meier plots and were tested for differences between groups by Peto-Peto Wilcoxon tests. BUN values are shown as medians and 20-80% ranges. Statistical differences between multiple groups for BUN were calculated by the Mann Whitney U tests between 2 groups with accounting for attrition (rats that were morbid were given BUN 120 mg dL^{-1}). T-tests were used for analyses of histology scores.

RESULTS

DEARE and mitigation in juvenile female rats

Juvenile female rats at 42 days of age (n=22, Table 1.1) were irradiated and a randomized group (n=9) was started on lisinopril from day 7 and continued (irradiation+lisinopril). The remaining rats (n=13) (irradiation only group) were housed together and both groups were followed head-to-head. Fig. 1.1 shows Kaplan Meier plots for morbidity that resulted from DEARE after 13 Gy leg-out PBI. The plots were censored for rats that were morbid from ARS before day 30 (~14% total from both groups) not shown in Fig. 1.1 since we are focusing on DEARE. But these are listed in Table 1.1.

Irradiated only rats (solid line) manifested two phases of injury. Morbid rats in the first phase from 30-60 days exhibited typical radiation pneumonitis with pleural effusion (see Table 1.1) at necropsy. The pleural effusions ranged from 5-8 ml/rat. Stained sections of lung are shown in Fig. 1.2. Irradiation resulted in congestion and substantial inflammation by infiltration of mast cells (stained brown). Mast cells are rarely present in lungs from nonirradiated rats (Fig. 1.2a) (Szabo et al. 2010). Only one morbid rat (at 60 days, Table 1.1) did not have pleural effusion but was emaciated with loss of body weight which is common in rats with severe radiation pneumonitis (Medhora et al. 2015).

In the second phase of lethal injury, from 100-114 days, irradiated only rats had high BUN levels ($> 120 \text{ mg dL}^{-1}$) that require euthanasia due to terminal radiation nephropathy (see Material and Methods). BUN values (medians with 20%-80% ranges) at 90 and 114 days, a measure of renal function, are shown in Fig. 1.3. All irradiated only rats were morbid by 114 days with kidneys that appeared pale, mottled and/or pitted and BUN $> 120 \text{ mg dL}^{-1}$ (values used for the 90-114 day time point in Fig. 1.3). Radiation-nephropathy was mitigated at 90 and 114 days by lisinopril ($p < 0.05$ vs irradiation+lisinopril for both time points).

In irradiated+lisinopril rats (dashed line), only one rat was euthanized at 57 days after radiation due to an injury in the right eye (Fig. 1.1, Table 1.1). There was no morbidity or gross organ damage or pleural effusion visible at necropsy in any rats in this group at 114 days. After all rats in the irradiated only group were morbid, surviving rats in the irradiation +lisinopril group were euthanized after measuring their BUN (Fig. 1.3, hatched bar). Lisinopril mitigated renal injury in irradiated rats. These results indicate that lisinopril enhanced survival and mitigated the lung and kidney injuries observed in the irradiation only group ($p = 0.0015$).

Nine percent of irradiated rats with ARS that were censored in Fig. 1.1, were morbid between 11-15 days (Table 1.1) corresponding to the time frame in which we have previously observed hematopoietic toxicity in our rats, implying that the bone marrow shielding was not 100% effective.

DEARE and mitigation in juvenile male rats

Juvenile males (n=15) at 42 days of age (Table 1.2) were irradiated and a randomized group (n=7) was started on lisinopril from day 7 and continued (irradiation+lisinopril), along with an irradiation only group (n=8). Fig. 2.1 shows Kaplan Meier plots for morbidity that

resulted from DEARE after 13 Gy leg-out PBI. The plots were censored for rats that were morbid from ARS before day 30 (1/15 total in both groups, not shown in Fig. 2.1 but listed in Table 1.2).

One irradiated only rat (solid line) became morbid at 62 days, with pleural effusion and abnormal lungs (Table 1.2). Six rats from this group developed lethal radiation nephropathy between 87-105 days, while one rat had severe weight loss and seizures but BUN at a non-lethal level. Stained sections of the lung from the morbid rat at 62 days is shown in Fig. 2.2. Images show inflammation by infiltration of mast cells (stained brown, Fig. 2.2b) during radiation pneumonitis and some alveolar wall thickening in the H&E stained section (see inset Figure 2.2d). Irradiated only rats had high BUN levels (median 120 mg dL⁻¹ by 90 and 114 days) that qualified for euthanasia (Fig. 2.3). Rats were morbid by 105 days with radiation nephropathy except one rat that had severe weight loss and seizures at 107 days (Table 1.2).

The irradiated+lisinopril rats (dashed line, Fig. 2.1) survived until termination at 114 days after all the irradiated only rats were morbid (Table 1.2). There was no gross organ damage or pleural effusion visible at necropsy in any rats in this group. All juvenile rats in the irradiation+lisinopril group had BUN<120 mg dL⁻¹ with the median (20%-80% ranges) of 46 (33-59) mg dL⁻¹ at 90 days and 54 (49-65) mg dL⁻¹ at 114 days. Taken together these results indicate that lisinopril enhanced survival and mitigated the injuries observed in the irradiation only group in juvenile male rats (Fig. 2.3).

One out of 15 irradiated juvenile male rats with exhibited ARS, and thus was censored at 11 days (see Table 1.2) in a time frame corresponding to bone marrow toxicity. This rat had been randomized to the irradiation+lisinopril group.

DEARE and mitigation in old female rats

A total of 18 old female rats (Table 2.1) between 483-651 days of age were irradiated with 13 Gy PBI, and n=7 of these were given lisinopril starting at day 7 and continued (irradiation+lisinopril). Seven additional rats served as nonirradiated controls to monitor health in normal old females, since we had no previous experience following rats this long (up to 730 days of age) i.e. 170 days after radiation. The results are plotted in the Kaplan Meier graph in Fig. 3.1. There was no difference in survival between the three groups.

These plots were censored for morbidity due to ARS by 30 days after radiation in a manner similar to juvenile rats. Irradiation only rats (solid black line) did not become morbid between 30-126 days and no rats having dyspnea were observed. This time covered the 39-80 days that corresponds to radiation pneumonitis in juvenile females (Fig. 1.1) and adult female rats (Zhang et al. 2008, Medhora et al. 2012, Fish et al. 2016). All irradiated, old, female rats developed lethal radiation nephropathy by 170 days (Fig. 3.1 and Table 2.1) as measured by BUN values (Fig. 3.2), except one rat, which was identified as moribund with bloat at 170 days (Table 2.1).

There was considerable ARS in this group of rats with only one hind limb shielded (i.e. no head shield, see Table 2.1), with 75% (3/4) of the irradiation only rats and 25% (1/4) of

irradiation+lisinopril rats becoming morbid at 10 days after exposure. To avoid bone marrow toxicity and in order to follow DEARE, the rats irradiated after these results, received additional shielding to the head to spare a larger volume of bone marrow (see Materials and Methods).

The earliest morbidity after 30 days in the irradiation+lisinopril rats (dashed line in Fig. 3.1) occurred at 122 days during anesthesia delivered for drawing blood to measure the BUN. This rat did not have lethal radiation nephropathy since the BUN was only 33 mg dL⁻¹. However, necropsy revealed >10 ml of pleural effusion around the lungs though the animal did not appear morbid before anesthesia (Table 2.1). A single morbidity at 168 days was caused by a tumor (Table 2.1). This rat also did not have a lethal BUN level. The lower BUN values from the irradiation+lisinopril group at 150 days (Fig. 3.2, hatched bar) as compared to irradiated only rats (solid bar) demonstrated mitigation of radiation nephropathy by lisinopril at 150 days after 13 Gy PBI (p=0.009).

Survival in nonirradiated old females was also plotted in Fig. 3.1 (grey line). These rats were censored at 150 days, about the time we began to observe morbidity in the irradiated only group, in order to harvest organs for histological comparison. Only one rat in this group was morbid before 150 days due to a severe lesion in the right uterine horn (visible on necropsy at 77 days).

The results of histology scoring of trichrome stained lung sections from rats in all 3 groups at 150-180 days are shown in Fig. 3.3. No severe lung injury was observed though there was some increase in blood vessel- (see black box and highlighted black inset in Fig. 3.3) and alveolar-wall thickness (green boxes), as well as increased numbers of foamy macrophages (red boxes) in the irradiated lungs. A composite score for these injuries only reached statistical significance in the radiation+lisinopril group (p 0.05), when compared to the nonirradiated controls (Fig. 3.3d). There was no difference in the histological score between the irradiation only versus irradiation+lisinopril groups. The relative areas with blue color in trichrome stained lung sections (representing collagen) were higher in irradiated only rats but not in the lisinopril group, though again, there was no statistical difference in collagen (blue) area between the two irradiated groups (Fig. 3.3e). These results show mild lung injury in irradiated rats which is consistent with the survival plots (Fig. 3.1) and absence of morbidity at the time corresponding to lethal radiation pneumonitis in younger females (~42-80 days after 13 Gy leg-out PBI).

The results of histology scoring of trichrome stained kidney sections from rats in all three groups at 150-180 days are shown in Fig. 3.4 a-c. The irradiated only rats demonstrate marked kidney injury with the presence of microscopic cysts, interstitial fibrosis, tubular proteinaceous casts and glomerular injury in the cortical and medullary regions (Figs. 3.4 b). A composite score for these injuries reached statistical significance in the irradiation only group as compared to nonirradiated controls (Fig. 3.4d). Histology injury scores were higher in the irradiation only kidneys versus those in the nonirradiated (p<0.002) and irradiation +lisinopril groups (p<0.002). Lisinopril markedly mitigated tubular (structural derangement and proteinaceous cast formation) and glomerular injury (sclerosis and mesangiolysis) in the

renal cortex and medulla (Fig.3.4 c). These results indicate marked mitigation of radiation nephropathy by lisinopril in older female rats.

Effect of age on DEARE in female rats

Comparison of the survival plots for DEARE after 13 Gy PBI in juvenile and old rats are shown together in Fig. 4. In order to focus on late effects to the lungs and kidneys only, all morbidity due to ARS prior to 30 days were censored. To enhance the comparison we added a plot representing young adult females (~84 days old at radiation as compared to 42 days for juvenile females and 483 days for old females) using historical results from a similar irradiation model [13 Gy leg-out PBI, reported in previous studies (Fish et al. 2016)]. The results show that all three groups respond differently ($p < 0.0001$ for young vs adult females, $p = 0.0010$ for young vs old females; $p = 0.0153$ for adult versus old females) and the older rats were less susceptible to radiation pneumonitis between days 39-80 (Fig. 4). Female rats of all ages that survived radiation pneumonitis developed lethal radiation nephropathy. Both phases of injury, pneumonitis and nephropathy appeared to develop later after irradiation in older animals. Old females were seen to be resistant to lethal pneumonitis from 13 Gy PBI.

DEARE and mitigation in geriatric male rats

Like the old females, retired male breeders were collected in small groups as they became available. A total of 24 geriatric male rats (Table 2.2) were irradiated with 13 Gy PBI, and $n=9$ of these were given lisinopril starting at day 7 and continued (irradiation+lisinopril). Nine additional rats served as nonirradiated controls. The results are plotted in Fig. 5.1. No rats were morbid from radiation pneumonitis through 80 days in this group (Fig. 5.1 and Table 2.2). The first morbidity occurred at 61 days in the irradiated only group and was caused by a tumor. Two rats died from anesthesia around 120 days (Table 2.2). These rats did not reveal any gross organ injuries at necropsy. Rats were censored at ~180 days at which time two irradiated only rats had radiation nephropathy with lethal BUN values. Additionally one rat had an enlarged heart and fibrotic lungs, while the remaining >30% (excluding censored ARS rats) appeared healthy (Fig. 5.1).

Irradiated geriatric males also developed ARS (Table 2.2). Total morbidity in this group without head shields before 30 days was 33% (3/9) with an additional two rats dying after delivery of anesthesia for hydration and not by ARS. The heads were shielded in rats irradiated after these results became available. One rat developed ARS at 7 days in spite of head shielding at a time corresponding to GI toxicity (Table 2.2).

In the irradiation+lisinopril group (dashed line, Fig. 5.1), two rats developed dyspnea at 69 and 83 days after irradiation (Table 2.2). No pleural effusions were observed on necropsy, though the lungs of two rats appeared fibrotic. None of the irradiated, geriatric, male rats given lisinopril developed lethal radiation nephropathy at termination at 180 days at which time ~57% of this group did not develop morbidity from DEARE (Fig. 5.1). The BUN levels in these rats (hatched bar, Fig. 5.2) indicated mitigation of radiation nephropathy by lisinopril ($p = 0.05$). Comparison of BUNs between old female (Figure 3.2) and male (Figure 5.2) rats in the irradiation only group showed decreased sensitivity of the males to radiation nephropathy at three time points: 90, 120 and 150 days ($p < 0.05$ and $n=5-8$ rats/group at each

time point, data not shown). However, there was no difference in BUNs between geriatric males versus females in irradiation+lisinopril groups at the same time points (data not shown), confirming that lisinopril mitigated radiation nephropathy in old rats from both sexes. Geriatric irradiated males treated with lisinopril (33%) also developed ARS between 10-12 days after 13 Gy leg-out PBI without head shielding (see Table 2.2). There were no differences in survival between the three groups, nonirradiated, irradiation only and irradiation+lisinopril geriatric males.

Nonirradiated, geriatric males (n=9, Fig. 5.1, grey line) were not morbid up to 42 days, at which time one animal was euthanized with a bloody pericardial effusion. At later times rats were morbid with fibrous lungs (n=2), abdominal infection (n=1), tumor (n=1) and seizures/tremors (n=1, Table 2.2). The remaining animals were censored at 180 days similar to the irradiated only group.

Total and differential blood cells counts were taken from nonirradiated juvenile and older rats to evaluate hematological and/or immune competence. The results are shown in Table 3. RBC ($p<0.001$) were increased while WBC ($p=0.006$) and lymphocyte ($p=0.003$) counts were lower in the old rats as compared to the young rats.

The histology results of lungs from rats in all three geriatric male groups at >173 days after irradiation are shown in Fig. 5.3. Although there were increases in vessel- (black box) and alveolar-wall (green box) thickness and macrophage infiltration (red box) in trichrome stained irradiated rat lung sections, these scores only reached statistical significance in the irradiation+lisinopril group when compared to the nonirradiated controls (Fig. 5.3 d, $p=0.05$). There was no difference between the irradiation only lungs and irradiated+lisinopril lungs for this end point. The relative area with collagen (blue color) in trichrome stained sections was higher in irradiation only geriatric males as compared to nonirradiated controls ($p=0.05$) but not in the irradiation+lisinopril geriatric rats, though again there was no difference in blue staining between the two irradiated groups. These results support the survival studies indicating mild lung injury with absence of lethal radiation pneumonitis in irradiated, geriatric males.

The results of histology scoring of trichrome stained kidney sections from geriatric male rats in all three groups at termination are shown in Fig. 5.4 a-c. The irradiated only rats demonstrated marked kidney injury with the presence of microscopic cyst, interstitial fibrosis, tubular proteinaceous casts and glomerular injury both in the cortical and medullary regions (Figs. 5.4 b). A composite score for these injuries reached statistical significance in the irradiation only group as compared to nonirradiated controls ($p<0.005$) or irradiated +lisinopril ($p<0.005$) (Fig. 5.4 d) groups. Similar to old female rats, these studies demonstrated that lisinopril mitigated tubular (structural derangement and proteinaceous cast formation) and glomerular injuries (sclerosis and mesangiolysis) in the renal cortex and medulla of irradiated, male, geriatric rats (Figs. 5.4 a-d).

Effect of age on DEARE in male rats

Comparison of the survival plots for DEARE after 13 Gy PBI in juvenile and old male rats are shown together in Fig. 6. In order to focus on late effects to the lungs and kidneys only,

the X-axis was plotted from 30-180 days after 13 Gy PBI. To enhance the comparison, we added a plot representing young adult males (84-90 days old at radiation, as compared to 42 days for juvenile males and 483 days for geriatric males) using a similar irradiation model (13 Gy leg-out PBI). The results show clear differences between survival of juvenile and geriatric males ($p=0.0036$), though older males did not appear to have different sensitivities or delay in manifestation of radiation pneumonitis between days 39-80 (Fig. 6) than the juvenile males. For juvenile vs adult males the p value was <0.0490 with $p=0.1004$ for adult vs geriatric males. Male rats of all ages that survived radiation pneumonitis developed lethal radiation nephropathy, though the frequency was lower than 100% up to 180 days in the geriatric group. In general, juvenile and old males had low frequencies of lethal radiation-pneumonitis after 13 Gy PBI.

DISCUSSION

We have examined the late effects of radiation on multiple organs in juvenile and old rats. The juvenile rats were irradiated before puberty while the old rats were reproductively senescent at the time of irradiation. Morbidity due to acute hematopoietic or gastrointestinal injuries were minimized by bone marrow sparing, hydration and antibiotics and any deaths occurring during the acute phase of injury to these organs were censored, to focus on delayed effects only. To our knowledge this is the first report of DEARE in multiple organs in these special populations in any species. The results show two classical phases of DEARE (lethal radiation-pneumonitis and nephropathy) in the juvenile rats (Figs 1 and 2). Radiation pneumonitis was confirmed by necropsy of morbid rats, revealing lung injury and pleural effusion between 39-60 days after exposure. Radiation nephropathy was observed after 90 days in juveniles and morbidity was confirmed by lethal BUN levels (Fig. 1.3 and 2.3). Both phases of DEARE in juvenile rats were mitigated by lisinopril (Figs. 1.1 and 2.1).

Unlike in the juvenile rats, there was little lethal radiation pneumonitis after 13 Gy PBI in old rats. Lisinopril did not mitigate the mild lung injury determined by histo-pathology at 150-180 days. One reason could be that these lungs were examined at a time corresponding to *recovery* from pneumonitis (as seen in younger rats, Zhang et al. 2008, Kma et al. 2012) and not during the peak of pneumonitis, so any mitigating effect of lisinopril during the period of injury would be missed. In fact, the Kaplan-Maier plots between nonirradiated, irradiated only and irradiated+lisinopril groups in old rats were not different for DEARE (Figs. 3.1 and 5.1) though Kaplan Meier plots between juvenile irradiated rats and those given lisinopril, were different from each other (Figs. 1.1 and 2.1). We believe this is due to lower severity of delayed injuries in the old rats by the same dose of radiation as previously suggested (Moulder and Fish 1997). The presence of more actively dividing cells in the organs of the growing population of juvenile rats could make them more sensitive to DNA damage induced by radiation in different organs.

Studies using higher doses of radiation to the lungs and kidneys only (whole thorax irradiation/isolated kidney irradiation) may be a better models than PBI with minimal bone marrow shielding to determine the threshold for lethal radiation pneumonitis and nephropathy in old rats. Models of localized irradiation to the lung are very relevant to cancer therapy. Depending on the volume of lung irradiated and the mean radiation dose to

the lung, cancer patients can respond with pneumonitis, but more importantly they also proceed to develop pulmonary fibrosis. Unlike the radiation to the whole volume of the lung of the rats, much higher doses than 13 Gy are used for radiotherapy, which is delivered to small volumes of lung without inducing lethality during pneumonitis in cancer patients. However, the localized high doses consistently induce fibrosis in the radiation field, months to years later. The 13 Gy PBI irradiation only group in our studies receive a fraction of the doses used in radiotherapy for lung cancer and do not survive long enough to manifest pulmonary fibrosis. Old rats and elderly cancer patients surely have a threshold sensitivity to radiation fibrosis in their lungs, even though old rats appear less sensitive to radiation pneumonitis than the juvenile rats in this study. Additionally, GI injury by doses 13 Gy PBI should be avoided in a thorax only irradiation model, since the gut will be shielded allowing sufficient animals to survive acute GI injury. Sufficient bone marrow will be spared to enable high rates of survival past ARS, while the kidney will not be in the field to induce lethal radiation nephropathy.

Radiation nephropathy was observed in all age groups of rats after 13 Gy PBI though earlier in the juvenile rats. BUN levels were higher in old females than in age-matched males at the same times after irradiation and lethal radiation nephropathy appeared earlier in juvenile females. Previously published reports indicate lethal radiation nephropathy in rats with doses as low as 7.8 Gy (Moulder et al. 2011). Though radiation nephropathy develops later than ARS and pneumonitis, it is induced by lower doses of radiation than acute GI or lung toxicities. In fact, the threshold for renal injury may be lower than 6 Gy in humans (Moulder 2014) making the kidney one of the most sensitive organs to radiation. In non-human primates, delayed radiation nephropathy developed by 100 days after 10-11 Gy (Cohen et al. 2017). This model also displayed acute kidney injury before 50 days after exposure, though BUN levels were never higher than 60 mg dL⁻¹. Mice (C57Bl/6) took 21 months to exhibit BUN up to 69 mg dL⁻¹ after 8.5 and 9.7 Gy total body irradiation (Unthank et al. 2015). Lisinopril, which mitigated radiation injury in all populations of rats tested in this study, also improved outcomes from lung and kidney injury in humans after radiation for hematopoietic stem cell transplant or lung cancer (Jenkins and Watts 2011, Jenkins and Welsh 2011, Cohen et al. 2012, Kharofa et al. 2012, Harder et al. 2015). Such information will be valuable to the FDA for approval of lisinopril via the Animal Rule.

These studies observed high incidences of ARS after 13 Gy PBI in old female and geriatric male rats. To enable sufficient numbers of animals to survive to express DEARE, additional shielding of the head along with one hind leg during irradiation was needed. Defects in the function of the hematopoietic stem cells (HSC) with age, particularly myeloid skewing with increased incidence of myeloid malignancies (Rossi et al. 2008) and osteoporosis are known. While a great deal of studies have documented decline in HSC function from intrinsic mechanisms primarily associated with accumulated genomic damage and DNA repair (Rossi et al. 2005, Rossi et al. 2007, Rossi et al. 2008, Warren and Rossi 2009) it is now also appreciated that alteration in HSC function can be the consequence of age-associated changes in the marrow microenvironment niches, (Rossi et al. 2008, Wagner et al. 2008, Van Zant and Liang 2012, Wagers 2012), although mechanisms are largely unknown. We observed high RBC counts but lower WBC counts in older rats as compared to the juvenile animals. Higher RBC counts in old rats has been documented previously (Glass and Gershon

1981). It is possible that a compromised immune system may protect geriatric rats from DEARE since infiltration of bone marrow-derived cells causes radiation pneumonitis. More detailed studies evaluating the hematopoietic potential, immune system and marrow distribution are needed to understand the causes of increased ARS and decreased DEARE in geriatric populations of rats.

One limitation of these studies is the lack of data on cardiac injury after radiation. The ACE inhibitor captopril attenuated radiation-induced cardiopulmonary damage when the heart was present in the field of radiation (van der Veen et al. 2015). It is becoming clear that lethal radiation pneumonitis involves cross-talk between irradiated lung and hearts (Medhora et al. 2015) and that pneumonitis is often a combined injury (Ghobadi et al. 2012, Ghobadi et al. 2012, Medhora et al. 2015, van der Veen et al. 2016). Observation of mild abnormalities such as small changes in heart size and mild pericardial effusions in some juvenile and old rats during necropsy are not listed in Tables 1&2 since they were not considered to be a major cause of morbidity. Studies to conduct systematic analyses by echocardiography and detailed structural and histological changes of the heart in juvenile and geriatric rats to better inform multiple organ injury from DEARE are needed. A 'Note' describing cardiac injury in adult female rats is included in this issue (Jacobs et al. Submitted 2018).

Our studies did not closely follow prolonged GI syndrome in rats as has been described in mice (Booth et al. 2012) or NHP models of DEARE (Shea-Donohue et al. 2016). However, unlike acute GI toxicity during ARS, our studies do not consider delayed GI injury to be a major cause of lethal DEARE after 13 Gy PBI in these rats. Muscle wasting was also not followed in these rats but described as a DEARE in NHPs (Cui et al. 2016). A drawback of a rat model is the absence of nausea and vomiting in rodents, which prevents assessment of the prodromal syndrome of ARS in humans (Williams et al. 2010, Singh et al. 2015). The rats in this study were not given personalized medical management as was provided to irradiated NHP (Garofalo et al. 2014, Unthank et al. 2015, Cohen et al. 2017). Individual medical management will likely be available to humans after a radiological attack or accident (Dorr et al. 2014). Lastly, the studies were conducted in a laboratory setting including only a single strain of rats, WAG/RijCmcr. This design will not account for effects of experimental factors, including, genetic diversity in response to radiation that has been observed in mice (Jackson et al. 2010, Jackson et al. 2011) and must be anticipated in humans.

In summary, these studies have described lethal DEARE after 13 Gy PBI with minimal bone marrow shielding in juvenile and old rats. Juvenile females are morbid from DEARE in multiple organs by 114 days after 13 Gy, if supportive care is provided and sufficient bone marrow is spared to mitigate acute GI and hematopoietic toxicities respectively. At 13 Gy, old female and geriatric male rats do not develop lethal radiation pneumonitis by 80-90 days after exposure, but may be more sensitive to ARS in the first 30 days as compared to juvenile rats. Lisinopril mitigates radiation pneumonitis in juvenile females, radiation nephropathy in juvenile females and males and radiation nephropathy in old rats of both sexes. Geriatric male rats develop less severe radiation nephropathy than age-matched females up to 150 days after radiation. In conclusion these studies provide insight into

differences in sensitivity to DEARE in special populations of rats, before and after their reproductive life spans and mitigation by lisinopril.

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1.2

1.3

Fig. 1.

1.1: DEARE and mitigation after 13 Gy leg-out PBI in juvenile female (42 days old) rats: Kaplan-Meier plots for time to morbidity after 13 Gy partial body irradiation with one leg shielded (13 Gy leg-out PBI, n=11 after censoring rats morbid from ARS, solid line) and 13 Gy leg-out PBI+lisinopril ($\sim 24 \text{ mg m}^{-2} \text{ d}^{-1}$, n=8 after censoring for ARS, dashed line) starting on day 7 and continuing are shown. All irradiated rats were given supportive care with subcutaneous hydration ($40 \text{ ml kg}^{-1} \text{ d}^{-1}$) and enrofloxacin ($10 \text{ mg kg}^{-1} \text{ d}^{-1}$) from days 1-14 and 3-7 respectively. The graph depicts two phases of injury, pneumonitis and nephropathy (grey shaded areas). All irradiated only rats were morbid by 114 days, at which time 88% of irradiated rats given lisinopril were alive ($p=0.0015$).

1.2: Lung injury during pneumonitis: Histological staining of a representative lung section at low magnification from a nonirradiated, young female rat at 80 days (historical control from a different study, panels a and c) and an irradiated juvenile female (b and d) that was morbid during radiation pneumonitis shows consolidation in alveolar spaces and heavy infiltration of mast cells, typical during radiation pneumonitis in WAG/Rij rats (Szabo et al. 2010). Mast cells (stained brown in a and b) were identified with anti-tryptase antibody and seen clearly in the magnified inset of the boxed area in b. The bar in (a) represents 100 μm .

Panels c and d were stained with hematoxylin and eosin. The boxed areas are magnified in the inset to highlight changes in alveolar wall thickness and cellular infiltrates in the lung, after radiation.

1.3: Mitigation of radiation nephropathy in juvenile females by lisinopril ($\sim 24 \text{ mg m}^{-2} \text{ d}^{-1}$). The graph shows medians and 20-80% ranges for blood urea nitrogen (BUN in mg dL^{-1} , on a log scale on Y-axis) in rats at 90 and 114 days after 13 Gy leg-out PBI. Black bars represent irradiated only rats and hatched bars represent irradiation+lisinopril rats. Asterisks (*) represent $p < 0.05$ as compared to irradiation only groups at the corresponding time. The normal BUN for nonirradiated rats ranges between 18-21 mg dL^{-1} . To account for attrition, rats with BUN $> 120 \text{ mg dL}^{-1}$ were euthanized and given a BUN of 120 mg dL^{-1} at the next time point. Numbers in the bars represent N in each group.

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2.3

Fig. 2.

2.1: DEARE and mitigation after 13 Gy leg-out PBI in juvenile male (42 days old) rats: Kaplan-Meier plots for time to morbidity after 13 Gy partial body irradiation with one leg shielded (13 Gy leg-out PBI, n=8, solid line) and 13 Gy leg-out PBI+lisinopril (~24 mg m⁻² d⁻¹, n=6 after censoring one rat for ARS, dashed line) starting on day 7 and continuing are shown. All irradiated rats were given supportive care with subcutaneous hydration (40 ml kg⁻¹d⁻¹) and enrofloxacin (10 mg kg⁻¹d⁻¹) from days 1-14 and 3-7 respectively. The graph depicts two phases of injury, pneumonitis and nephropathy (grey shaded areas). All

irradiated only rats were morbid before 114 days, at which time 100% of irradiated rats given lisinopril were alive ($p < 0.05$).

2.2: Lung injury during pneumonitis: Histological staining of a representative lung section from a nonirradiated male rat (a and c), and an irradiated (young) male rat that was morbid during radiation pneumonitis (b and d) shows infiltration of mast cells (stained brown, see magnified in inset in b), typical during radiation pneumonitis in WAG/Rij rats (Szabo et al. 2010). Mast cells were identified with anti-tryptase antibody in a and b. The bar in a represents 100 μm . Panels c and d were stained with hematoxylin and eosin. The boxed areas are magnified in the inset to highlight changes in alveolar wall thickness and cellular infiltrates in the lung, after radiation.

2.3: Mitigation of radiation nephropathy in young males by lisinopril ($\sim 24 \text{ mg m}^{-2} \text{ d}^{-1}$). The graph shows medians and 20-80% ranges for blood urea nitrogen (BUN in mg dL^{-1}) in rats at 90 and before 114 days after 13 Gy leg-out PBI. Black bars represent irradiated only rats and hatched bars represent irradiation+lisinopril rats. Asterisks (*) represent $p < 0.05$ as compared to irradiation only groups at the corresponding time. The normal BUN for nonirradiated rats ranges between 18-21 mg dL^{-1} . To account for attrition, rats with BUN 120 mg dL^{-1} were euthanized and given a BUN of 120 mg dL^{-1} at the next time point. Numbers in the bars represent N in each group.

3.1

3.2

3.3

3.3

3.4

3.4

d

Fig. 3.

3.1: DEARE and mitigation after 13 Gy PBI in older female (483-651 days old) rats:

Kaplan-Meier plots for time to morbidity after irradiation [n=6 after censoring for ARS (Table 2.1), solid black line] and 13 Gy PBI+lisinopril ($\sim 24 \text{ mg m}^{-2} \text{ d}^{-1}$, n=6 after censoring for ARS, dashed line) starting on day 7 and continuing and nonirradiated controls (n=6, after censoring for ARS, solid grey line) are shown. Rats in all three groups were given supportive care with subcutaneous hydration ($40 \text{ ml kg}^{-1} \text{ d}^{-1}$) and enrofloxacin ($10 \text{ mg kg}^{-1} \text{ d}^{-1}$) from days 1-14 and 3-7 respectively. There was no lethal radiation pneumonitis and no difference in survival between the three groups. The plots were censored for morbidity before 30 days and after 170 days.

3.2: Mitigation of radiation nephropathy in old females by lisinopril ($\sim 24 \text{ mg m}^{-2} \text{ d}^{-1}$). The graph shows medians and 20-80% ranges for blood urea nitrogen (BUN in mg dL^{-1}) in rats at approximately 150 days after 13 Gy PBI. Black bar represents irradiation only rats and hatched bars represent irradiation+lisinopril rats. Asterisk (*) represent $p=0.009$ as compared to the irradiation only group. The normal BUN for age-matched, nonirradiated rats ranges between $15\text{-}18 \text{ mg dL}^{-1}$ (horizontal grey bar). To account for attrition, rats with BUN $\geq 120 \text{ mg dL}^{-1}$ at or before 150 days were euthanized and given a BUN of 120 mg dL^{-1} . Numbers in the bars represent N in each group.

3.3: Minimal lung injury at 150 days in old female rats irradiated with 13 Gy PBI. Trichrome staining of representative lung sections from nonirradiated (a); 13 Gy PBI only (b) and; 13 Gy PBI+lisinopril (c) old female rats at 150-180 days (past the time corresponding to radiation pneumonitis by similar doses of irradiation in juvenile females). There is a modest and variable increase in thickness of some vessel walls (black boxes) and alveolar walls (green boxes) as well as in the number of infiltrating macrophages (red boxes) in b and c though the alveolar spaces remain largely open. Mild fibrosis was seen after radiation by the increase in blue color in (b) and (c) as compared to nonirradiated lung (a). The bar in (a) represents $100 \mu\text{m}$.

Graphical representation of the results from composite histology scores of lungs from the three groups of geriatric rats are shown in (d), while blue staining quantitation is shown in (e). Both are expressed as mean percent control $\pm$ SEM. Grey bars represent nonirradiated females ($n=3$); black bars for irradiation only ($n=4$) and; hatched bars for irradiated +lisinopril ($n=3$) groups respectively. * represents $p < 0.05$ versus nonirradiated rats.

3.4: Kidney injury and mitigation by lisinopril in old female rats irradiated with 13 Gy PBI and comparison with nonirradiated controls. Representative photomicrographs of kidney cortex (upper panel) and medulla (lower panel) from nonirradiated (a); 13 Gy PBI (b); 13 Gy PBI+lisinopril (c) groups are shown. Colored arrows show normal or damaged glomeruli (black), tubules (yellow) and tubular casts (red). The bar in (a) represents $100 \mu\text{m}$. Note damaged glomeruli and tubules with increased number and size of casts in (b).

Graphical representation of the results from composite histology scoring of kidneys from the three groups of geriatric rats are shown in (d). Mean percent control scores $\pm$ SEM are represented by grey bars for nonirradiated old females ($n=3$); black bars for irradiation only ($n=4$) and; hatched bars for irradiation+lisinopril ($n=3$) groups respectively. *represent $p < 0.002$ versus nonirradiated rats, # represents $p < 0.002$ versus 13 Gy PBI rats. Note damage by radiation and mitigation of renal injury by lisinopril in old female kidneys.

Fig. 4.

DEARE after 13 Gy PBI in female rats irradiated at different ages: In order to focus on late effects to the lungs and kidneys only, all morbidity due to ARS prior to 30 days were censored. Kaplan-Meier plots for time to morbidity between 30-180 days after irradiation and supportive care, consisting of subcutaneous hydration ($40 \text{ ml kg}^{-1} \text{ d}^{-1}$) and enrofloxacin ($10 \text{ mg kg}^{-1} \text{ d}^{-1}$). Two phases of injury, pneumonitis due to lung morbidity and nephropathy by renal morbidity are shaded in grey. Note morbidity by lethal radiation pneumonitis in juvenile and adult rats but not in old females. All three groups exhibited radiation nephropathy. $p < 0.0001$ for juvenile vs adult rats; $p = 0.0010$ for juvenile versus old rats; $p = 0.0153$ for adult ($n = 15$) versus old rats. Latency for lethal lung and kidney injury (DEARE) increase with age at radiation.

5.1

5.2

5.3

5.3

5.4

5.4

Fig. 5.

5.1: DEARE and mitigation after 13 Gy PBI in geriatric male rats: Rats that were morbid from ARS prior to 30 days have been censored. Kaplan-Meier plots for time to morbidity by DEARE after 13 Gy PBI (n=9, solid black line) and 13 Gy PBI+lisinopril ($\sim 24 \text{ mg m}^{-2} \text{ d}^{-1}$, n=7) starting on day 7 and continuing (dashed line) and nonirradiated controls (n=9, solid grey line) are shown. Rats in all three groups were given supportive care with subcutaneous hydration ($40 \text{ ml kg}^{-1} \text{ d}^{-1}$) and enrofloxacin ($10 \text{ mg kg}^{-1} \text{ d}^{-1}$) from days 1-14 and 3-7 respectively. There was no difference in survival between the groups. The plots were censored for morbidity before 30 days and after 180 days. Note the high incidence of morbidity in nonirradiated (control) geriatric males.

5.2: Mitigation of radiation nephropathy in geriatric males by lisinopril ($\sim 24 \text{ mg m}^{-2} \text{ d}^{-1}$). The graph shows medians and 20-80% for blood urea nitrogen (BUN in mg dL^{-1} , on a log scale on Y-axis) in rats at approximately 180 days after 13 Gy PBI. Black bars represent irradiation only rats and hatched bars represent irradiation+lisinopril-treated rats. Asterisk (*) represents p 0.05 as compared to irradiation only rats. The normal BUN for age-matched,

nonirradiated rats ranges between 17-19 mg dL⁻¹ (horizontal grey bar). To account for attrition, rats with BUN 120 mg dL⁻¹ were euthanized and given a BUN of 120 mg dL⁻¹ at 180 days. Numbers in the bars represent N in each group.

5.3: Modest lung injury in geriatric male rats irradiated with 13 Gy PBI. Histology of trichrome stained representative lung sections is shown from nonirradiated (a); 13 Gy PBI (b); 13 Gy PBI+lisinopril (c) geriatric male rats at >170 days. There is modest increase in thickness of some vessel walls (black box) and alveolar walls (green box), as well as, in the number of infiltrating macrophages (red box), though the alveolar spaces are open. Mild fibrosis is seen after 13 Gy PBI, by the increase in blue color in (b) and (c) as compared to a nonirradiated lung in (a). The bar in (a) represents 100 µm.

Graphical representation of the results from composite histology scoring of lungs from the three groups of geriatric rats is shown in (d), while fibrosis (area stained blue) is shown in (e). Both are expressed as mean percent control±SEM. Grey bars represent nonirradiated, geriatric males (n=3); black bars for irradiation only (n=4); and hatched bars for irradiation +lisinopril (n=4) males respectively. * represents p 0.05 versus nonirradiated rats.

5.4: Histology of kidneys in geriatric controls as well as male rats irradiated with 13 Gy PBI with and without lisinopril. Representative photomicrographs of kidney cortex (upper panel) and medulla (lower panel) from nonirradiated (a); 13 Gy PBI (b); and 13 Gy PBI+lisinopril (c) rats is shown. Colored arrows point to normal or damaged glomeruli (black), tubules (yellow) and tubular casts (red). The bar in (a) represents 100 µm. Note glomerular injury, tubular casts and interstitial fibrosis (blue color) in (b) indicating renal injury.

Graphical representation of the results from composite histology scoring of kidneys from the 3 groups of geriatric, male rats are shown in (d). Mean percent control scores+SEM are represented by grey bars for nonirradiated males (n=3); black bars for irradiation only (n=4); and hatched bars for irradiation+lisinopril (n=4) groups respectively. * represents p<0.005 versus nonirradiated rats, # represents p<0.005 versus 13 Gy PBI rats. Note mitigation of renal injury in geriatric male kidneys by lisinopril.

Fig. 6.

DEARE after 13 Gy PBI in male rats irradiated at different ages: Rats that were morbid from ARS prior to 30 days have been censored. Kaplan-Meier plots for time to morbidity between 30-180 days after irradiation with supportive care consisting of subcutaneous hydration ($40 \text{ ml kg}^{-1} \text{ d}^{-1}$) and enrofloxacin ($10 \text{ mg kg}^{-1} \text{ d}^{-1}$). Two phases of injury, pneumonitis due to lung morbidity and nephropathy by renal morbidity are shaded in grey. $P=0.0036$ for juvenile versus geriatric male rats, $p=0.0490$ for young versus adult male rats and $p=0.1004$ for adult versus geriatric male rats. Note the short latency for lethal kidney injury in young rats after radiation as compared to their geriatric counterparts.

Table 1

Juvenile female (1.1) and male (1.2) necropsy results. The table shows the cause of morbidity as determined by necropsy or BUN levels in morbid rats that were irradiated at 42 days of age (juveniles) with 13 Gy leg-out PBI. Rats that were not morbid are marked as 'Term' when the study was terminated (after all irradiated only rats were morbid). Rats with acute radiation syndrome (ARS) that occurred before 30 days after irradiation were considered to have bone marrow (BM) toxicity since the time of morbidity corresponding to bone marrow injury after recovery from gastrointestinal injury that occurs before 9-10 days.

1.1:

Radiation	Lisinopril	Days in study	N	Major Findings
13 Gy leg-out PBI	No	11	1	ARS (BM)
13 Gy leg-out PBI	No	15	1	ARS (BM)
13 Gy leg-out PBI	No	39-58	6	Lung/effusion
13 Gy leg-out PBI	No	60	1	>20 % weight loss
13 Gy leg-out PBI	No	106	1	Kidney (BUN>120 mg dL ⁻¹)
13 Gy leg-out PBI	No	114	3	Kidney (BUN>120 mg dL ⁻¹)
13 Gy leg-out PBI	Yes	15	1	ARS (BM)
13 Gy leg-out PBI	Yes	57	1	Eye injury
13 Gy leg-out PBI	Yes	120	7	Term

1.2:

Radiation	Lisinopril	Days in study	N	Major findings
13 Gy leg -out PBI	No	62	1	Lung/effusion/fibrotic
13 Gy leg -out PBI	No	87-105	6	Kidney (BUN>120 mg dL ⁻¹)
13 Gy leg -out PBI	No	107	1	Seizures, weight loss
13 Gy leg -out PBI	Yes	11	1	ARS (BM)
13 Gy leg -out PBI	Yes	114	6	Term

BUN=blood urea nitrogen in mg dL⁻¹.

Table 2

2.1: Older female necropsy results. The table shows the cause of morbidity as determined by necropsy or BUN levels in morbid, old female rats that were irradiated with 13 Gy PBI. Rats that were not morbid are marked as 'Term' when the study was terminated or censored. Rats with acute radiation syndrome (ARS) that occurred before 30 days after irradiation were considered to have bone marrow (BM) toxicity since the time of morbidity corresponding to bone marrow injury after recovery from gastrointestinal injury that occurs before 9-10 days.

Radiation	Lisinopril	Head Shield	Days in study	N	Major findings
0	No	No	3	1	Alveolar edema, hemorrhage
0	No	No	77	1	Prolapsed uterus
0	No	No	150	5	Term
13 Gy PBI	No	No	3	1	Aspiration of food
13 Gy PBI	No	Yes	10	1	Effusion, fibrotic lung
13 Gy PBI	No	No	10 to 13	3	ARS (BM)
13 Gy PBI	No	Yes	126	1	Kidney (BUN>120 mg dL ⁻¹)
13 Gy PBI	No	No	150	1	Kidney (BUN>120 mg dL ⁻¹)
13 Gy PBI	No	Yes	165	2	Kidney (BUN>120 mg dL ⁻¹)
13 Gy PBI	No	Yes	170	2	Bloat, fibrous lungs (n=1)
13 Gy PBI	Yes	No	10	1	Kidney (BUN>120 mg dL ⁻¹) (n=1)
13 Gy PBI	Yes	Yes	122	1	ARS (BM)
13 Gy PBI	Yes	Yes	168	1	Effusion, fibrotic lung
13 Gy PBI	Yes	No	170	3	Mammary tumor
13 Gy PBI	Yes	Yes	170	1	Term
13 Gy PBI	Yes	Yes	170	1	Term

2.2: Geriatric male necropsy results. The table shows the cause of morbidity as determined by necropsy or BUN levels in geriatric male rats that were irradiated with 13 Gy PBI. Rats that were not morbid are marked as 'Term' when the study was terminated or censored. Rats with acute radiation syndrome (ARS) that occurred before 30 days after irradiation were considered to have bone marrow (BM) toxicity at 10 days or gastrointestinal (GI) injury that occurs before 9-10 days.

Radiation	Lisinopril	Head Shield	Days in study	N	Major findings
0	No	No	42	1	Pericardial effusion, fibrous lungs
0	No	No	115	1	Severe weight loss, fibrous lungs
0	No	No	133	2	Testicular tumor (n=1)
0	No	No	166	1	Seizures, tremors (n=1)
0	No	No			Abdominal infection

Radiation	Lisinopril	Head Shield	Days in study	N	Major findings
0	No	No	173	1	Enlarged heart, fibrous lungs
0	No	No	180	3	Term
13 Gy PBI	No	No	4	2	Anesthesia death
13 Gy PBI	No	Yes	7	1	ARS (GI)
13 Gy PBI	No	No	11 to 14	3	ARS (BM)
13 Gy PBI	No	Yes	61	1	Tumor
13 Gy PBI	No	No	119 & 122	2	Anesthesia deaths
13 Gy PBI	No	Yes	180	1	Enlarged heart, fibrotic lungs
13 Gy PBI	No	No	180	1	Kidney (BUN>120 mg dL ⁻¹)
13 Gy PBI	No	Yes	180	1	Kidney (BUN>120 mg dL ⁻¹)
13 Gy PBI	No	No	180	3	Term
13 Gy PBI	Yes	No	10 & 12	2	ARS (BM)
13 Gy PBI	Yes	Yes	69	1	Enlarged heart, fibrous lungs
13 Gy PBI	Yes	No	83	1	Fibrous lungs, enlarged esophagus
13 Gy PBI	Yes	Yes	180	1	Enlarged heart, fibrous lungs
13 Gy PBI	Yes	No	180	3	Term
13 Gy PBI	Yes	Yes	180	1	Term

BUN=blood urea nitrogen in mg dL⁻¹.

Table 3

Total & differential blood cell counts in nonirradiated juvenile and old rats. Total & differential blood cell counts in juvenile and old rats show that hemoglobins were elevated, but total WBC counts and lymphocytes were lower in old rats (mixed gender) relative to that of their juvenile counterparts.

Groups (N)	RBC 10 ⁶ /ml	WBC 10 ³ /ml	Lymphocytes 10 ³ /ml	Neutrophils 10 ³ /ml	Monocytes 10 ³ /ml
Young (3 female, 3 male)	5.778±0.292	6.250±0.468	5.185±0.815	0.995±0.444	0.030±0.050
Old (2 female, 4 male)	8.420±0.761 *	5.367±0.423 #	3.703±0.476 \$	1.377±0.363	0.110±0.080

* p (young vs old)=0.0000126,

p=0.00643,

\$ p=0.00323.