

## 2-0, 3-0 Desulfated Heparin does not Affect Radiation Injury Induced Mortality but Reduces Radiation Combined Skin-burn Injury Induced Survival in Mice

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### Abstract

Many radiation events have involved a high incidence of radiation combined injuries. Victims of radiation events succumb to serious infections as a consequence of bacterial translocation and sepsis. Exacerbation of the risk of infection by radiation combined burn injury (RCBI) further heightens vulnerability. There are currently no suitable countermeasures that exist for RCBIs. We evaluated 2-0, 3-0 desulfated heparin (ODSH), an anti-inflammatory and anticoagulant agent as a potential countermeasure to RCBI. Female B6D2F1/J mice (12-week) were subjected to 9.5 Gy (LD70/30 for RCBI) whole-body bilateral <sup>60</sup>Co gamma-photon radiation (0.4 Gy/min), followed by dorsal skin burn injury under anesthesia (~15% total-body-surface area burn). Mice were injected subcutaneously with ODSH (25 mg/kg every 12 h; days 1-2 and 17.5 mg/kg every 12 h; days 3-7) or vehicle (sterile saline of equal volume) for 7 days post-injury and further administered topical gentamicin (0.1% cream; days 1-10) and oral levofloxacin (100 mg/kg; days 3-16). Mice were euthanized on day 30 following water consumption, body mass and survival analysis. Our data showed ODSH had no effect on radiation injury (RI)-induced mortality (45% ODSH vs. 45% VEH; n=20). However interestingly, ODSH treatment significantly reduced survival after RCBI (12% ODSH vs. 41% VEH; n=22, p<0.05). Furthermore, ODSH did not affect water consumption or body mass accrual after RI or RCBI. ODSH was not able to counteract the negative alterations in hematology, splenocytes, or bone marrow cell counts after RI or RCBI. These data illustrate that ODSH in combination with antibiotic treatments, may not be a mitigating countermeasure for RCBI.

**Keywords:** Combined injury; Countermeasure; Hematology; ODSH; Radiation injury; Survival study

### Abbreviations:

ACE: Angiotensin Converting Enzyme; CI: Combined Injury; LD: Lethal Dose; ODSH: 2-0, 3-0 Desulfated Heparin; RCBI: Radiation Combined Skin-burn injury; RI: Radiation Injury; TBI: Total-Body Irradiation; VEH - Vehicle

### Introduction

Radioisotope use in medicine has increased the dissemination of radioactive materials and patient exposures. The majority of cancer patients receive radiotherapy at some point during the course of their disease, which can result in a significant exposure [1]. Most concerning is the possible threat of terrorist groups using nuclear or radiological weapons for inflicting mass casualties, which would include almost simultaneous wound, burn, blast, and radiation injuries. Responses to such threats have mainly focused efforts on radiation exposure effects alone. This supposition may be unrealistic especially when making the assumption that radiation injury will occur in the absence of other injuries, particularly when considering terrorist incidents and natural disaster events such as Fukushima. Radiation exposure combined with many kinds of other injuries, ranging from blast trauma to infection, often results in a negative synergistic response more harmful than the sum of the individual

injuries. Only recently has there been an appreciation of the practical consequences of radiation combined injury, as well as an understanding that the body's response to radiation combined trauma may be different from the responses to radiation or physical injury alone [2-4]. Nearly 60-70% of the casualties after a nuclear detonation sustain a combined injury, burns and/or wounds in addition to radiation exposure, significantly increasing their risk of morbidity and mortality [5,6]. Animal studies clearly demonstrate that non-lethal burns [7-9] and wounds [6-10] exacerbate acute radiation syndrome. Human studies similarly demonstrate that burns and wounds complicate the morbidity and mortality of personnel exposed to ionizing radiation [5,11]. Moreover, radiation-combined injuries (CI) contribute to increased susceptibility to infection and higher mortality when compared to radiation-only injuries (RI) [6]. Mechanisms responsible for these interactions are not fully understood or characterized. Furthermore, no evidence-based clinical guidelines exist for rehabilitation or recovery of individuals with such injuries.

In this study, we sought to evaluate 2-0, 3-0 desulfated heparin (ODSH), an anti-inflammatory and mild anticoagulant agent as a potential countermeasure to radiation combined skin-burn injury. ODSH is produced by the desulfation of unfractionated heparin, reducing its anticoagulant effect to <5% of the potency of unfractionated heparin, yet retaining the anti-inflammatory properties. In mammals heparin is exclusively seen in mast cells residing in the connective and mucosal tissues [12]. This phenomenon suggests that endogenous heparin may have a physiological role in the

regulation of inflammatory responses. Because of this, there have been a considerable number of clinical studies demonstrating the positive anti-inflammatory roles of heparin, and its potential for therapeutic applications [12,13]. Preliminary in-house studies (unpublished data) have demonstrated that mice treated with ODSH delayed radiation-induced mortality and significantly increased 30 day survival. ODSH has yet to be fully evaluated in a radiation combined skin-burn injury (RCBI) animal model as proposed in this study.

## Materials and Methods

### Animals

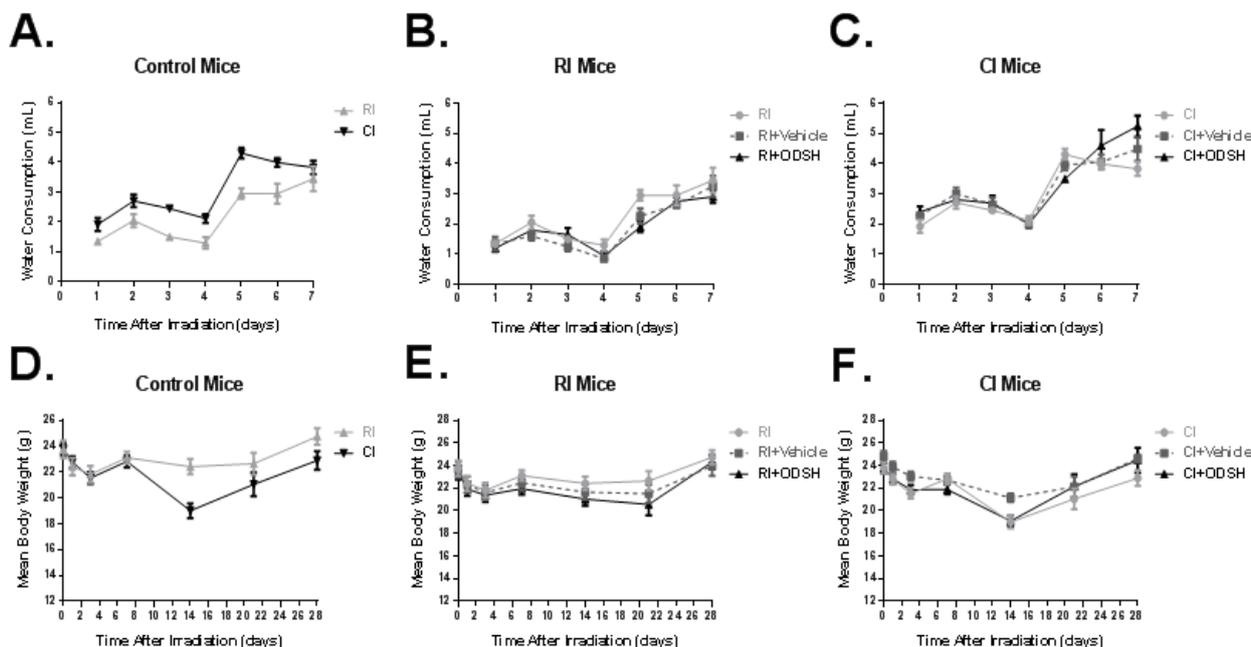
B6D2F1/J female mice (Jackson Laboratory, Bar Harbor, ME) at 12 to 16 weeks of age were maintained in an animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) in plastic micro-isolator cages with hardwood chip bedding. Commercial rodent 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 06:00 (light) to 18:00 (dark) full-spectrum lighting cycle was in operation for the holding room. Mice were assigned into four injury groups: sham (n=20), burn injury (n=20), radiation injury (n=20) and radiation combined burn injury (n=22); with vehicle or ODSH treatment. The Armed Forces Radiobiology Research Institute (AFRRI) Institutional Animal Care and Use Committee (IACUC) reviewed and approved all animal procedures involved in this experiment.

### Gamma irradiation

Mice were given 9.5 Gy (LD70/30 for RCBI) whole-body bilateral <sup>60</sup>Co gamma-photon radiation [7], 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 x 2.5 cm acrylic phantoms to ensure uniform electron scattering. The mapping of the radiation field was performed with alanine/EPR dosimetry [14]. 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 ± 1.8% over all of the 120 compartments. Calibration of the dose rate 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 x 2.5 cm custom designed metal template positioned centrally over the shaved dorsal skin surface. Mice received a 12 second burn from ignited 95% ethanol (0.5 mL) [8].



**Figure 1:** Subcutaneous administration of ODSH (25 mg/kg every 12 h; Days 1-2 and 17.5 mg/kg every 12 h; Days 3-7) did not improve volume of 7-day water consumption (A-C) or 28-days body mass loss (D-F) after whole-body ionizing irradiation alone (RI) or when combined with skin burn injury (CI) in female B6D2F1/J mice. N=20-22 per group. Vehicle and ODSH groups received additional topical gentamicin (0.1% cream; Days 1-10) and oral levofloxacin (100 mg/kg; Days 3-16) treatment. Data are expressed as mean ± SEM.

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, CA) and buprenorphine (0.05 mg/kg), immediately after skin burn injury to alleviate pain. Four hours post-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 and ODSH treatment

For vehicle and ODSH treated animal groups, gentamicin sulfate 0.1% cream (Perrigo, NY) was applied topically each day for 10 days to the skin burn injury from days 1 to 10. Levofloxacin oral solution (Hi-Tech Pharmacal, NY) at a dose of 100 mg/kg in 0.2 mL was administered orally (p.o.) each day for 14 days from days 3 to 16. ODSH (2-0, 3-0 desulfated heparin) was obtained from Pyramid Laboratories (Costa Mesa, CA) and administered subcutaneously (25 mg/kg every 12 h; days 1-2 and 17.5 mg/kg every 12 h; days 3-7) in sterile 0.9% saline as vehicle.

### 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. Mice body weights were measured on days 0, 1, 3, 7, 14, 21 and 28. Mice water consumption levels were assessed from days 1 to 7. On day 30, all surviving mice were anesthetized by isoflurane inhalation. Blood samples were collected by cardiac puncture for hematological analysis. Spleens and bone marrow from femurs were collected after cervical dislocation.

### Hematological, spleen and bone marrow analysis

Blood samples were collected in EDTA KE/1.3 tubes (Sarstedt, NC) and assessed with the ADVIA 2120 Hematology System (Siemens, IL). Complete blood cell differential analysis was conducted using the peroxidase method and the 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 counted using an automated hemocytometer.

### Data analysis

Data are expressed as mean  $\pm$  SEM. Data sets were analyzed by ANOVA with a Bonferroni correction for multiple comparisons. A p value of  $\leq 0.05$  was considered significant.

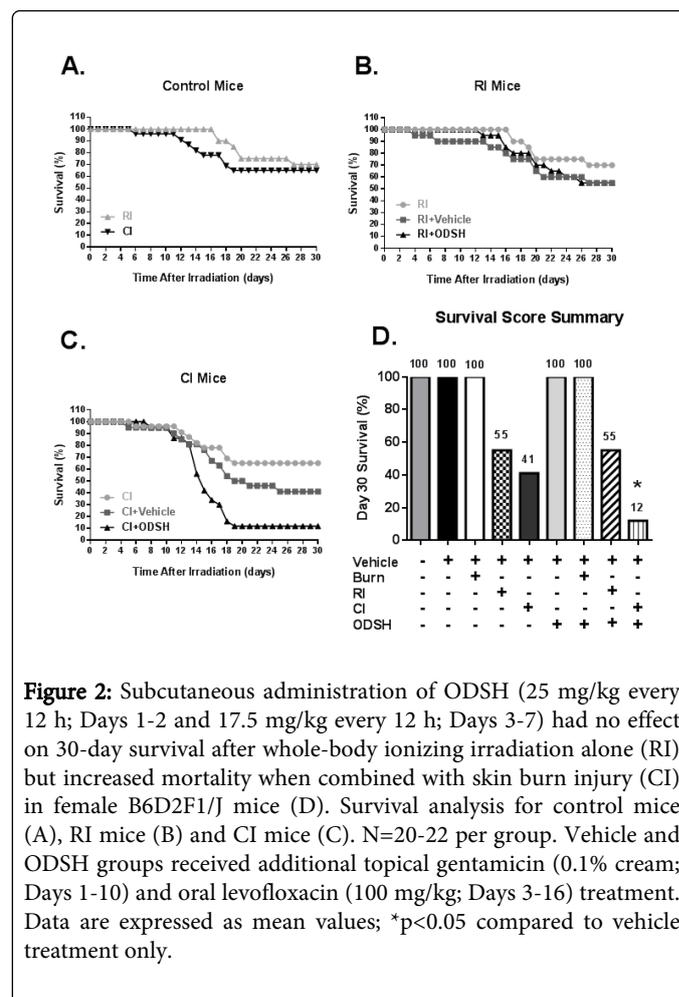
## Results

### Survival, water consumption and body weight analysis

We conducted 7-day water consumption and 28-day mean body mass analysis in our study. These data demonstrated that subcutaneous ODSH treatment administered twice daily for seven days did not significantly improve overall volume of water consumption (Figure 1B and 1C) or improve overall body mass loss (Figure 1E and 1F) after whole-body RI alone or when combined with skin burn injury. Indeed, the ODSH treatment produced lower average body mass for RI mice (Figure 1E). As expected, when compared to CI groups, RI group mice exhibited lower daily water consumption during the initial phases (days 1-6) of the study and higher body mass

during the later phases (days 14-28) of the study (Figures 1A and 1D). We previously observed skin burn trauma alone did not result in mortality over the 30-day observation period. As demonstrated in Figure 2A, skin burn following RI decreased survival to 65%, which was lower than survival observed in RI mice (70%).

In RI mice, vehicle treatment decreased RI-induced survival to 55% (Figure 2B). However, treatment with ODSH had no effect on 30-day survival which remained at 55% also (Figure 2D). In CI mice, vehicle treatment decreased survival to 41% (Figure 2C) and ODSH treatment significantly decreased survival further to 12% on day 30 (Figure 2D;  $p < 0.05$ ).



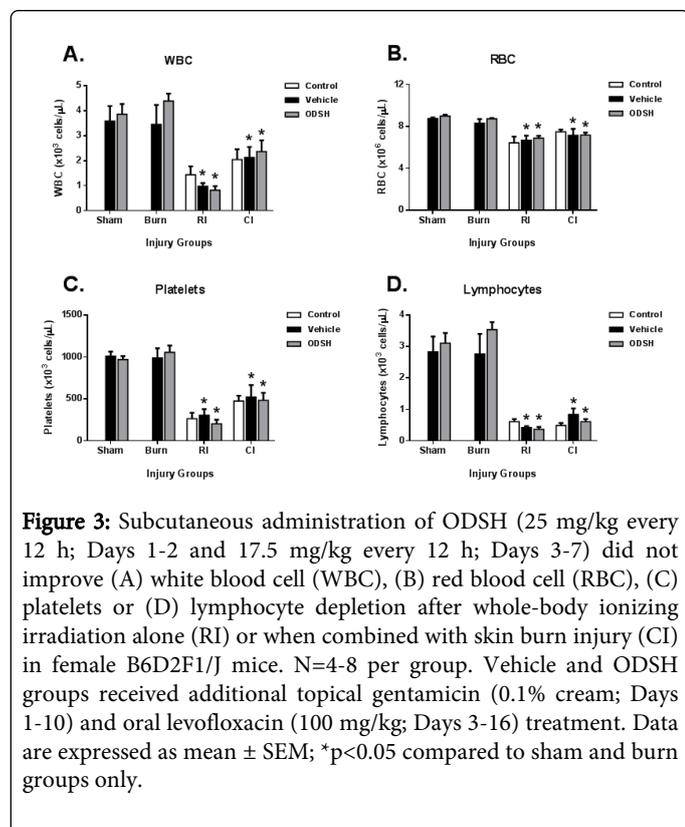
**Figure 2:** Subcutaneous administration of ODSH (25 mg/kg every 12 h; Days 1-2 and 17.5 mg/kg every 12 h; Days 3-7) had no effect on 30-day survival after whole-body ionizing irradiation alone (RI) but increased mortality when combined with skin burn injury (CI) in female B6D2F1/J mice (D). Survival analysis for control mice (A), RI mice (B) and CI mice (C). N=20-22 per group. Vehicle and ODSH groups received additional topical gentamicin (0.1% cream; Days 1-10) and oral levofloxacin (100 mg/kg; Days 3-16) treatment. Data are expressed as mean values; \* $p < 0.05$  compared to vehicle treatment only.

### Hematological analysis

Blood collected from surviving mice following the 30-day survival period was subjected to complete blood cell differential analysis. These data showed that both RI and CI mice had significantly decreased numbers of white blood cell (WBC), red blood cell (RBC) and platelets compared to sham (Figure 3). Skin burn injury alone did not affect WBC, RBC or platelet counts compared to sham (Figure 3). WBC depletion observed in the RI and CI groups was mainly due to diminished lymphocyte counts (Figure 3D). ODSH treatment did not significantly alter numbers of WBC, RBC and platelets in sham or skin burn injury mice, compared to controls. Furthermore, ODSH treatment did not counteract the decreases observed in WBC, RBC

and platelet counts in RI and CI mice compared to controls (Figure 3). This suggests ODSH did not promote hematopoietic recovery for RI and CI mice.

The detailed mechanisms which contribute to the pathophysiology of both RI and CI remain to be determined.

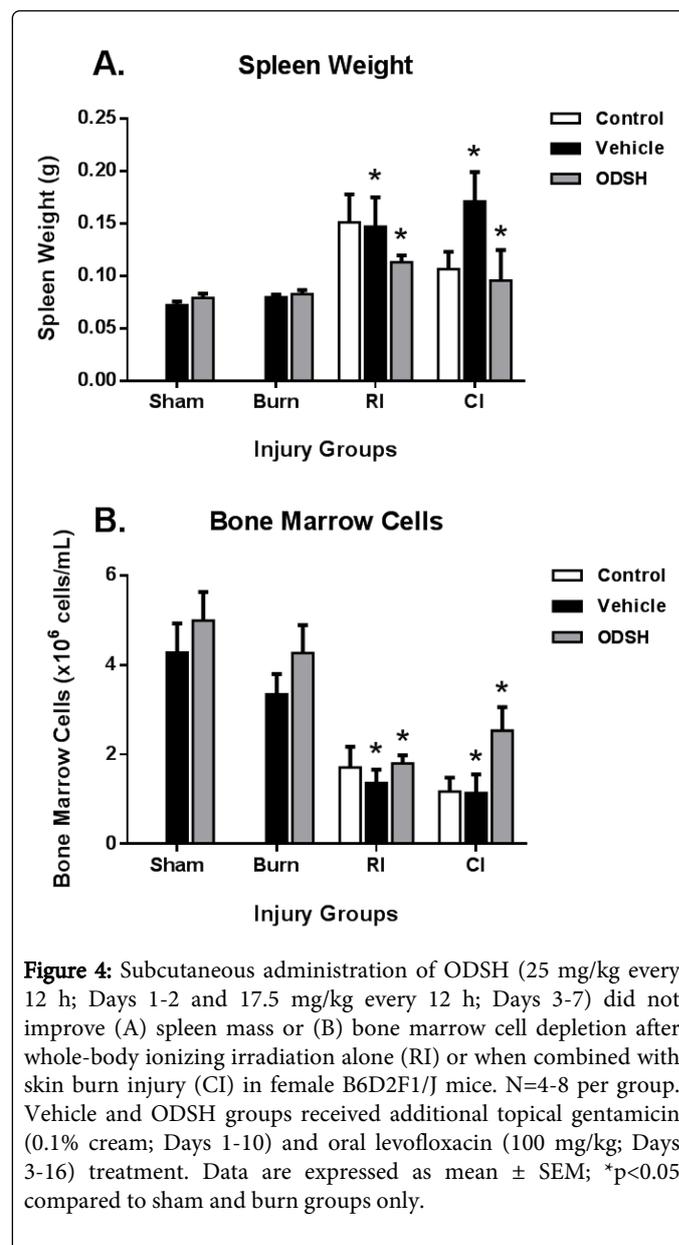


### Spleen and bone marrow analysis

In surviving mice, spleen and bone marrow were evaluated. Both RI and CI mice demonstrated significantly increased spleen mass (splenomegaly) compared to sham (Figure 4A). In contrast, RI and CI mice resulted in significantly lower bone marrow cell counts compared to sham (Figure 4B). In skin burn injury mice, no overall significant differences in spleen mass or bone marrow cell counts were observed compared to sham. Moreover, ODSH treatment failed to improve bone marrow cell counts and mitigate splenomegaly in both RI and CI mice (Figure 4).

### Discussion

There is an urgent need to develop reliable countermeasures against both RI and CI. We evaluated the drug ODSH as a possible candidate for use after radiation combined skin-burn injury. Our study demonstrated that skin burn injury following RI significantly increased mortality and diminished hematopoietic supporting factors in a fashion similar to that of radiation injury alone. However, subcutaneous ODSH administration, significantly increased mortality for CI groups while having no effect on mortality for RI groups. This implies that ODSH treatment may exert its actions in a different manner between the two injury models (RI vs. CI) and suggested ODSH treatment, in combination with topical and systemic antibiotic treatments (using the dosing regimen in this investigation), may not be a suitable countermeasure for radiation combined skin-burn injury.



Heparin is a heterogeneous mixture of polysaccharide molecules with a mean molecular weight between 12–30 KDa containing 200 to 300 disaccharide units of glycosaminoglycan chains [15]. Although heparin has been used as an antithrombotic drug for more than 60 years, it also has many other biological activities. It has been proved to be an anticoagulant, anti-inflammatory, antiangiogenic, antiproliferative and antimetastatic [16,17]. It has been reported that heparin can inhibit the activation of a variety of inflammatory cells [18,19]. This effect has been partially attributed to the binding and resulting neutralization of inflammatory mediators and enzymes secreted during an inflammatory response directed for the stimulation of inflammatory cells [12]. Heparin has also been shown to inhibit the release of certain enzymes and cytotoxic mediators released from cells involved in the inflammatory response process and subsequent tissue

damage, and from cells involved in the remodeling process. Such mediators have included elastase [20], eosinophil peroxidase [21], cathepsin G [22], eosinophil cationic protein [23], major basic protein [24] and specific chemokines and cytokines [25]. Both antimetastatic and anti-inflammatory effects of heparin are assumed to be partly due to its selectin blocking capacity. Selectin is an adhesion molecule, which recruits blood cells to the activated endothelium. As heparin exhibits strong antithrombin mediated anticoagulant activities, the risk of bleeding may limit its therapeutic use in inflammation or cancer treatment [26]. As such heparin has been chemically modified to molecules that retains its anti-inflammatory property while attenuating its anticoagulant activity. The structurally modified heparin with well controlled biological functions would be a potential candidate for anti-inflammatory therapeutic approaches [27]. The modified desulfated heparin entity known as ODSH elicits its anti-inflammatory activity by efficiently blocking leukocyte adhesion and improving wound and burn healing through increased capillary circulation and endothelial cell repair [28-30]. Our study evaluated ODSH within a mice CI model utilizing a skin-burn trauma.

In vitro studies using HUVEC cells have utilized controlled release formulations of desulfated heparin to exert anti-inflammatory properties via E-selectin inhibition [31]. It is thought that this effect was only evident due to reaching optimal cellular concentrations of desulfated heparin using a chitosan microsphere formulation [31]. Another in vivo study conducted in a rat burn wound model utilized desulfated heparin within controlled delivery system microspheres embedded within a collagen matrix wound dressing [32]. This aimed to deliver desulfated heparin in a controlled manner in order to mitigate inflammatory events and speed up recovery of burn wounds [32]. ODSH has been used to ameliorate inflammatory neurological injury in a rat transient middle cerebral artery occlusion model [33]. The study demonstrated that ODSH significantly decreased anticoagulant activity while also simulating anti-inflammatory effects. Importantly, the study demonstrated that ODSH could potentially be administered at a sufficient dose for providing postischemic anti-inflammatory neuroprotection, yet without an increased risk of intracerebral hemorrhage. Reports on the use of heparin in radiation-induced injuries are rare. Although one study did report that long term treatment of patients with anticoagulants following radiation therapy for cerebral tumors resulted in arrest and reversal of small-vessel endothelial injury (the fundamental lesion of radiation necrosis) and produced clinical improvement of radionecrosis in patients [34]. Taking these studies into consideration, we believe that the failure of ODSH to improve survival of RI mice in our study was probably due to not delivering the therapeutically relevant sustained concentration of ODSH to illicit the beneficial anti-inflammatory properties. Furthermore the increased mortality observed for the CI mice could be explained by both not delivering the therapeutically relevant ODSH dose in a controlled manner and fundamental mechanistic differences between the two injury models. Such differences in the mechanisms between RI and CI have been highlighted previously and need further investigation [35-38].

In summary we have demonstrated that ODSH treatment, administered twice daily subcutaneously post-injury for seven days, had no effect on mortality in RI mice but adversely increased mortality in mice with radiation combined skin-burn trauma. Whether this difference is as a result of differences between the mechanisms contributing to RI and CI cannot be determined by this present study. However, our findings do suggest that the drug ODSH in combination with topical and systemic antibiotic treatments may not be a suitable

treatment to mitigate RCBI. Further work will be required to assess the effect of differing ODSH concentrations and dosing regimen on mice survival for the different injury models and investigate specific differences between the mechanisms responsible for the injury models used in this study (RI vs. RCBI).

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## Disclaimer

The views, opinions, and findings contained herein are those of the author and do not necessarily reflect official policy or positions of the Armed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences, Department of the Navy, Department of Defense, the National Institutes of Health, nor the United States Government. The study was conducted at the Armed Forces Radiobiology Research Institute, Bethesda, Maryland, USA. This study was approved and monitored by the AFRRI, Institutional Animal Care and Use Committee.

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