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Study logistics that can impact medical countermeasure efficacy testing in mouse models of radiation injury

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Abstract

Purpose: To address confounding issues that have been noted in planning and conducting studies to identify biomarkers of radiation injury, develop animal models to simulate these injuries, and test potential medical countermeasures to mitigate/treat damage caused by radiation exposure.

Methods: The authors completed an intensive literature search to address several key areas that should be considered before embarking on studies to assess efficacy of medical countermeasure approaches in mouse models of radiation injury. These considerations include: (1) study variables; (2) animal selection criteria; (3) animal husbandry; (4) medical management; and (5) radiation attributes.

Results: It is important to select mouse strains that are capable of responding to the selected radiation exposure (e.g. genetic predispositions might influence radiation sensitivity and proclivity to certain phenotypes of radiation injury), and that also react in a manner similar to humans. Gender, vendor, age, weight, and even seasonal variations are all important factors to consider. In addition, the housing and husbandry of the animals (i.e. feed, environment, handling, time of day of irradiation and animal restraint), as well as the medical management provided (e.g. use of acidified water, antibiotics, routes of administration of drugs, consideration of animal numbers, and euthanasia criteria) should all be addressed. Finally, the radiation exposure itself should be tightly controlled, by ensuring a full understanding and reporting of the radiation source, dose and dose rate, shielding and geometry of exposure, while also providing accurate dosimetry. It is

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important to understand how all the above factors contribute to the development of radiation dose response curves for a given animal facility with a well-defined murine model.

Conclusions: Many potential confounders that could impact the outcomes of studies to assess efficacy of a medical countermeasure for radiation-induced injuries are addressed, and recommendations are made to assist investigators in carrying out research that is robust, reproducible, and accurate.

Keywords

Radiation; mouse strain; confounders; dosimetry; husbandry

Introduction

The Radiation and Nuclear Countermeasures Program (RNCP) within the National Institute of Allergy and Infectious Diseases (NIAID) at the US National Institutes of Health (NIH) was created in 2004, in response to a congressional mandate to initiate a program to develop approaches to assist in diagnosis and treatment of radiation injuries in the aftermath of a radiological or nuclear public health emergency. Since that time, the RNCP has been involved in funding animal model development, biomarker determination, identification of targets for radiation injury, and assessment of medical countermeasures (MCMs) for possible efficacy in mitigation/treatment. Many of these studies have been carried out in mouse models as it is the least sentient among the lower mammals frequently used in the laboratory setting. Extensive similarities in anatomy, physiology and genetics have allowed numerous inferences about human biology to be drawn from murine experimentation. The advanced knowledge of mouse genetics and the availability of numerous genetically modified mouse models greatly facilitate functional studies. Moreover, their low maintenance cost (as compared with other mammalian experimental models), high reproductive rates and short life cycle are substantial advantages of the mouse model. In reviewing the literature involving MCM efficacy testing, it is apparent that radiation dose response relationship (DRR) survival curves in murine strains are extremely steep, and therefore, it can be difficult to accurately reproduce results across different laboratories. There are many seemingly minor variables that can have a dramatic effect on the findings. In fact, the NIH has recently acknowledged issues of poor reproducibility in biomedical research, particularly in preclinical studies using animal models (Collins and Tabak 2014). Through NOT-OD-16-011¹, the NIH has notified the research community that it now requires applicants to address: (1) the scientific premise forming the basis of the proposed research, (2) rigorous experimental design for robust and unbiased results, (3) consideration of relevant biological variables, and (4) authentication of key biological and/or chemical resources. Possible solutions to improve the kind of reproducibility now required by the NIH include inclusion of both sexes in animal studies, division of litters among treatment groups, and thorough documentation of all husbandry variables (Kilkenny et al. 2010).

¹<https://grants.nih.gov/grants/guide/notice-files/not-od-16-011.html>.

As a funding agency, it is critical that the RNCP understand issues surrounding the conduct of studies using animal models of radiation injury to ensure harmonization and comparison between different laboratories. Furthermore, understanding the gaps allows RNCP to implement strategies like scientific meetings and funding opportunities to address the issues. Therefore, this manuscript aims to critically and fully investigate confounding factors that should be considered when designing a radiation study in mice. Literature regarding other animal models of radiation injury is also considered. Among the issues that will be addressed for their possible role in the variability of results are confounders that have been broadly grouped into several categories: (1) study variables; (2) animal selection criteria; (3) animal husbandry; (4) medical management; and (5) radiation attributes (Table 1).

Study variables

Exposure models

The most commonly used quantitative estimate of radiation sensitivity in an animal model is the median lethal dose or LD₅₀. A 30-day observation period is the standard interval used in tabulating mortality from the hematopoietic (H) acute radiation syndrome (ARS) in the mouse and the total body irradiation (TBI) necessary to kill 50% of the exposed animals within this period is called the LD_{50/30} dose. This end-point is often used to determine radiation sensitivity of a strain and the efficacy of a hematopoietic MCM in improving survival. Similarly, other LD₅₀ curves can be generated based on varying organ sensitivities (e.g. LD_{50/15} for the gastrointestinal (GI) syndrome, or LD_{50/180} for lung injuries). These radiation exposures can be TBI, but for GI and lung models, partial-body irradiation (PBI) of the animal might be preferred. These PBI exposures might involve shielding some portion of the bone marrow (e.g. PBI 5% or 2.5% sparing) or targeting a particular organ (e.g. whole thorax lung irradiation (WTLI)). LD₅₀ metrics are reliable; however, there are confounders inherent to the study animal that should be taken into consideration when designing experiments, including (1) age, (2) gender, (3) strain selected (e.g. genetic background, intercellular pathways to respond to MCMs, inbred vs. outbred), and (4) seasonal variations in animals supplies and vendors.

Circadian considerations

In addition to the process of irradiating animals, there are also considerations of the time of day that these and other actions are conducted. It has long been known that there exist circadian variations in all species in response to many external stressors. This effect is observed in mice (that are normally nocturnal animals) and needs to be considered for two reasons: (1) the potential effect of daytime lighting in an animal facility on circadian rhythms, and (2) the effect of the timing of administration of radiation. The impact of the latter variation in the radiation exposure of animals is well documented (Rubin 1981; Duncan et al. 1983; Haus 2002; Palombo et al. 2015). Chrono-radiosensitivity of mice has been recognized since the early 1960s (Pizzarello et al. 1963). In mice, mortality was shown to be higher when radiation was delivered during the night time hours (Nelson 1966) and/or during the early light hours (Pizzarello et al. 1964). Chrono-radiosensitivity has more recently been reported in C57BL/6 mice, with the finding that mice irradiated between 11

am to 1 pm were more radioresistant than mice irradiated in the morning or later in the afternoon (Plett et al. 2012). For this reason, it is important that studies be designed such that animals are irradiated within a window of several hours on any given day, and that the same time of day is selected for all irradiations conducted within a study.

Environmental considerations

Environmental variables are of extreme importance in influencing LD_{50/30} estimations. Radiation doses in the range used for MCM efficacy survival studies cause severe radiation injury, making the mice more sensitive to relatively minor deleterious environmental factors; therefore, factors related directly to the construction and engineering of a small rodent facility should be addressed before embarking on a new animal study. These could include: lighting (which could influence circadian rhythms), cool temperatures (Kuskin et al. 1959) (cold temperatures can lead to a state of hibernation that could be radioprotective (Cerri et al. 2016; Tinganelli et al. 2019), and warm temperatures attributed to seasonal variations. For example, in a comparative study of mouse strains, survival after radiation exposure, when administered during the summer months, was decreased compared to exposure during other months (Roderick 1963). The author attributed the difference to heat variations, since the animal rooms were not maintained at constant temperature. Even rarely considered factors like ambient electromagnetic fields in the facility and mechanical vibration (both of which protect against oxidative stress) (DiCarlo et al. 2001) could impact results.

Statistics

In any efficacy study, outcomes between a test article group and a placebo control group will be compared. As outlined in US Food and Drug Administration (FDA) Guidance (US FDA 2015), important statistical considerations are protection against bias, and selection of the appropriate prospective statistical tests for primary and secondary endpoints. This is also directly governed by the NIH policy on robustness and reproducibility. Protection against bias is achieved in various ways and should be followed as much as possible. The various elements of statistical design for efficacy studies include randomization, clear definitions of criteria used to provide supportive care or to select animals for euthanasia, and blinding of study personnel. Blinding is imperative for adequate and well-controlled studies that would be used for consideration for drug approval; however, personnel or financial limitations may restrict blinding for exploratory studies. When blinding is not possible, randomization and documentation of objective treatment and removal criteria are especially important. Another critical study consideration to ensure appropriate statistical outcomes is selection of sample size for each treatment. It is important that the number of animals used for each treatment provide statistical power to detect a significant change ($p < .05$) in the study outcome. If other outcomes, such as measurements taken throughout a study, are important for the study, sample size selection should also take into account animals that will succumb to radiation exposure during the survival study, thus decreasing the number of animals and, therefore, the number of data points. Consequently, when considering late effects of radiation, which usually rely on PBI models with significant early mortality, the number of animals should reflect the reality that some will succumb to acute hematopoietic and GI syndromes and not make it to the time when the late effects become manifest, and therefore will not be considered for the final endpoint.

Animal selection

Perhaps the most significant variable that should be considered when initiating a radiation exposure study in mice is selection of animal attributes. These selections impact the outcome of a study, as detailed below.

Age

The age at which an animal is irradiated can have a profound influence on its radiation response, as major changes in mortality are found with different ages within a given strain. In early studies comparing the LD_{50/30} following TBI at birth, or 2 weeks to up to 90 weeks, researchers (Abrams 1951; Lindop and Rotblat 1959) demonstrated that mice had higher resistance to radiation lethality at birth and 2 weeks than at 4 weeks of age. At ages above 4 weeks, mice demonstrated an increasing LD_{50/30} to a maximum in young adult-hood, which remained at a plateau for a variable length of time, finally declining in old age (>70 weeks) (Sacher 1957; Crossfill et al. 1959; Trujillo et al. 1962). There is no complete agreement when the plateau of maximum resistance is attained, and this time frame appears to be strain-dependent. In general, resistance increases from the time of weaning to about 3 or 4 months of age. Comparing LD₅₀ estimates of animals exposed during the period of rapidly changing radiation sensitivity can lead to misleading results. For consistency in results, the routine use of experimental mice at least 3–4 months of age is preferable. On the other hand, irradiated rats were found to be more sensitive at infancy (0–3 weeks) and grew more radiation resistant with age (4–40 weeks), while elderly rats (>65 weeks) were more sensitive than adult rats (Hursh and Casarett 1956; Jones et al. 1969). A comprehensive review on the impact of age on radiation sensitivity in different species (hamsters, dogs, lamb and sheep) and interrogating endpoints other than survival (hematological end-points, brain injury, and immune pathways) has been published (Stricklin et al. 2018). A comparable age-dependent sensitivity to radiation relationship has not been established for humans due to the limited number of people in the different age groups under consideration.

Sex

As far as survival on day 30 following TBI, the sex of mice appears to have relatively little effect on resistance to single doses of radiation in the TBI model, and LD_{50/30} estimates appear to be consistent in both sexes in several strains studied. In studies using both sexes of a mouse strain, 30-d survival data are often pooled since there is no statistical difference in lethality due to the sexes (Grahn and Hamilton 1957; Plett et al. 2012). In fact, the International Commission of Radiological Protection based its recommendations for human protection standards on population average rather than sex. Similarly, the LD_{50/60} for humans of ~4.5 Gy is based on a composite estimate. Recently, it was reported that DRR curves for male and female nonhuman primates (NHPs) in a whole thoracic lung irradiation (WTLI) model were not significantly different (LD_{50/180} of 10.28 Gy and 10.27 Gy in females and males, respectively (Thrall et al. 2019)). In WTLI studies, there was no difference in 180-day survival between males and females in C57L/J mice. In contrast, a clear sex difference was observed in the C57BL/6J mice exposed to WTLI, with females 3.54 times more likely to succumb to mortality compared to 1.62 in males (Jackson et

al. 2016). A marked sex-bias has also been observed when other endpoints are queried (Narendran et al. 2019). Historically, mouse studies resulted in mean survival times of 20–40 days after radiation exposure. A marked sex difference in survival time was observed, with females dying significantly earlier, but no difference in 60-day survival between the sexes was noted (Sacher and Grahn 1964). This difference was abolished by ovariectomy of the female mice (Hamilton et al. 1963), suggesting that the radiation sensitivity was related to endocrine function. Other researchers have demonstrated a sex-difference in radiation-induced gene and protein expression, and global genome DNA methylation in male and female mice exposed to acute or chronic TBI (Kovalchuk et al. 2004; Kovalchuk et al. 2004; Pogribny et al. 2004; Silasi et al. 2004). Also noted were altered microRNA expression patterns in irradiated hematopoietic or brain tissue (Ilnytsky et al. 2008; Koturbash et al. 2011), differential metabolites and cytokines (Jones et al. 2019), and rates of genome damage (Stojkovic et al. 2016). Using metabolomics, sex-differences were also reported in biomarkers from 7 Gy-irradiated NHPs (Pannkuk et al. 2015). Consistent with these preclinical findings are data from individuals exposed during the Chernobyl nuclear accident, which indicate that females are at increased risk for long-term health effects compared to men receiving the same radiation dose. These exposures resulted in adverse instances of blood-based diseases, reproductive effects, thyroid dysfunction, differences in sex-ratio, and a higher cancer incidence (Narendran et al. 2019). Because NIH policy stipulates the use of both male and female subjects in experimental designs, it is important that each institute commit to establishing DRR curves for both sexes of the organism used, and to include both sexes in pilot studies for end-points other than survival.

Mouse strain

Genetic variation is one of the major factors influencing radiation resistance among different mouse strains. Early researchers (Henshaw 1944) reported a difference in the acute response of C3H and LAF₁ mice to x-ray irradiation. Since then, several investigators have confirmed findings of major strain differences in radiation response (Reinhard et al. 1954; Kallman and Kohn 1956; Grahn and Hamilton 1957; Kohn and Kallman 1957; Grahn 1958; Grahn and Sacher 1958; Frölen et al. 1961; Yuhas et al. 1966). A comparison of LD_{50/30} values for 10 inbred strains of mice showed that the LD_{50/30} radiation dose ranged from <570 cGy in BALB/cJ to 657 cGy for the moderately sensitive C57BL/6J mice to 734 cGy for the most resistant 129/J strain (Storer 1966). Because of these genetically controlled variations, inbred mouse strains provide an ideal model for determining the nature of radiation response. Other studies described survival of 27 inbred mice exposed to daily low-dose x-rays (Roderick 1963). Some of these strains were resistant to radiation-induced mortality while others were highly susceptible. Generally, these differences are attributed to inherited genes, and influence the response of the organism to ionizing radiation. The most commonly used strains for TBI survival studies are BALB/c, C3H/HeN, B6D2F1, and C57BL/6 (Williams et al. 2010), with LD_{50/30} estimates ranging between 6.5 and 9 Gy. Radiation sensitivity of hybrid strains usually falls between the two parental strains, though closer to the more resistant parent (Frölen et al. 1961).

Inbred mouse strains have a high degree of genetic homozygosity that allows for fewer organisms in an experimental arm, and reproducibility of experimental end-points are more

consistent. In contrast, outbred mice are genetically diverse, requiring more mice per group in a given experiment, and reproducibility is sometimes hindered due to the inherent genetic differences; however, it has recently been demonstrated that outbred mice do not, in fact, display more trait variability than inbred mice (Tuttle et al. 2018). Some advantages of using outbred mice lie in the improved fecundity (3–9 pups per inbred litter vs. 12 pups per litter in the outbred CD-1 strain), large size, and the fact that the DRR curves for outbred mice are often more gradual². Fewer studies have been reported for radiation specific experiments using outbred mice; however, that trend is changing. For example, immunological responses to radiation and radiation combined injury were recently studied in CD-1 mice (an outbred strain from Charles River), since the genetic composition of an outbred strain may be more comparable to the human response (Tajima et al. 2013). In addition, a study done comparing ICR (outbred) and C57BL/6N mice found that the radiation-induced mortality seen for the inbred mice was generally higher than what was observed for the outbred strain (Ryu et al. 2016).

Another issue that impacts inbred strains is genetic drift, where there is a constant tendency of genes to evolve despite the lack of external forces. Genetic drift occurs randomly due to spontaneous transient or permanent mutations (Silver 1995). Genetic drift is significant in small animal colonies, since they are more prone to spontaneous mutations (Russell and Russell 1996), with new mutations becoming fixed in the coding sequence every 6–9 generations. These variations can translate to phenotypic difference impacting the neurons, metabolism or immunity, and hence influence radiation sensitivity.

Current advances in genetic engineering allows for development of novel mouse models (e.g. genetically-modified) that can mimic human responses to ionizing radiation. While knockout and other modified mice can provide a wealth of information regarding the mechanism of action of an MCM, for advanced development of an anti-radiation drug, testing in the more commonly used strains such as the C57BL/6 or C3H/HeN can help ensure accurate assessment of the effectiveness of the MCM, despite the inherent genetic variations. For instance, a chemically synthesized fibroblast growth factor analog, FGF-P, is an effective mitigator of GI syndrome in BALB/c, NIH Swiss, C3H/HeN and C57BL/6 mice, demonstrating its application across different phenotypes (Zhang et al. 2010). Similarly, filgrastim (Neupogen[®], granulocyte-colony stimulating factor, or G-CSF), which is FDA approved to treat neutropenia following a radiological or nuclear incident, is also effective in improving survival in four strains of mice with different radiation sensitivities (Satyamitra et al. 2017).

Vendors

Few published studies were identified in the literature that specifically compared the effect of radiation on organisms acquired from different vendors or barriers, although there are indications that this variable can lead to different outcomes across laboratories (Garrett et al. 2019). In one study, when mice from two different vendors (both inbred and outbred mice) were irradiated at several different radiation dose levels, there was a significant different

²-Orschell CM, Sampson CH, Chua HL, Katz BP, Macvittie TJ, Plett PA. Outbred (J:DO) mice as a model of the hematopoietic acute radiation syndrome (H-ARS). 40th Annual Meeting of the European Radiation Research Society, Dublin, Ireland, 2013.

in sensitivity between the two companies, especially for the outbred mouse strain (Ryu et al., 2016). In further support of this finding are physiological studies in other disciplines that compared mice from two different vendors, and demonstrated different findings relating to general gut composition (Ericsson et al. 2015; Rasmussen et al. 2019). The divergent phenotype between the same mouse strains from different vendors was attributed to the gut microbiome, which is known to modulate the phenotype of models that mimic human disease.

Animal husbandry

In terms of care of animals within a facility, there are many components that could lead to different outcomes in what is otherwise a well-planned experiment. Because biology can be chaotic, it is important to identify variables that can be controlled, to ensure that the experimental outcome can be traced back to the actual variables under consideration.

Diet

The food provided to the mice is a variable that is routinely overlooked in terms of potential variability in radiation study outcomes (Augenlicht 2014), although it is clear that it can have an effect on a wide variety of rodent studies (Turner et al. 2002; Svendsen et al. 2012; Giles et al. 2016; Rasmussen et al. 2019). Providers of rodent chow (e.g. Charles River³, Taconic⁴, LabDiet⁵) routinely make the composition of their diets publicly available; however, it is incumbent on researchers to ensure that the content of the feed will not impact the radiation exposure study. For example, many diets include known radiation injury protectants/mitigators, such as soy products (Ohara et al. 2001; Hillman et al. 2011; Abernathy et al. 2017; Landauer et al. 2019), vitamin A (Seifter et al. 1988; Harapanhalli et al. 1994; Roche et al. 2015; Changizi et al. 2019), vitamin C (Narra et al. 1994; Konopacka et al. 1998; Kanter and Akpolat 2008; Satyamitra et al. 2011; Mortazavi et al. 2015; Roche et al. 2015; Zangeneh et al. 2015; Rostami et al. 2016; Alexander et al. 2018; Jafari et al. 2018), vitamin D (Langberg et al. 2009; Gavrilov et al. 2010; Marampon et al. 2016) and vitamin E (Ross et al. 1983; Shaheen and Hassan 1991; Empey et al. 1992; Nair et al. 2003; Satyamitra et al. 2003; Erol et al. 2004; Singh et al. 2013). In addition, certain estrogens (Wang et al. 2005; Fucic and Gamulin 2011), which have been shown to influence the degree of damage from radiation exposure, may also be found in rodent chow. There is also evidence that active components in compounds in feed can affect outcomes. These include flaxseed⁶ (Bhatia et al. 2007; Christofidou-Solomidou et al. 2011; Christofidou-Solomidou et al. 2012; Pietrofesa et al. 2014), resveratrol (Velio lu-O ünç et al. 2009; Zhang et al. 2017), melatonin (Karbownik and Reiter 2000; El-Missiry et al. 2012; Reiter et al. 2012; Zetner et al. 2016) (which has been linked to observed circadian variations in radiation injury responses) (Ijiri and Potten 1988, 1990), isoflavones including genistein (Landauer et al. 2003; Weiss and Landauer 2003; Day et al. 2008; Landauer et al. 2019), ocimum flavonoids (Uma Devi et al. 1999; Uma Devi and Satyamitra 2004; Nayak and Devi 2005),

3. <https://www.criver.com/products-services/research-models-services/preconditioning-services/custom-diets?region=3611>

4. <https://www.taconic.com/quality/animal-diet/>

5. <https://www.labsupplytx.com/labdiet/lab-diet-rodent-5001/>

6. <https://www.exoticanimalsupply.com/about-us/what-we-do/rodent-pellets-5663>

phytoestrogens (Brown and Setchell 2001), green tea polyphenols (Ding et al. 2015) and selenium (Lee et al. 2017; Son et al. 2017; Bagheri et al. 2018). For example, chow formulations from Lab Diet (5021, 5L0B, 5001, 5015, and 5020) contain vitamins A, D, and E as well as omega-3 fatty acids. In addition, rodent diets are modified by the supplier from time to time, which can lead to challenges in repeating a study at a later date (Pellizzon 2016). Furthermore, limiting the provision of food (caloric restriction), either intentionally or not (e.g. animals might experience difficulty accessing nutrition post-irradiation), can impact biological responses to radiation exposure (Parsons 2009), beyond simple weight loss or failure to gain weight. It is important to make sure that feed is provided *ad libitum*. Inability to access food could occur for several reasons, for example, the inability of the animal to reach the food (due to general weakness and difficulty rearing up to access the food location) or difficulties in gnawing the pellets. It is known that radiation exposure can lead to dental injury in rodent models, making it difficult for the animals to consume hard chow (Pearson and Phelps 1981). The extent of damage to the incisors of C57BL/6 mice (and body weight changes) has been linked to radiation dose and was seen when exposures exceeded 10 Gy.

Gel-packs and other nutritional approaches

After irradiation, animals may experience sickness and severe weakness, which can reduce their water intake and food consumption as early as day one post-exposure. Since dehydration and starvation contribute to mortality, strategies to provide nourishment are needed. One approach is to provide wet chow in Petri dishes at the bottom of the cages. Studies have demonstrated that provision of wet chow allows adequate feeding and hydration of the animals during the critical periods (Booth et al. 2012; Hankenson 2014; Plett et al. 2012). Because of a reduction in water intake from the sipper tube, if drug is provided *ad libitum* in the water, there could be a decrease in the dose of drug ingested by the mice. Therefore, it is important to prepare the wet food with the medicated water. Studies have demonstrated that this method allows adequate feeding and hydration of the animals during the critical periods (Booth et al. 2012; Hankenson 2014; Plett et al. 2012). Another approach is the use of nutritionally supplemented gel packs. In studies where these were provided, animals consumed less pelleted food; however, one study suggests that use of gel packs can influence radiation study outcomes. In the study by Moccia et al. (2010) both hydration and nutritional (calorie-supplemented) gels were studied in a 30-day radiation survival mouse model. Mice exposed to 8.5 Gy of ^{60}Co TBI that were provided gel-based nutrition (in addition to pelleted food and water) had lower survival (e.g. a reduction of 25% for the nutritional gel) than those animals not offered additional support, although the difference was not statistically significant. Therefore, more studies are needed to carefully evaluate the use of these supplements. Furthermore, gel packs can provide a vehicle for MCM administration. In a study comparing consumption of acetaminophen, it was shown that dosing the drug simultaneously in gel packs and the drinking water achieved effective drug concentrations (Christy et al. 2014). The advantages of self-administration via drinking water and gel packs together with the stress reduction due to decreased handling and restraint need to be taken into consideration for each experiment. However, it is important to consider and document the exact composition of the gel pack, as these vary between brands and can obscure results depending on the measurements being made and the mechanisms

of action of the drug under consideration. Being aware of these issues and addressing them early can help minimize variability between studies due to differences in animal feed and feeding.

Native flora/fauna in an animal facility

Differences between facilities in terms of the presence of known, but not necessarily infectious, microbiological species/contaminants can also lead to difficulties comparing outcomes of radiation experiments across institutions. The contribution of the microbiome has only recently been recognized as a factor in the progression of radiation injuries or an element of the damage response. For example, germ-free mice have been found to be resistant to radiation exposures in general (Wilson and Piacsek 1962; van Bekkum 1968) and in radiation-induced GI injury specifically (Crawford and Gordon 2005), with fewer apoptotic cells than animals that are not raised germ-free. In addition, gnotobiotic animals in a facility with defined flora can have different radiation responses than the same strain of animal reared in another facility⁷.

Housing

Proper housing should provide the appropriate physical and social environment, as well as adequate space to promote the health and well-being of the animals. Evidence from a wide number of studies suggest that housing conditions before and after the in-life portion of a study must be taken into consideration to ensure that they do not obscure the obtained results (Lyte et al. 2005). Perhaps the most recognized variable in experiments conducted in all areas of preclinical research is the impact of single versus group housing for the animals. The National Academies' *Guide for the Care and Use of Laboratory Animals* establishes that animals should be housed with the goal of maximizing species-specific behaviors while minimizing stress-induced behaviors (National Research Council Committee 2011). For rodents, it is important that caging allows them to have social interactions, normally through housing in compatible pairs or groups. This social component, in addition to the elevated cost of single housing, space limitations in animal facilities for the increased number of cages needed, and the ease of handling multiple mice at once per cage, are a strong driver of group housing. However, arguments can also be made against group housing, since it could contribute to differences seen between experiments due to competition for space, food, social rank, as well as spread of disease and infection due to cross-contamination within cages. Emotional interactions such as huddling and fighting can also have a significant impact. In the case of males, this arrangement can lead to a high-stress environment, and subsequent fighting that can result in injuries that lead to confounding results in the study. For example, an increase in glucocorticoids (known to have an anti-inflammatory effect) have been observed in animals in group-housing, and could contribute to the immune response of the animals to certain diseases (Christian and Williamson 1958). These considerations are also important in experiments involving radiation exposure. Work done with female rats exposed to increasing levels of radiation showed a significant shift in the mortality curve for grouped housed animals compared to singly housed animals (Hahn and

⁷Sedlacek RS, Rose E, Suit HD. Abstract: Gnotobiotic mice and techniques for radiation biology. 35th Annual Meeting of the Radiation Research Society, p. 84, Philadelphia, PA, 1987.

Howland 1963). For lower radiation exposures, the percent survival was higher in singly caged groups. In higher radiation exposures, the onset of mortality occurred later in the singly caged animals. In another study, comparing male and female rats exposed to the same levels of radiation, there was no effect of housing on the mortality rate in males (Ader and Hahn 1963); however, group-reared females had a significantly greater mortality rate than those singly-housed. In addition, there is a risk of observing cage-specific effects, when animals that have received the same treatment are housed together. This effect can be mitigated by the randomization of different treatment groups within the same cage. Therefore, housing conditions are highly context-dependent and thus must be considered for each individual experiment. Furthermore, detailed documentation provided in a publication is important for subsequent analysis and reproducibility (Kappel et al. 2017).

Bedding

It is also possible that less-considered variables, such as the type and cleanliness of bedding used, could influence a study. Nesting material is important for the thermoregulation of rodents (Gaskill et al. 2012; Maher et al. 2015; Moehring et al. 2016). Therefore, the type of bedding used must be carefully selected to provide adequate insulation (Hess et al. 2008) and reduce possible impacts on assessment of drug efficacy, especially in pharmacological and toxicological studies where endpoints might be dependent on core temperatures (Gordon 2004). In addition to the type and amount of bedding, the cage change frequency must be regulated to maintain a balance in hygiene, minimize bio-mass accumulation and reduce cage disturbances (Rosenbaum et al. 2009). Another consideration includes contaminants present in the bedding which could affect pharmacologic responsiveness and lead to discrepancies in drug metabolism (Smith et al. 2004).

Handling of animals

Handling mice during procedures involves physical manipulation that conflicts with normal posture and movement. This added stress is known to affect the behavior and physiology of animals, and thus is commonly singled out as a potential source for variation in animal experiments (Gouveia and Hurst 2013). Handling stressors encompass a range of activities that are necessary to provide careful and appropriate attention to rodents within a facility (Balcombe et al. 2004), such as restraint during administration of pharmaceuticals, medical management, blood sampling, animal tracking approaches, and telemetry. Studies with irradiated mice, bled weekly for 30-days, showed significantly reduced survival times for the bled mice when compared to controls (Plett et al. 2012). While the total amount of drawn blood and fluids replenished via injections can play a role in the outcome of the mice, the additional stress caused by the handling in itself has negative impacts that can outweigh these other effects, especially at higher radiation doses (Booth et al. 2012; Plett et al. 2012). Together, these data suggest that special care needs to be taken when planning experiments with extensive testing of irradiated mice, since these animals are more sensitive to stress effects than their non-irradiated counterparts. Furthermore, the added stress from aggressive MCM administration schedules, such as daily injections and intrusive procedures like oral gavage, can also affect DRR curves. An extensive analysis of studies performed in the well-characterized H-ARS, TBI model over a 10-year period has shown that manipulation of mice during the acute phase (e.g. day 1–day 30) of the radiation response leads to increased

lethality (Plett et al. 2015). Importantly, these effects are more notable in higher doses of radiation, when mice tend to exhibit more severe symptoms of radiation sickness and are more susceptible to stress and infections. Therefore, to control for these effects, studies should minimize handling and use the appropriate vehicle controls to account for these effects across different treatment groups. Dose regimen and sampling schedules should also be taken into consideration when choosing radiation doses. For this purpose, complete DRR curves should be generated using increasing doses of radiation, to select the appropriate dose for the survival study. Alternatively, two or more radiation doses would need to be used in the study, to include a lower dose to allow for the possibility of increased lethality due to handling stress. For more invasive procedures and to enable thorough sample collection, satellite groups can be included in the experimental design.

Gender and characteristics of animal caretaker

Even the gender of the animal caretaker, responsible for basic colony maintenance, and/or invasive procedures, could potentially influence study outcomes. Although not a direct manipulation of the macro- or microenvironment of the animal, studies have shown that the gender and other personality characteristics of the person handling the animals can influence the results of the study, particularly if hormone responses might influence the outcome (Chapman et al. 2018; Daniali and Flaten 2019). In a recent paper (Sorge et al. 2014), it was shown that exposure of rodents to male, but not female experimenters resulted in pain inhibition of the animals, due to induced physiological stress resulting in stress-induced analgesia. Other studies have shown that how the caretaker treats the animals (e.g. gentle or aggressive handling) can modify responses to different stimuli (Neely et al. 2018). These results demonstrate that one must carefully monitor all the environmental factors interacting in the laboratory setting and experimental set-up. Even though some of these factors may have unpredictable effects, it is important to have all the information documented in the methods section of every publication, so that experiments can be more easily replicated (Chapman et al. 2018).

Interventions

Anesthesia

The use of some forms of anesthesia prior to radiation exposure and during other procedures to prevent animal movement has been linked to changes in the effects of radiation exposure (Langendorff and Koch 1954). For example, some injectable drugs, as well as certain inhaled anesthetics can have an impact on survival when used in radiation experiments (Keizer and van Putten 1976; Conere et al. 1986). In studies conducted in larger animals, the use of anesthetics such as ketamine (an NMDA receptor antagonist) and acepromazine (a phenothiazine tranquilizer) decreased levels of radiation-induced citrulline in the circulation as compared to unanesthetized animals (Bujold et al. 2016). The use of pentobarbital was found to offer radiation protection to the lung in a murine model of radiation-induced pneumonitis (Down et al. 1983), and a decrease in brain injuries resulting from whole-brain irradiation were observed with the use of pentobarbital or lidocaine (Oldfield et al. 1990). In the latter study, these anesthetics were compared to either no medications, or to ketamine. Unlike earlier reports, researchers found that while ketamine was not radio-protective in

their hands, the use of either pentobarbital or lidocaine increased survival. In addition, while anesthetized, animals experience a drop in body temperature if not closely regulated (Caro et al. 2013), which could have an impact on radiation injuries (Levan et al. 1970). Other interventions, which more closely constitute standard animal care and medical management, will be discussed in greater detail below.

Route of administration

In addition, how medical management (e.g. antibiotics and fluids) is provided, as well as the route of administration selected for an MCM (e.g. oral, subcutaneous (sc), intravenous (iv), intramuscular (im), intraperitoneal (ip) and retro-orbital) can make interpretation of results and comparison to other outcomes problematic. In addition, using routes of administration that would not normally be used to provide drugs to humans during a mass casualty radiation public health emergency (e.g. ip) should be avoided if conducting MCM efficacy studies. As mentioned in the handling section, dosing schedules that require frequent manipulation of the animals can also increase lethality. This can be exacerbated with invasive administration procedures such as orally administered drugs. For example, while oral gavage allows for precise dosage of the drug, the stress associated with animal restraint and repeated disturbance to the esophageal cavity in an already-injured GI tract can obscure MCM-induced survival benefits. Experimental considerations include dosing in drinking water and/or via gel packs (discussed above) or even coating gavage needles with sucrose to pacify mice and induce swallowing (Hoggatt et al. 2010). The potential issues surrounding the selection of administration route can be especially challenging when cellular therapies are being tested, as these usually require iv administration for proper localization and engraftment of the cells to the targeted location. While tail vein injections are most common for iv injections, the technical challenge of this method makes retro-orbital injections an attractive alternative (Yardeni et al. 2011; Schoch et al. 2014). Additionally, retro-orbital injections are less stressful on the mice and are easier to perform in darker pigmented mice where tail veins are harder to visualize. Studies comparing hematopoietic stem cell engraftment (Leon-Rico et al. 2015), tumor uptake (Kim et al. 2010) and therapeutic agents (Steel et al. 2008) administered through the retro-orbital venous sinus and lateral tail vein showed no significant difference between the two methods, providing compelling evidence for the use of retro-orbital injections for administration of cellular therapies. It is important to note that irradiated mice, with a compromised hematopoietic system and fragile vasculature, may be more susceptible than unirradiated mice to differences between these methods, thus rigorous training of study personnel and pilot studies are warranted to determine which route is most efficacious in radiation experiments.

Medical management and MCM testing

When designing mouse studies for MCM efficacy testing, researchers must recognize that many variables can influence the outcome and should prepare for the proper comparison methods. Because study sites differ in such aspects as feed, watering procedures, room environment, IACUC requirements, and bacterial populations, any of which can influence the radiation DRR, it is important to consider how these variables could affect study

outcomes and site-to-site comparisons. In this section, we discuss some of the variables that should be considered.

Water source

In addition to considerations surrounding selection of feed, sites may also vary in the water provided to the animals. Water acidification (pH 2.5–3.0) has been one method used by many animal care facilities to reduce bacterial load in water bottles. Other sites may use an automatic watering system without additional acid treatment, rather than static bottles filled with acidified water. Researchers (Langgartner et al. 2017) tested acidified water vs. normal tap water in mice and found that water acidification altered thymus and adrenal weights. In addition, in a non-obese diabetic mouse model, water acidification was associated with reduced gut flora diversity (Sofi et al. 2014). It is not clear if there is a best system for radiation studies, but water treatment should be considered a variable, and if possible, tested.

Antibiotics use

The Infectious Disease Society of America recommends antibiotic therapy for patients with radiation-induced neutropenia (Freifeld et al. 2011), and in patients, antibiotic therapy is considered standard of care (Waselenko et al. 2004; Dainiak 2018). In the NHP irradiation model, a medical management regimen that includes antibiotics (starting with enrofloxacin and progressing, depending on antibiotic efficacy) has improved survival (Farese et al. 2012). For patients, broad antibiotic coverage is sought, and fluoroquinolones and amoxicillin/clavulanate are recommended (Dainiak 2018). Mouse models have been used to test various antibiotic regimens, and the work by Brook et al. (2004) showed that levels of *Enterobacteriaceae* that have the potential to translocate from the gut into the bloodstream could be reduced using quinolones. Similar to what is recommended for patients, penicillin administration helped prevent infections from *Streptococcus spp.* These and other studies were used as the basis of testing the effects of various antibiotic regimens on survival in a mouse H-ARS model (Plett et al. 2012). In these studies, three regimens: doxycycline þ neomycin, ciprofloxacin, and levofloxacin added to drinking water and wetted chow were tested. All three regimens increased mean survival time after radiation exposure, which could widen the window of opportunity for an MCM to act. In a mouse model of GI-ARS, researchers (Booth et al. 2012) also showed that fluoroquinolone antibiotics in drinking water improved survival. Another possible use for antibiotics is to avoid complications that would impair evaluation in mouse models. An example of this is the occasional appearance of a ‘swollen muzzle’ syndrome following high levels of TBI typical of testing for efficacy in a GI model. This visual and lethal outcome, which appears to be a sign of opportunistic infections (Booth et al. 2012), can be ameliorated by the use of antibiotics, although it is important to understand if the use of a selected antibiotic can also impact radiation lethality studies, as certain antibiotics (e.g. ciprofloxacin) are also known mitigators (Fukumoto et al. 2014). Acquiring mice from facilities with more stringent barrier procedures could also reduce swollen muzzle syndrome, as well as monitoring health reports from these particular facilities (Garrett et al. 2019). Despite these data and the attractiveness of modeling the human experience, researchers may also want to consider antimicrobial stewardship when deciding whether to routinely provide antibiotics in all radiation studies. Bacterial resistance in a vivarium or in the general population is an issue, and it is recommended that antibiotics

should be used judiciously (Narver 2017). For routine testing of MCMs, antibiotics may not be necessary unless requested by regulatory agencies.

Euthanasia criteria

Criteria for mouse studies will normally be driven by each site's Institutional Animal Care and Use Committee (IACUC), which will determine which observations are considered routine and feasible. If animals are weighed periodically, weight loss is often used as a single criterion (e. g., 30% loss of baseline weight) or combined (e.g. 15–20% loss of baseline weight) with other scored appearance and behavioral criteria. Other criteria used include hunched posture, decreased activity, withdrawn behavior, squinted or closed eyes with or without discharge, lack of grooming, reduced apparent body temperatures (cold to the touch, cyanosis of snout and extremities), and signs of dehydration (tenting of skin and loss of elasticity) (Booth et al. 2012; Plett et al. 2012; Bunin et al. 2020). Investigators need to have clear scales for each observation to be as objective as possible and to reduce bias.

Statistical considerations

The FDA Animal Rule states that the primary endpoint is 'generally the enhancement of survival or prevention of major morbidity ... ' (21 CFR 314.610(a)(3)⁸ and 21 CFR 601.91(a)(3)⁹). For these studies, animals are followed for a predetermined observation period, with mortality occurring at various times. Data to show survival can be plotted on a Kaplan-Meier plot of percent (or fraction of) survival vs. time, based on the idea that once an animal has died, it is censored (removed) from the study. Two types of models that are used for survival analysis include log-rank test and logistic regression, such as Cox proportional hazards model (Bewick et al. 2004). NIAID has had experience with log-rank (Bunin et al. 2020) and logistic analysis (Plett et al. 2012; Landes et al. 2012) described the application of Cox proportional hazards regression analysis as applied to radiation studies. Statistical tests can also be applied to secondary endpoints, such as blood counts over time. When comparing groups, it is important to consider data censored because of animal deaths, and although means may be compared, median comparison may be more appropriate (Hankey et al. 2015). Because proper statistical evaluations can be complex, even during early research and development, investigators need to collaborate with statisticians who can help with techniques to reduce bias, determine group sizes based on expected outcome, and design the appropriate statistical models for analyses. Both the NIH and FDA will expect a statistical analysis plan in studies they review for funding or approval, so it is important to have strong and documented statistical considerations in place.

Radiation attributes

Radiation exposure considerations

Reproducible data are of the utmost importance to the NIH, and are necessary for the development of MCMs to be approved/licensed by the US FDA. A fundamental requirement for reproducibility in the field of radiation biology is the harmonization of radiation

⁸. <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=314.610>

⁹. <https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=601.91>

exposure parameters of small animal systems used to test MCMs for efficacy. Achieving harmonization is highly dependent on a standardized reference dosimetry. To help meet the needs and standardize the field of radiobiology and radiotherapy, external beam dosimetry protocols have been developed by the American Association of Physicists in Medicine (AAPM). Moreover, the AAPM partners with the National Institute of Standards and technology (NIST) and Accredited Dosimetry Calibration Laboratories (ADCLs) to provide NIST-traceable reference standards for calibration (Ibbott et al. 2008).

Institutions should establish a robust dosimetry method using these tools along with the appropriate phantoms and radiation sensors, as has been done in several NIH-funded consortia. (Kazi et al. 2014). To address this need within NIAID-funded studies and harmonize exposures across laboratories that utilize a variety of irradiators (e.g. x-ray (250–320 kVp), ^{137}Cs , ^{60}Co , single, small animal radiation research platforms (SARRP), and medical linear accelerators (LINACs)), a workshop was conducted in 2016 by the NIAID, which brought together experts in the field, including radiation physicists and biologists who work with radiation. The workshop provided a forum to share, evaluate, and discuss challenges of current dosimetry methods used for animal radiation studies at NIAID-funded institutions. Using programmatic experience and discussions from this meeting, a list of parameters requiring special attention has been compiled, which should be considered before irradiating an animal. These include an acknowledgement of the appropriateness and limitations surrounding the source of the radiation, type of irradiator, exposure field (e.g. size, volume, uniformity), dose rate (cGy/min) and total dose delivered, geometry of the radiation exposure, animal placement, and devices and phantoms used for monitoring and reporting. Each of these parameters are critical since a small difference in any one variable can modify the DRR of a model system as well as result in an increase in experiment variability (MacVittie et al. 2012; Kazi et al. 2014; Plett et al. 2015; Thrall et al. 2015). This is especially true for mouse models, as their small size leaves little room for error in terms of properly assessing the radiation exposure administered. Each of these aspects is discussed in more detail below.

Types of irradiators and radiation sources

Small animal irradiation studies typically use self-shielded gamma irradiators (^{137}Cs or ^{60}Co), x-ray irradiators or LINAC systems in combination with small-animal adapted micro-computed tomography image-guided radiation therapy techniques, similar to those intended for diagnostic or therapeutic human use (Spiegelmann et al. 1993; Solberg et al. 1994; Jaffray 2007; Yoshizumi et al. 2011). Both gamma (γ) and x-ray irradiation sources produce a field of high energy electromagnetic radiation that carry and transfer their energy to a specified target (Yoshizumi et al. 2011). Radiation exposures generated from γ -emitting sources originate from a sealed radionuclide source, ^{137}Cs or ^{60}Co , are monoenergetic on the order of megavolts (MV). While γ -irradiators are reliable, the source must be maintained and disposed of properly; therefore, most γ -irradiators use ^{137}Cs due to its relatively long half-life of 30 years as compared to ^{60}Co (5.27 years). However, a ^{137}Cs removal program initiated in 2014 by the Department of Energy's National Nuclear Security Administration's Office of Radiological Security (NNSA ORS) may impact accessibility to these irradiators for research¹⁰. The NNSA ORS has facilitated the replacement of

^{137}Cs irradiators housed in hospitals and research centers with non-radioactive alternatives, such as x-rays. This replacement program is set to be complete by 2020. Termed the Cesium Irradiator Replacement Project, this security measure intends to address concerns about radiation security and safety in the US. X-rays are produced using electricity and high-voltage x-ray tubes. X-ray irradiators do not require maintenance of a radiation source and can be easily turned on or off to produce a poly-energetic beam (10–120 kilovolts, kV) similar to the kilovoltage irradiators used in medical radiography. X-ray irradiators such as the orthovoltage and megavoltage irradiators have higher energy capabilities that operate at 130–320 kV and 6–18 MV, respectively. The ability to penetrate tissues is dependent on the x-ray tube energy; greater x-ray energy will yield greater tissue penetration (Yoshizumi et al. 2011). In all cases, the x-ray tube energy and beam current must be considered, as these will affect the dose delivered. Another benefit of use of x-rays is the ease of use for methodologies involving PBI exposures, where bone marrow shielding is required.

Animal setup (geometry, restraints, and shielding)

The design of each irradiation device is variable and dependent on the manufacturer, but most can be adapted to experimental requirements. Animal number, size, shape and shielding requirements are some of the parameters that will likely require adjustments. Animal containment chambers or ‘jigs’ can be made to accommodate several animals per run, and lead shielding blocks can be used to prevent exposure to certain areas of the animal’s body. Even with this kind of shielding in place, it is still possible to have some radiation exposure (albeit much less than the part of the animal fully in the field) to the tissue under the lead (Fish et al. 2018). In addition, the position and geometry of the radiation beam to the animal can also be adjusted. A plexiglass pie plate jig or a polymethyl methacrylate cage is commonly used for TBI or PBI, but each can vary in size to accommodate a few to multiple mice per jig. In some cases, anesthetized mice of varying quantities may be lined up in a row for radiation exposure, or special jigs can be created to isolate or shield certain limbs. In all cases, one must consider the uniformity of exposure; the geometry of the exposure (e.g. anterior to posterior, lateral, or head to toe), and the use of shielding to obtain 2.5% or 5% shielded bone marrow, WTLLI, hemi-body exposure, etc. In addition, when reporting data from radiation studies, it is important to state how the radiation dose was estimated; for example, free-in-air versus midline to tissue (explored in more detail below).

Shielded models are often dependent on custom-made jigs and arrangements of animals that can be unique to each site. Given the variety of setups, it is critical to use radiographic film and nanodots to determine the amount of radiation leakage around the lead shielding and calculate the correct percentage of bone marrow being spared (Hammersberg et al. 1998). Other parameters that can influence the amount of radiation exposure to certain parts of the body include the presence (and location) of air or loading holes on the jig. If these face the source of radiation, it may lead to sensitivity of the exposed area. For example, some instances of the ‘swollen muzzle’ syndrome (discussed above) (Garrett et al. 2019) could be due to the presence of air holes at the top of a radiation pie jig setup, where the radiation

10. https://www.energy.gov/sites/prod/files/migrated/nnsa/2017/11/f45/ors_cirp_brochure_r18_web.pdf

exposure comes from above. This geometry could allow the animals to poke their noses outside of the enclosure, leading to a higher than anticipated radiation exposure to that area (personal communication, Michelle Lambert, Children's Hospital of Philadelphia, 2017).

Beyond the actual setup, other confounders that should be considered are: (1) whether the animals are exposed to radiation from a c source in a unilateral, simultaneous bilateral or sequential bilateral configuration, and (2) whether a rotating table is used, since rotational devices have been shown to maintain a more accurate and consistent dose distribution by helping to minimize radiation hotspots in the radiation field (Brady et al. 2012; Brodin et al. 2016).

Radiation dose and dose rate

The nature of radiation exposure in the context of a nuclear accident or terrorist attack cannot be fully predicted. What is known; however, is that the exposure will be heterogeneous and the subsequent biological effects could differ based on body position, random shielding, differential dose rate, distance from the source, and radiation intensity. Radiation exposure victims will have variable responses to treatment due to time of administration and other factors such as random shielding during exposure that would likely spare a portion of tissue-regenerating stem and progenitor cells that could enhance recovery (MacVittie et al. 2012). It is for these reasons that accuracy and validation is needed to ensure consistency between the expected and actual radiation dose delivered.

Radiation dose- and time-dependent relationships that dictate mortality and morbidity are achieved through stringent experimental verification. Animal models have been developed through RNCP funding to reflect the onset, latency, severity, and dose- and time-dependent relationships of ARS. In particular, H-ARS, GI-ARS, and lung delayed effect of acute radiation exposure (DEARE) mouse models have been developed to test MCM efficacy (MacVittie 2012). For example, a mouse DRR was established using six radiation doses ranging from 775 to 900 cGy (estimated 0–100% lethality) with a ^{137}Cs radiation source at $0.97\text{--}1.03\text{ Gy min}^{-1}$ (dose rates represent ^{137}Cs decay over a 2.7-year study period (Plett et al. 2012)). These data determined the range of lethal doses for subsequent studies. Stability of the DRR is essential for accurate MCM efficacy studies and is assessed by monitoring 'drift' in the expected survival of control groups. A lethal dose for 50% of the population in a 30-day survival study ($\text{LD}_{50/30}$), for example, could translate to $50\% \pm 20\%$ (i.e. 40% to 60% survival) in the control group. However, if survival is greater than $\pm 20\%$, the ability to assess the potential efficacy of an MCM with this model may be problematic (Plett et al. 2015). Unfortunately, steepness of mouse DRR curves mean that institutional DRRs may need to be reestablished periodically and verified with conditions representative of planned studies; however, this may not be needed if survival in the vehicle-treated group at a given radiation lethality remains consistent with the original DRR. It may also be important to determine separate DRRs for each sex and with vehicle administration in the manner that the MCM will be delivered.

Dosimetry devices and phantoms

Accurate dosimetry is dependent on a reference standard to measure the dose rate of the irradiator, and the measurement of the specific dose delivered to a mouse within a specific setup and radiation type. The manufacturer usually initially calibrates irradiators, and dosimetry should be repeated on a regular basis (usually yearly) by well-trained technicians. Ideally, a NIST-traceable reference should be used to ensure the irradiator is working properly and emitting the expected free-in-air energy¹¹. The second component measures midline to tissue exposure using an ionization chamber, radiochromic film, optically stimulated luminescence dosimeters (OSLDs), or thermal luminescence dosimeters (TLDs) to accurately determine the actual dose at a specified radiation position (Yoshizumi et al. 2011). Therefore, to obtain accurate dosimetry for animal studies, it is essential that a radiation health physicist properly and regularly calibrate radiation devices using the geometry planned for the experiment.

Dose verification should also be completed with in-run or in vivo dosimetry for each study, using air-filled ion chambers, silicon diode systems, radiographic or radiochromic films, TLDs, OSLDs, MOSFETs (metal-oxide semiconductor field-effect transistor), diamond, alanine, or gel detectors. For more accurate in-run dosimetry measurements, mouse phantoms with embedded TLDs can be placed in a similar position alongside laboratory mice to measure the absorbed radiation dose delivered for each individual setup (Welch et al. 2015; Seed et al. 2016; Welch et al. 2017). In some cases, such as with mouse lung studies, true tissue-equivalent phantoms provide more reliable dosimetry measurements for small animal radiation experiments. The lack of lung, ribs and muscle in a phantom can lead to significant differences in entrance and target tissue dose rates as compared to euthanized rats and mice with implanted detectors and corresponding phantom measurements (McGurk et al. 2012), which emphasizes the importance of understanding the limitations and benefits of the phantom used. Mouse-like phantoms have evolved from water-filled bags into anthropomorphic phantoms that offer a practical and anatomically accurate measurement of radiation exposure using physical and computational measurements from strategically embedded TLDs (Knoll 2000; Perks et al. 2015; Seed et al. 2016; Welch et al. 2017). Three-dimensional (3D) printing has allowed for more detailed phantoms that can mimic the scattering and absorption differences dependent on the external mouse anatomy (Bache et al. 2015; Bentz et al. 2016). Moreover, 3D printing has also allowed for the construction of anatomically accurate mouse phantoms that account for areas of inhomogeneity such as bone and organs (Welch et al. 2015; Welch et al. 2017). These tools are helping advance the area of radiobiology and removing some of the error from pre-clinical studies, crucial for MCM development for ARS.

It is generally accepted that institutions that apply quality assurance and control procedures to all radiation studies have more reliable outcomes. Unfortunately, it can be difficult to find detailed dosimetry methodologies in radiation research publications, making it impossible for other researchers to repeat or to make valid comparisons with these studies. Therefore, it is critical not only that these factors are considered during the planning and execution of a

11. <https://www.nist.gov/programs-projects/dosimetry-standardization-radiobiology-archive>

radiation efficacy study, but also that they are reported in the published work. It is important to learn from the laboratories that do publish these details and researchers should assess the available resources at their institution that will allow them to perform proper quality assurance. The most reliable first step begins with careful oversight by an in-house radiation physicist who can conduct reliable dosimetry and provide guidance to experimental design. This is more valuable than relying on historical dosimetry calibrations or data supplied by the device manufacturer.

In 2016, the RNCP program held a meeting to seek input from the research community to establish a portfolio-wide dosimetry harmonization effort to address confounders associated with variations in radiation exposure studies and dosimetry assessments across laboratories funded by the NIAID. In 2019, NIAID released a Request for Proposals contract solicitation to assess the current state of radiation dosimetry within the RNCP and work with program awardees to ensure accurate dosimetry at each research site. Ultimately, the RNCP hopes to develop a consistent means of comparison and reproducibility across institutes, enabling better comparisons of data generated by different laboratories.

Conclusions

Because the outcome of small animal studies to address the impact of a mitigator on radiation-induced injury can be important go/no-go decision points during the assessment of biomarkers or development of MCMs to treat radiation injuries, it is especially important that all the potential confounding variables be considered in study designs. This includes not only variables surrounding the animals themselves and their care, but also factors concerning the radiation source and its attributes. It is incumbent on funding agencies, as much as possible, to ensure that research carried out with government support considers the potential impact of these variables on study outcomes. By the same reasoning, journals could require authors to document important scientific study variables in their manuscripts, to enhance reproducibility. By calling attention to these issues, the authors hope this publication will alert researchers to potential variables that might be encountered, so that appropriate planning can be done before the study is initiated. If the potential confounders delineated here are addressed in a way to minimize their impact, the reliability of the research and study outcomes can be improved.

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Table 1.

Important confounders to consider when planning murine radiation studies.

Confounder	Potential Impact	Mitigation Strategy
<i>Study variables</i>		
Exposure model	High	Review literature to select appropriate model for study and Identify optimum radiation exposure dosing for the model
Timing of irradiation	High	Irradiate animal cohorts at the same time of day and within a 3–4 hour window
Statistics	Moderate	Minimize bias with blinded studies, ensure animal numbers in each study arm are sufficient to provide statistical power
<i>Animal variables</i>		
Age at time of irradiation	High	Use animals consistently within a narrow age range (1–2 weeks) and be aware that younger and older animals may have different radiation sensitivity
Sex	High	Conduct studies in both male and female animals and compare data
Strain	Moderate	Select strain appropriate for study design (e.g. prone to develop endpoint of interest), establish radiation dose response curves for selected exposure model
Seasonality	Low	Transport conditions at different times of year may impact study outcomes so plan studies with this information in mind
Vendor	Moderate	When possible, order animals from one breeding room at one vendor site
<i>Husbandry variables</i>		
Diet and nutritional access	Moderate	Investigate chow ingredients and possible impact of known dietary mitigators; be aware of dental injuries and assist animals unable to access standard chow
Housing (single vs. group)	Moderate	Group animals in cages when possible; combine animals from different treatment arms in the same cage to avoid bias
Animal handling	Moderate	Minimize handling of irradiated animals; consider weighing less frequently
Use of anesthesia	Moderate	Minimize anesthesia use, irradiate within a jig so not needed during exposure
Route of administration	Moderate	Minimize number of injections; rotate injection volumes and sites; Limit gavage use post-irradiation
<i>Medical management</i>		
Water (e.g. acidified)	Moderate	Determine facility water source and report in publications
Antibiotics use	Moderate	Understand potential mitigation of radiation lethality with selected antibiotic(s)
Euthanasia criteria	High	Establish prior to study; Individuals assessing animal status Should be independent
<i>Radiation variables</i>		
Source	Low	Ensure same irradiator/radiation type is used throughout a study. Be aware of irradiators used in other studies when attempting comparisons
Exposure geometry	Medium	Consider directionality and orientation of animals relative to radiation source; Report exposure geometries in publications
Dose rate	Medium	Maintain a consistent dose rate across studies; avoid very high or very low dose rates for MCM efficacy studies to enable comparison with other research
Shielding	High	Measure exposures in shielded areas with dosimetry; Report details of shielding in publications
Radiation dosimetry	High	Consider pre-/in-run-/post-exposure dosimetry as appropriate, to ensure correct dosing; report methods in publications