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Captopril Increases Survival after Whole-Body Ionizing Irradiation but Decreases Survival when Combined with Skin-Burn Trauma in Mice

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Past and recent radiation events have involved a high incidence of radiation combined injury where victims often succumb to serious infections as a consequence of bacterial translocation and subsequent sepsis. The risk of infection is exacerbated in radiation combined skin-burn injury (RCI), which increase vulnerability. Furthermore, no suitable countermeasures for radiation combined skin-burn injury have been established. In this study, we evaluated captopril as a potential countermeasure to radiation combined skin-burn injury. Captopril is an FDA-approved angiotensin-converting enzyme inhibitor that was previously reported to stimulate hematopoietic recovery after exposure to ionizing radiation. Female B6D2F1/J mice were whole-body bilateral ^{60}Co gamma-photon irradiated (dose rate of 0.4 Gy/min) with 9.5 Gy ($LD_{70/30}$ for RCI), followed by nonlethal dorsal skin-burn injury under anesthesia (approximately 15% total-body surface-area burn). Mice were provided with acidified drinking water with or without dissolved captopril (0.55 g/l) for 30 days immediately after injury and were administered topical gentamicin (0.1% cream; day 1–10) and oral levofloxacin (90–100 mg/kg; day 3–16). Surviving mice were euthanized on day 30 after analyses of water consumption, body weight and survival. Our data demonstrate that, while treatment with captopril did mitigate mortality induced by radiation injury (RI) alone (55% captopril vs. 80% vehicle; $n = 20$, $P < 0.05$), it also resulted in decreased survival after radiation combined skin-burn injury (22% captopril vs. 41% vehicle; $n = 22$, $P < 0.05$). Moreover, captopril administration via drinking water produced an uneven dosage pattern among the different injury groups ranging from 74 ± 5.4 to 115 ± 2.2 mg/kg/day. Captopril treatment also did not counteract the negative alterations in hematology, splenocytes or bone marrow cellularity after either radiation injury or radiation combined skin-burn injury. These data suggest that captopril may exert its actions differently between the two injury models (RI vs. RCI) and that captopril dosing, when combined with topical and systemic antibiotic treatments, may not be a suitable countermeasure for RCI.

INTRODUCTION

The widespread use of radioisotopes in medicine increases the dissemination of radioactive materials and patient exposures. The fact that more than 50% of cancer patients receive radiotherapy at some point during the course of their treatment represents another significant source of exposure (*1*). Of great concern is the possibility that terrorist groups could use nuclear or radiological weapons to inflict mass casualties that would include simultaneous wound, burn, blast traumas and radiation injuries. Several studies have modeled many exposure scenarios with a focus on the effects of radiation exposure alone. However, a more complete model scenario should take into account that radiation injury (RI) will occur in the presence of other injuries particularly when considering terrorist incidents

Radiation exposure combined with many other types of injuries, ranging from blast trauma to infection, often results in a negative synergistic response more harmful than the sum of the individual injuries. However, some studies have shown that this is not always the case (*2–4*). There has recently been a growing appreciation of the practical consequences of radiation combined injury (RCI) as well as an understanding that the body's response to radiation combined injuries may be different from the responses to radiation or physical injury alone (*5–7*). It is known that nearly 60–70% of the casualties after a nuclear detonation sustain combined injuries involving burns and/or wounds in addition to radiation exposure, significantly increasing their risk of morbidity and mortality (*6, 7*). Animal studies clearly demonstrate that burns (*8–10*) and wounds (*7–11*) exacerbate acute radiation syndrome. Data from humans studies similarly demonstrate that burns and wounds complicate the morbidity and mortality of individuals exposed to ionizing radiation (*6, 12*). Moreover, radiation combined injuries contribute to increased susceptibility to

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infection and higher mortality compared to radiation injury alone (*6*). The mechanisms of these interactions are not fully understood. Furthermore no evidence-based guidelines exist for rehabilitation or recovery of individuals with such injuries.

Captopril is mainly used to treat hypertension, congestive heart failure and renal failure. More interestingly, captopril has been reported to mitigate the effects of total-body irradiation by reducing mortality and stimulating hematopoietic recovery in mice studies when administered in drinking water (*13, 14*). In this study, we evaluated captopril, an FDA-approved angiotensin-converting enzyme (ACE) inhibitor as a potential countermeasure to radiation combined skin-burn injury.

MATERIALS AND METHODS

Animals

Female B6D2F1/J mice (Jackson Laboratory, Bar Harbor, ME) 12 to 16 weeks of age were maintained in an animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) in plastic microisolator cages with hardwood chip bedding. Commercial rodent chow and acidified potable water were provided *ad libitum*. Animal holding rooms were maintained at 20–26°C with 30–70% relative humidity using at least 10 changes per hour of 100% conditioned fresh air. A 12 h light-to-dark (06:00–18:00) full-spectrum lighting cycle was maintained in the holding room. Mice were assigned to one of four injury groups: sham-injury ($n = 20$); skin-burn injury ($n = 20$); radiation injury ($n = 20$); and radiation combined skin-burn injury ($n = 22$), each treated with either vehicle, captopril or no treatment. Only vehicle and captopril treated animal groups received antimicrobial treatment. Captopril was administered via drinking water at a concentration of 0.55 g/l. The Armed Forces Radiobiology Research Institute (AFRRI) Institutional Animal Care and Use Committee (IACUC) reviewed and approved all animal procedures involved in this study.

End points for the animal survival study were determined when there was death from the sequelae of combined injury or euthanasia for moribund mice or survivors after mitigation of the sequelae of radiation injury and combined injury. To minimize animal suffering, pain or distress, moribund mice were considered to have arrived at the study end point. Such animals were euthanized by CO_2 inhalation. Mice that survived for more than 30 days after exposure were also euthanized using CO_2 inhalation. Animals were scored twice daily throughout the survival study using an IACUC-approved Rodent Intervention Score Sheet to assess their well-being and clinical status. To minimize suffering of the animals the analgesics acetaminophen and buprenorphine were administered when appropriate and procedures conducted under methoxyflurane anesthesia to reduce pain and distress. All study end points were approved by the IACUC. Less than 5% of the study animals died without humane euthanasia as a result of acute radiation syndrome (ARS), which has rapid onset and is difficult to predict and characterize. Since survival, time to death and systemic biomarker collection were used as screening tools in this study and the goal of this project was to evaluate the efficacy of antimicrobial agents together with drug therapies to mitigate ARS and to promote survival from radiation combined injury, alternative study end points were not judged to be appropriate.

Gamma Irradiation

Mice were exposed to 9.5 Gy ($LD_{70/30}$ for RCI) whole-body bilateral ^{60}Co gamma-photon radiation (*8*), delivered at a dose rate of 0.4 Gy/min, while held in vertically stacked, ventilated, four-compartment, acrylic plastic boxes that provided electron equilibrium during irradiation. Empty compartments within the boxes were filled with 7.5×2.5 cm acrylic phantoms to ensure uniform electron scattering. The mapping of the radiation field was performed with alanine/EPR dosimetry (*15*). The mapping provided dose rates to water within the core of the acrylic phantoms in each compartment of the mouse rack on that specific day. The field was uniform within $\pm 1.8\%$ over all of the 120 compartments. Dose rate calibration with alanine was traceable to the National Institute of Standards and Technology (NIST) and the National Physics Laboratory of the United Kingdom. Sham-irradiated mice were placed in the same acrylic restrainers, taken to the radiation facility and restrained for the time required for irradiation.

Skin-Burn Injury

Skin surface burn injuries were performed on the shaved dorsal surface of mice. Animals receiving skin burns were anesthetized by methoxyflurane inhalation. A 15% total-body surface-area skin burn was performed within 1 h of irradiation using a 2.5×2.5 cm custom-designed metal template positioned centrally over the shaved dorsal skin surface. Mice received a 12 s burn from ignited 95% ethanol (0.5 ml) (*9*). All mice subjected to the skin-burn injury and their controls were administered 0.5 ml sterile 0.9% saline intraperitoneally (i.p.), which contained analgesics, acetaminophen (150 mg/kg, Cadence Pharmaceuticals, San Diego, CA) and buprenorphine (0.05 mg/kg), immediately after skin-burn injury to alleviate pain. Four hours after injury mice were given a second dose of acetaminophen (150 mg/kg, i.p.). Additional analgesic doses were considered during the 30-day study duration.

Antimicrobial Treatment

For vehicle and captopril-treated groups, gentamicin sulfate 0.1% cream (Perrigo®, Bronx, NY) was applied topically each day for 10 days to the skin-burn injury from day 1 to 10. Levofloxacin oral solution (Hi-Tech Pharmacal Co. Inc., Amityville, NY) at a dose of 100 mg/kg in 0.2 ml was administered orally (p.o.) each day for 14 days from day 3 to 16.

Survival Monitoring and Measurements of Body Weight and Water Consumption

Animals were monitored at least twice daily for their general health and survival for 30 days. Body weights were measured on day 0, 1, 3, 7, 14, 21 and 28. Water consumption levels were assessed from day 1 to 7. On day 30, all surviving mice were anesthetized by isoflurane inhalation. Blood samples were collected by cardiac puncture for hematological analysis, and after cervical dislocation, spleens and bone marrows from femurs were collected.

Hematological, Spleen and Bone Marrow Analysis

Blood samples were collected in EDTA KE/1.3 tubes (Sarstedt, Newton, NC) and assessed with the ADVIA 2120 Hematology System (Siemens Industry Inc., Hoffman Estates, IL). Complete blood cell differential analysis was conducted using the peroxidase method and light scattering techniques as recommended by the manufacturer. Spleens were collected from each euthanized mouse and weighed. Bone marrow cells from femurs were harvested and washed with 3 ml 1X phosphate buffered saline (PBS). The cells were then centrifuged at 800*g* for 6 min, resuspended in 3 ml 1X PBS and then counted using an automated hemocytometer.

Data Analysis

Data are expressed as mean ± SEM. Data sets were analyzed by one-way ANOVA with a Bonferroni correction for multiple comparisons. A P value of ≤ 0.05 was considered significant.

TABLE 1
Mean Captopril Dose per Day per Animal Weight over 30-Day Survival for Different Injury Groups

Injury groups	Mean captopril dose (mg/kg/day)
Sham	101 ± 2.3
Burn	115 ± 2.2*
RI	74 ± 5.4*
RCI	106 ± 5.1**

Notes. Four different groups of female B6D2F1/J mice were studied: Sham-injury; Burn (skin-burn injury alone); RI (whole-body ionizing radiation injury alone); and RCI (radiation combined skin-burn injury). Data are expressed as mean ± SEM; *$P < 0.05$ compared to other groups; **$P < 0.05$ compared to the RI group.

RESULTS

Captopril Drug Dosing

The dose of captopril in the current study was based on previous studies where successful mitigation of the effects of radiation injury in mice were reported (*13, 14, 16*). Since captopril was administered via drinking water, the mean dose per mouse over the 30-day survival study was dependent upon mean water consumption. Using this approach, calculated values (Table 1) showed that an uneven dosage pattern among the different injury groups existed. Mice in the skin-burn injury group received the highest average daily dose (115 ± 2.2 mg/kg/day, $P < 0.05$) while mice in the RI group received the lowest average daily dose (74 ± 5.4 mg/kg/day, $P < 0.05$). Furthermore, mice in the RCI group received a significantly greater average daily dose of captopril (+43%) compared to RI mice. The maximum captopril dose used in adult humans for clinical purposes is approximately 6 mg/kg/day in divided doses, which is much lower than the doses reported in our study above (*17*).

Survival, Water Consumption and Body Weight Analysis

We conducted 7-day water consumption analysis and 28-day mean body weight analysis in our study. These data demonstrated that captopril treatment in drinking water did not significantly improve overall volume of water consumption or improve overall body weight loss after whole-body irradiation alone (Fig. 1B and E) or when combined with skin-burn injury (Fig. 1C and F). Indeed, captopril treatment produced lower daily water consumption for RI mice (Fig. 1B). As expected, both the radiation injury and combined radiation injury groups exhibited lower daily water consumption during the initial phases (day 1–4) of the study and lower body weights during the later phases (day 14–28) of the study (Fig. 1A and D).

As expected, the skin-burn injury did not result in mortality over the 30-day observation period, however, the radiation combined skin-burn injury decreased survival to 47%, which was higher than survival observed in RI mice (30%) (Fig. 2A). In RI + vehicle-treated mice, survival decreased to 20% [Fig. 2D (4th column from the left)], significantly lower than both sham-injury and skin-burn injury groups (Fig. 2B). Treatment with captopril, however, significantly increased 30-day survival to 45% [Fig. 2D (8th column from the left); $P < 0.05$ vs. RI + vehicle]. In RCI mice, vehicle treatment decreased survival to 41% [Fig. 2C and D (5th column from the left)] and captopril treatment significantly decreased survival further to 22% at day 30 (Fig. 2D (9th column from the left); $P < 0.05$).

Hematological Analysis

Blood collected from surviving mice after the 30-day survival period was subjected to complete blood cell differential analysis. These data showed that both RI and

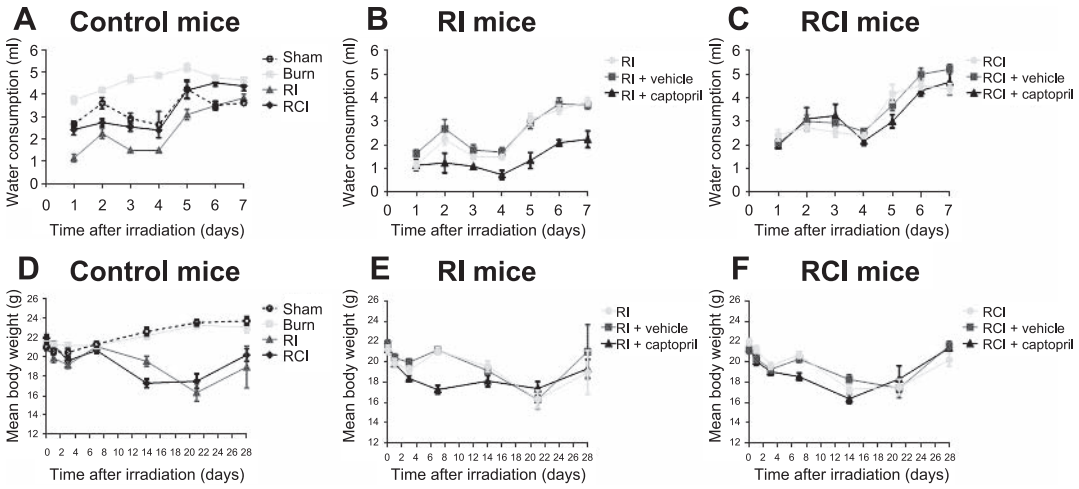

FIG. 1. Captopril treatment in drinking water (0.55 g/l) did not improve volume of 7-day water consumption (panels A–C) or 28-day weight loss (panels D–F) after whole-body ionizing irradiation alone (RI) or when combined with skin-burn injury (RCI) in female B6D2F1/J mice. N = 20–22 per group. Vehicle and captopril groups received additional topical gentamicin (0.1% cream; day 1–10) and oral levofloxacin (90–100 mg/kg; day 3–16) treatment. Data are expressed as mean ± SEM.

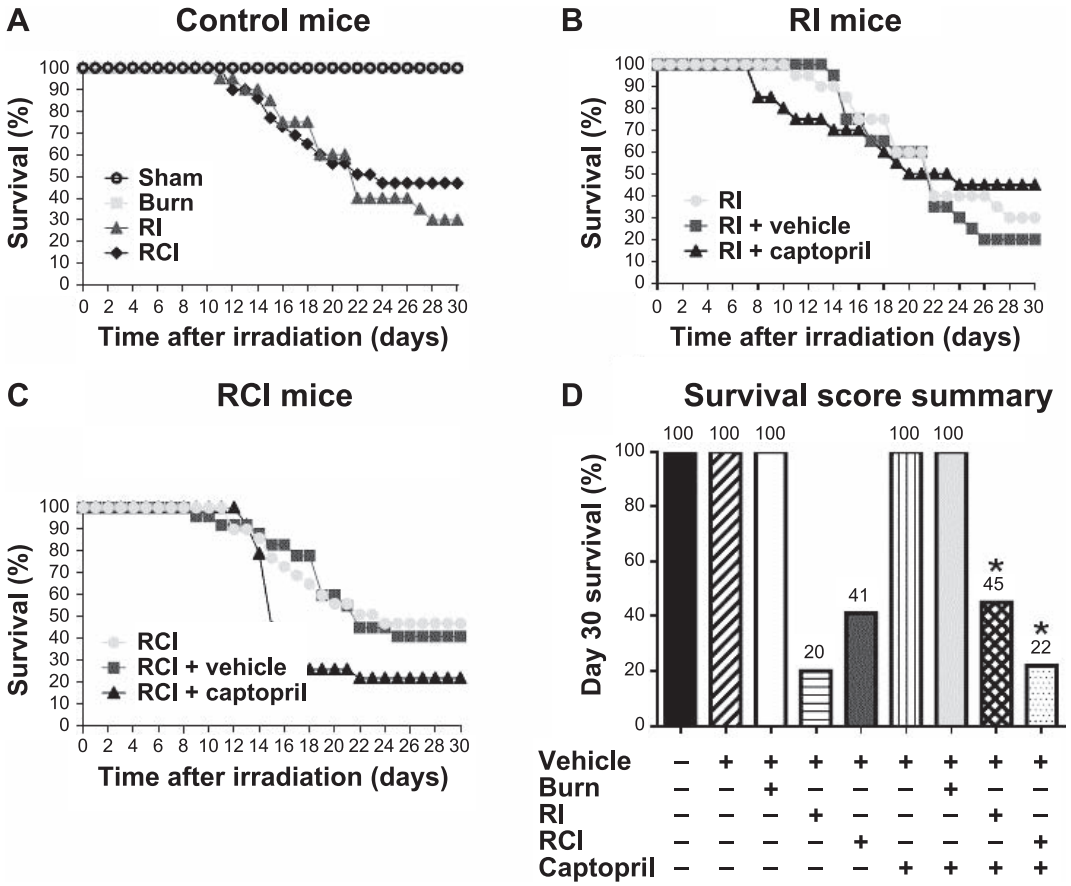

FIG. 2. Captopril treatment in drinking water (0.55 g/l) improved 30-day survival after whole-body ionizing irradiation alone (RI) but not when combined with skin-burn injury (RCI) in female B6D2F1/J mice (panel D). Survival analysis for control mice (panel A), RI mice (panel B) and RCI mice (panel C). N = 20–22 per group. Vehicle and captopril groups received additional topical gentamicin (0.1% cream; day 1–10) and oral levofloxacin (90–100 mg/kg; day 3–16) treatment. Data are expressed as mean values; $*P < 0.05$ compared to vehicle treatment only.

RCI mice had significantly decreased numbers of white blood cells (WBC), red blood cells (RBC) and platelets compared to sham-injury mice (Fig. 3). Skin-burn injury alone did not affect WBC, RBC or platelet counts compared to sham-injury counts (Fig. 3). WBC depletion observed in the radiation injury and radiation combined injury groups was mainly due to diminished lymphocyte counts (Fig. 3D). Captopril treatment did not significantly alter the WBC, RBC and platelets counts in sham-injury or skin-burn injury groups compared to controls. Furthermore, captopril treatment did not counteract the WBC, RBC and platelet count decreases observed in RI and RCI mice compared to controls (Fig. 3), suggesting that captopril did not stimulate hematopoietic recovery for RI and RCI mice by day 30.

Spleen and Bone Marrow Analysis

In surviving mice, spleen and bone marrow were evaluated after euthanasia. Spleen weight in both RI and RCI mice was significantly increased (splenomegaly) compared to sham-injury mice (Fig. 4A). In contrast, RI and RCI mice had significantly lower bone marrow cell counts compared to sham-injury mice (Fig. 4B). In skin-burn injury mice, no overall significant differences in spleen weight or bone marrow cell counts were observed compared to sham-injury mice (Fig. 4). Moreover, captopril treatment failed to improve bone marrow cell counts and mitigate splenomegaly in both RI and RCI mice (Fig. 4).

DISCUSSION

In the midst of a pressing need to develop reliable countermeasures for RI and RCI, we evaluated the drug captopril as a possible candidate for use after radiation combined skin-burn injury. Our study showed that radiation combined skin-burn injury significantly increased mortality and diminished hematopoietic supporting factors in a fashion similar to that of radiation injury alone. However, captopril administration via drinking water significantly increased mortality in the RCI groups, and significantly decreased mortality in the RI groups without stimulating hematopoietic recovery. The improvement in survival is in agreement with previously reported observations in irradiated C57 black female mice (*13, 14, 16*). These findings

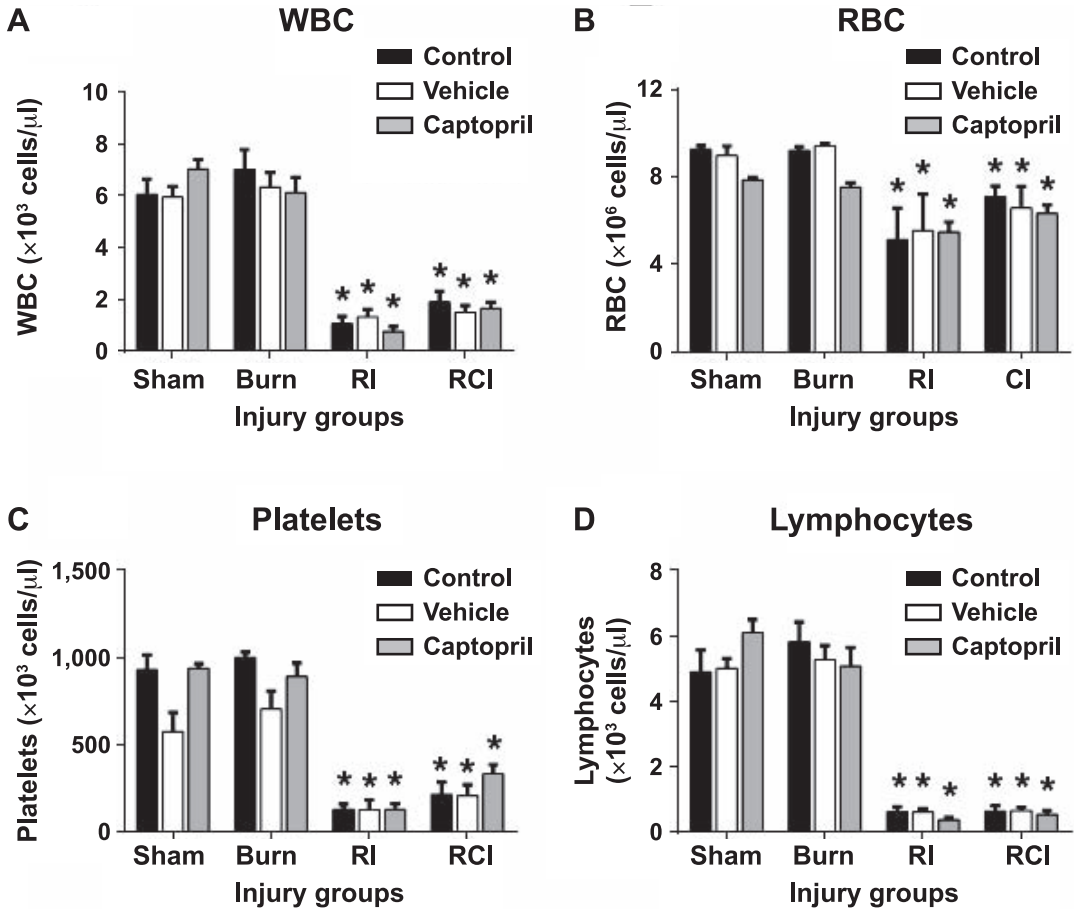

FIG. 3. Captopril treatment in drinking water (0.55 g/l) did not improve white blood cells (WBC) (panel A), red blood cells (RBC) (panel B), platelets (panel C) or lymphocyte depletion (panel D) after whole-body ionizing irradiation alone (RI) or when combined with skin-burn injury (RCI) in female B6D2F1/J mice. N = 4–8 per group. Vehicle and captopril groups received additional topical gentamicin (0.1% cream; day 1–10) and oral levofloxacin (90–100 mg/kg; day 3–16) treatment. Data are expressed as mean ± SEM; *$P < 0.05$ compared to sham-injury and skin-burn injury groups only.

suggest that captopril treatment may exert its actions in a different manner between the two injury models (RI vs. RCI) and that in combination with topical and systemic antibiotic treatments, captopril may not be a suitable countermeasure for whole-body radiation exposure combined specifically with skin-burn trauma. The detailed mechanisms that contribute to the pathophysiology of both radiation injury and combined injury remain to be determined.

Captopril is an ACE inhibitor, which causes decreased activation of the vasoconstrictor angiotensin II and decreased inactivation of the vasodilator bradykinin, bringing about benefits in hypertension and congestive heart failure (*18*). ACE inhibitors have been used extensively for treatment of breast cancer, leukemia and lymphoma to prevent cardiac complications associated with chemotherapy regimens (*19*). Captopril has been demonstrated to modify a variety of late-effect radiation-induced tissue injuries, including kidney, lung, skin and heart (*20*). In mice, studies have shown captopril administration to improve survival for RI with possible hematopoietic recovery (*13, 14, 16*). In contrast, other studies have shown that captopril used in a rat model, administered in drinking water at a dose of 0.30 g/l for 7 days prior to total-body irradiation, produced detrimental hematological effects (*21*). This suggested that irradiated rats and mice have different responses to the drug captopril. It remains unclear, however, whether mitigation of radiation injury in mice is a result of ACE inhibition or indirect regulation of other cytokines or factors. The non-ACE inhibitor actions of captopril may explain some of the findings in our study and would therefore merit further investigation in future studies. Furthermore, relatively few studies are available which have assessed the effect of captopril treatment on combined injury. One study, using a rat model of multiple-site combined radiation injuries (lung and skin), showed captopril treatment to specifically improve recovery from radiation-induced skin dermatitis (*2*). However, in our combined injury model, captopril administration significantly increased mortality, suggesting that captopril may be inappropriate for mitigating the effects of whole-body irradiation combined with skin-burn

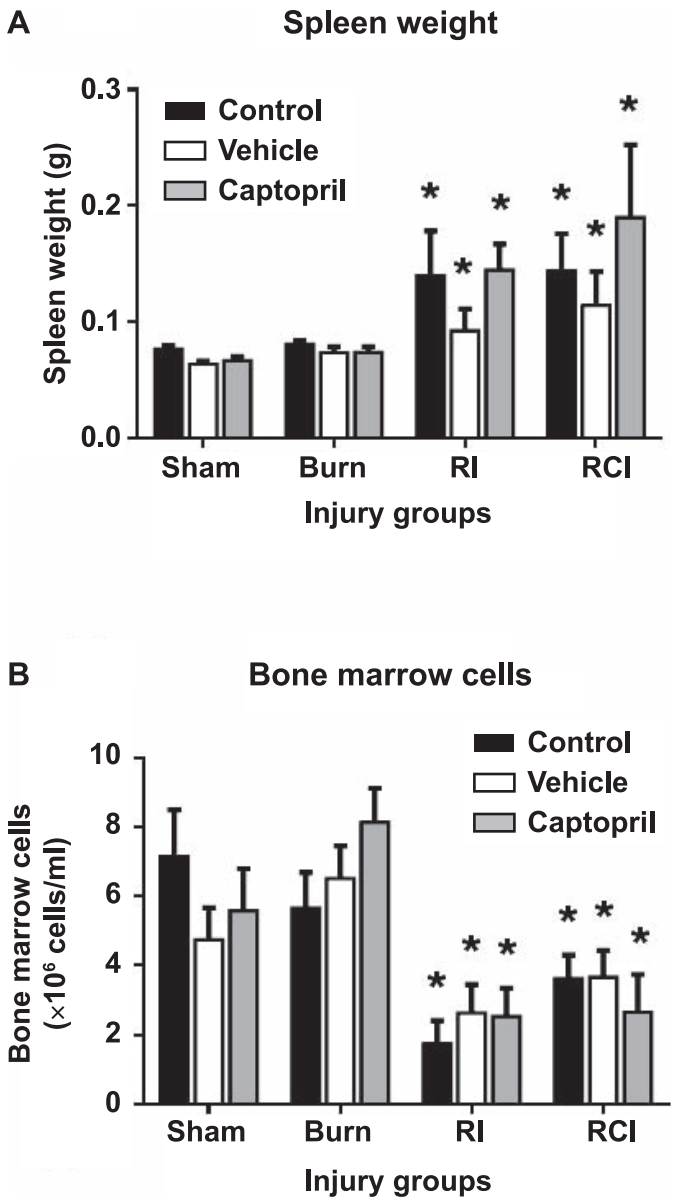

FIG. 4. Captopril treatment in drinking water (0.55 g/l) did not improve spleen weight (panel A) or bone marrow cell depletion (panel B) after whole-body ionizing irradiation alone (RI) or when combined with skin-burn injury (RCI) in female B6D2F1/J mice. N = 4–8 per group. Vehicle and captopril groups received additional topical gentamicin (0.1% cream; day 1–10) and oral levofloxacin (90–100 mg/kg; day 3–16) treatment. Data are expressed as mean ± SEM; **P* < 0.05 compared to sham-injury and skin-burn injury groups only.

injury. Moreover, our findings could highlight a difference between the mechanisms responsible for radiation injury versus combined injury. Another more plausible explanation for this difference in survival outcome could be the fact that as the amount of captopril administered via drinking water directly correlated with overall water consumption, mice in the CI group received a significantly higher mean daily captopril dose compared to mice in the RI group (Table 1). This showed an uneven dosage pattern among the different injury groups, suggesting that the lower average daily dose (74 ± 5.4 mg/kg/day) may be optimal for the reduced mortality observed in the RI group and such a consequence requires further investigation. Skin-burn injury has been shown to produce significant transepidermal water loss leading to increased thirst and water consumption (*22*), a point that also needs to be addressed for future drug dosing in RCBI experiments.

Complicating the pathology of the healing mechanisms associated with radiation injury is the extent of an additional injury on the body surface, such as a wound or burn (*23*). It has been demonstrated that total-body irradiation followed by wounding will reduce acute immune responses (*10*). This has been noted by decreases in inflammatory cells and impaired cellular functions (*23*). It has also been demonstrated that exposure to radiation will affect the healing remodeling process by inhibiting cellular proliferation. This alteration can result in a reduction in the number and function of vascular endothelial cells, fibroblasts and a delay in re-epithelialization (*11, 24*). In the acute phase of ARS, skin healing is impaired or prevented resulting in chronic wounds (*24*). It has been well documented in animal injury models with burns or wounds that mortality increases after exposure to nonlethal doses of radiation (*10, 25–27*). Animals subjected to radiation exposure and wounding exhibited increased susceptibility to infection (*7*), delayed skin healing (*11*) and increased mortality (*11, 27–29*). When comparing the effects of RCI models with RI models, combined injury generally demonstrates increased risk of infection (*7*), contributing to a higher incidence of morbidity and mortality. These differences are due to the impairment of the immune system from exposure to radiation (*30, 31*). Ironically, the wounding of mice 24 h prior to exposure to radiation actually improved 30-day survival compared to animals exposed to radiation alone (*10, 32*). This phenomenon is believed to be due to an increase in clonogenic myeloid elements (*26*). Further research is necessary to differentiate and understand fully the detailed processes contributing to animal injury models for both radiation injury and combined injury.

In summary, we have demonstrated that captopril treatment administered in drinking water after injury reduced mortality in mice with radiation injury, but adversely increased mortality in mice with radiation combined skin-burn injury. Whether this difference is a result of differences between the mechanisms contributing to radiation injury and combined injury cannot be determined by the current study. However, the findings from this study of an animal model of radiation exposure combined with skin-burn trauma suggest that the drug captopril in combination with topical and systemic antibiotic treatments may not be a suitable countermeasure against RCBI. Further work will be required to assess the effect of different captopril concentrations and other ACE inhibitors on mouse survival for the different injury models and to investigate differences among the mechanisms responsible for the injury models (RI vs. RCBI) used in this study.

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