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Refining bone marrow ablation and reconstitution in mice

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Abstract

This report presents findings from a group of UK-based researchers with expertise in the use of animal models for bone marrow ablation and reconstitution. The primary aim is to facilitate the implementation of the Three Rs (Replacement, Reduction and Refinement), with an emphasis on refinement. Bone marrow ablation and reconstitution procedures are performed for a number of different purposes and conducted predominantly in mice. These procedures can induce significant suffering, classified as "severe", Category E or Category D/E under European, US and Canadian legislation, respectively. Although severity categorization is not mandated in countries such as Australia and New Zealand, legislation still requires that the level of animal suffering must be minimized to the greatest extent possible. This report identifies specific animal welfare issues and proposes practical measures aimed at reducing both animal use and suffering.

INTRODUCTION

Bone marrow ablation and reconstitution procedures are performed for a number of different purposes, including studies of immune system function, aging and cancer biology. The procedure involves the ablation of hematopoietic tissues within live mice, using irradiation or chemotherapy, followed by reconstitution using stem cells derived from bone marrow from source animals.[1,2] These animals are commonly referred to as recipients and donors, and this report adopts these terms to reflect common usage.

This resource provides practical guidance on refining procedures involving bone marrow ablation and reconstitution in mice, referring primarily to the use of irradiation, unless stated otherwise. "Refinement" in this context means reducing suffering and improving welfare throughout the animals' lives by reviewing and enhancing all aspects, including scientific procedures, housing, husbandry, care and humane killing. This report complements the literature by providing information on good practice that is often not included in publications. The intended audience includes researchers, animal technologists, ethics or animal care and use committees, veterinarians, funding bodies, regulators and anyone designing or reviewing studies involving bone marrow ablation and reconstitution worldwide.

The topic was chosen because it can lead to severity classified as "severe", Category E or Category D/E under European, US and Canadian legislation, respectively. In countries where severity categorization is not required by law, such as Australia and New Zealand, the role of the Animal Ethics Committee (AEC) must take into consideration the levels of suffering. For example, NHMRC guidelines state that particular justification to the AEC must be provided for activities that involve "severe compromise to animal wellbeing". In bone marrow ablation and reconstitution many adverse events, including mortality, are avoidable. Following this flow diagram (Figure 1), and implementing the information on refinement at each stage, will enable good planning and practice to prevent "severe" suffering.

> *Note*: The 'R' of Replacement, within the 3Rs concept of Replacement, Reduction and Refinement, requires methods not involving living animals to be used wherever possible. This is also a legal requirement in most countries. This guidance assumes that there is currently no scientifically viable alternative approach to achieving the scientific objectives.

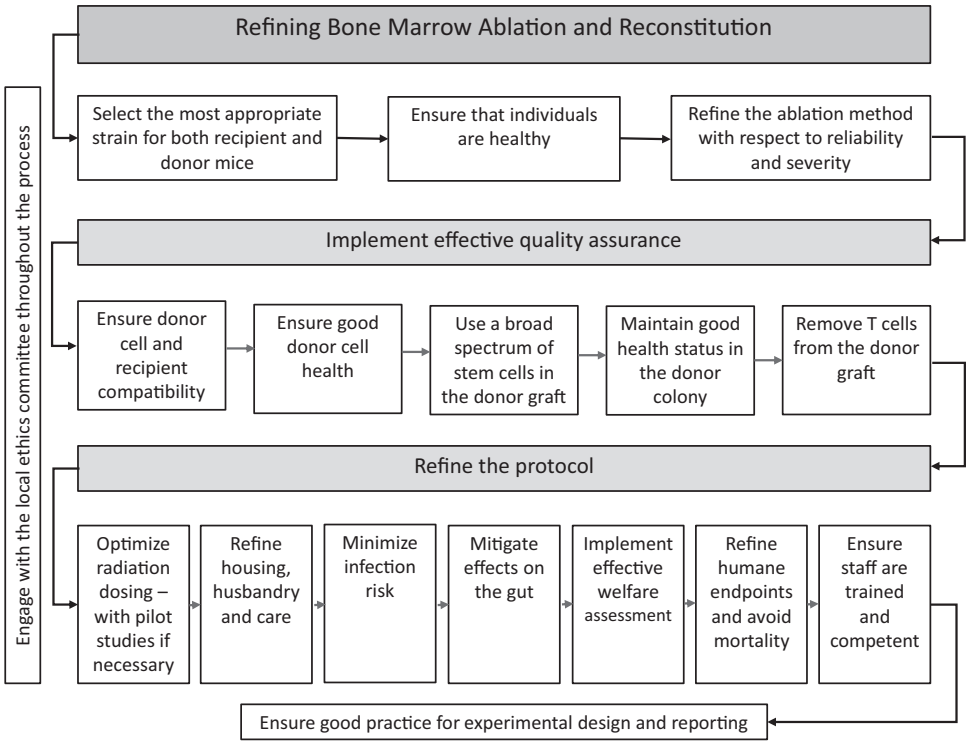

Figure 1. A flow diagram of how to refine animal models of bone marrow ablation and reconstitution at each stage.

SELECT THE MOST APPROPRIATE STRAIN WITH RESPECT TO RESILIENCE

Mouse strains can vary significantly regarding their resilience to radiation and/or their response to reconstitution, and researchers may select mouse strains based on their research goals and the desired level of sensitivity to radiation effects. Sensitivity to gamma irradiation across all strains can also vary depending on the specific endpoints being measured and the experimental conditions. Doses should be adjusted accordingly to mitigate radiation sickness as much as possible.[2]

The C57BL/6 strain of mice is one of the most commonly used in research and is known for its relative resistance to gamma irradiation. This strain has been extensively characterized, and its genetic background makes it a robust model for various studies.[3] However, it may be appropriate to use less resistant strains, for example, if they are the most appropriate disease model for the study. While it is generally possible to reduce the dose of irradiation, this approach will require a harm–benefit analysis as immune compromised inbred strains also have inherent welfare problems and require specialist care. For example, BALB/c mice are sensitive to whole body irradiation, and immune compromised mice are more sensitive than wild type strains to gamma irradiation.[4] The SCID strain is also characterized by its compromised immune system, making it more susceptible to radiation-induced effects.[5] This strain is commonly used in research focused on studying the effects of irradiation without interference from a fully functional immune response.

There may be a tendency to use male mice, as they are generally larger than females within a given strain, based on the assumption that larger animals are better able to cope with ablation and reconstitution. However, this introduces sex-related bias into experiments. It is now widely accepted that both sexes should be used in experimental designs, except in studies on single-sex mechanisms or diseases such as prostate cancer.[6] It is more important to ensure that individuals are healthy.

Careful consideration should be given to the scientific and animal welfare implications of using different strains, with input from animal technologists, the attending veterinarian and AEC as appropriate. If it is not feasible to select more resilient strains or animals for scientific reasons, the procedures should be refined in other ways, such as adjusting radiation protocols.

ENSURE THAT INDIVIDUALS ARE HEALTHY

It is important to ensure that individual mice are physically fit for the protocol. A clinical examination should be performed before irradiation and animals excluded if they do not pass predetermined criteria. For example, minimum weights of 18 g for male mice, and 16 g for females, are suggested unless there is special scientific justification for smaller or younger animals. There is also some evidence that antigen presenting cells in recipient mice mediate an increased incidence of Graft Versus Host Disease (GVHD) as the recipients age.[7] We therefore suggest an optimal age range of 7–12 weeks for performing bone marrow ablation, unless there is special scientific justification for using older animals.

During the course of bone marrow ablation, recipient mice will be immunocompromised and therefore prone to opportunistic infections. A colony housed in facilities with good biosecurity, pathogen exclusion policies and effective monitoring for potential pathogens is essential for reproducibility over time and meaningful comparisons of experiments between units. Knowledge of the prevailing spectrum of gut microbiota is also increasingly seen as important for many areas of immunological study.

REFINE THE ABLATION METHOD

In general, bone marrow ablation is performed using ionizing (gamma) radiation, x-ray irradiation or chemotherapy, e.g. using busulphan. In each case, DNA strands are broken, leading to cell necrosis or apoptosis. Rapidly dividing cells, such as those in the bone marrow and gut epithelium, are most affected. While the bone marrow is the target tissue, off-target cell death in the gut is likely to produce side effects. The use of ionizing radiation is becoming less common in some countries due to human health concerns, although it produces more consistent outcomes, which is preferable from an ethical perspective. Although both x-ray irradiation and chemotherapy are less established methods than ionizing radiation, x-ray irradiation is more reliable than chemotherapy, so may be preferable on ethical grounds, as fewer animals will be needed. The authors are not aware of any differences in severity between x-ray irradiation and chemotherapy.

QUALITY ASSURANCE

Effective quality assurance measures in the selection of donor and recipient animals, and throughout the grafting process and after-care, will help to reduce both animal suffering and wastage.

Donor cells and recipient compatibility

The need for immunocompatibility between the cell source and recipient's genetic strain applies to both background strains and any genetically altered lines created from these. Only mice of the same background, for at least 10 generations, should be used to produce bone marrow chimaeras. If the donor cell source and recipient are genetically divergent, the resulting graft is termed allogeneic. Recipients of allogeneic grafts are at risk from GVHD, where source cells attack recipient tissues, especially the skin, liver, gut and lymphopoietic system. This is most severe when all major histocompatibility loci are mismatched. Additionally, sex-mismatched donors and recipients, especially female-to-male transplants, can lead to chronic inflammation in male reproductive organs, as female immune cells react against male-specific antigens, damaging tissues such as the testis and prostate.[8] Same-sex transplants do not trigger this immune response, suggesting a clear benefit in matching donor and recipient sexes.

In some instances, allogeneic grafts are used deliberately to study the pathology of GVHD.[9] Unintended GVHD is preventable and should not happen. There is a risk of "accidental" allogeneic grafts if source cells from one facility are given to recipients from another, even if the mouse strains appear to be the same. This may be caused by genetic drift, which can occur in inbred mice to the extent that an amendment is required to the usual strain suffix, for example C57BL/6J. Commercial breeders offer screens to assess genetic divergence from parent strains, and may add a further suffix to denote separation from the parent stock, e.g. C57BL/6J/Babr. If units do not do this, there will be a risk that spontaneous mutations or divergence may not be detected. Less well-managed colonies may also not refresh pedigrees to maintain genetic integrity, for example, through the use of frozen embryos or imports from the parent colony. Opportunist mating between individuals of the wrong strain can also occur. All of these situations can, and should, be prevented. If unintended GVHD occurs, a full systems review should be initiated immediately to ensure that processes are in place to stop re-occurrence. In cases where genetic divergence cannot be determined, strains should be back-crossed for a minimum of eight generations before creating a chimaera.

If the host's (recipient's) array of immune cells have been insufficiently ablated prior to reconstitution, then allogenic grafts may be attacked and rejected, resulting in

host versus graft disease (HVGD). The dose of radiation that is both effective and safe is therefore relatively narrow.

Cell source and health status

Good donor cell health and good handling *in vitro* are vital to ensure both graft viability and sterility. For fresh isolation of bone marrow, preparations with < 90% viability indicate that the cells are in poor condition, so ideally new preparation of bone marrow should be performed. If this is not possible, injecting a higher number of cells can compensate to some degree.

Cells can be frozen and stored, or transported, to achieve reduction in overall animal usage, for example, by freezing bone marrow and storing in liquid nitrogen for transport and thawing before use (Box 1). However, this process may result in a loss of up to 50% of the cells, which must be planned for. Transport between facilities also requires careful attention to biosecurity and biological contamination of donor cells, to prevent wastage, experimental artifacts and recipient sickness due to infection transmission. There may also be a risk of incompatibilities due to genetic drift in different facilities, so vials should be screened by an importing unit using PCR tests to ensure compatibility of presumed syngeneic grafts. There may be issues with customs and border controls when transporting between countries, which can lead to delays and cells being damaged or destroyed. Transporting bone marrow in intact bones in media, which can survive for up to > 16 h, can mitigate some of these effects. However, after 40 h of storage in the bone, a dramatic and significant loss of mature cells is observed.[10]

Box 1. Cryopreservation protocol

- Freeze cells in cryo-protective media and store in liquid nitrogen.
- Transport in intact bones if possible, ensuring biosecurity and minimizing contamination.
- Use PCR tests to confirm genetic compatibility of donor cells before use.
- Thaw frozen cells in a 37°C water bath.
- Wash cells twice in cold phosphate buffered saline.
- Centrifuge washed cells at 400 *g* for 5 min to eliminate DMSO.
- Filter cells through a sterile 40-μm strainer.
- Resuspend cells in sterile media at a concentration of $1.0–5.0 \times 10^7$ in sterile media and inject 100 μL intravenously.

If frozen fetal liver cells are used for reconstitution, these should be frozen in cryoprotective media. For example, fetal liver cells can be frozen in RPMI 1640, 10% fetal bovine serum (FBS), and 5% dimethyl sulphoxide (DMSO), then stored in liquid nitrogen. There are animal welfare and ethical implications of FBS, and until non-animal sera are available, efforts should be made to reduce the proportion of FBS when freezing fetal liver cells. To prepare cells for injection, they should be thawed in a 37°C water bath, washed twice in 50 mL cold phosphate buffered saline, then centrifuged at 400× *g* for 5 min to eliminate DMSO. The cells should then be filtered through a sterile 40-μm strainer to remove clumps and to ensure smooth administration. For optimal reconstitution, the cells should be resuspended in a sterile media at a concentration of $1.0–5.0 \times 10^7$. The injection volume should typically range from 100 to 200 μL injected intravenously per recipient, depending on the experimental needs. It is crucial to inject the cells immediately after preparation to maintain viability and prevent sedimentation. Sterile techniques should be used throughout the process to minimize contamination risks.

Before donor cells are imported, the receiving institution should perform an ethical due diligence assessment to ensure that animal housing, husbandry, care, experimental oversight and humane killing standards at the donor establishment are at least as good as their own. This prevents the tacit support of poor standards of animal welfare (see also Box 2).

Suitability of grafts

Using as broad a spectrum (as experimentally possible) of hematopoietic stem cells in the donor graft helps to ensure recipient survival and wellbeing. For example, T-cell lineage depleted grafts may reduce the incidence of

Box 2. Welfare of the "donor" mice

The welfare of the "donor" (source) mice used for reconstitution is also an important ethical issue. These animals will spend their lives in a laboratory setting and may be genetically altered, possibly with a harmful phenotype. Housing, husbandry and care should be tailored and refined to improve the animals' quality of life, and the method of killing should cause the least suffering possible. Reducing animal numbers, wastage and the duration of their lives in the laboratory will also help to minimize ethical issues and harms to animals.

GVHD, but may also lead to recipient immune incompetence. Where pluripotent hematopoietic stem cells populations are to be engrafted, young adult mice (6–12 weeks) are suitable donors, as the number of hematopoietic stem cells decreases with age.

Colony health

It is critical to assess the health status of colonies providing donor animals for hematopoietic stem cells, in the same manner as the recipients. As well as bacterial and parasitic infections, source tissues may be contaminated with intracellular viruses, which are not removed by media washes or filtration. These viruses could then infect recipients, and from there the rest of an in-contact colony. Viruses with a tropism for immune tissues, such as parvoviruses, noroviruses and mouse hepatitis virus (a coronavirus) represent particular risks.

When donor animals and recipient mice are housed in different facilities, the risk of an incompatible transfer, either for genetic or pathogen contamination reasons, is higher than when donor and recipients are co-housed. Colony managers at recipient facilities should not allow living tissues, such as hematopoietic cells intended for inoculation into living animals, to enter a facility unless the disease-free provenance of the donor animals has been established. Aliquots of the donor cells should also be screened by PCR for the presence of pathogen DNA. At the same time, a small aliquot can be cultured without antibiotics to check for both sterility and viability.

Removal of T cells

Removal of T cells from the donor graft reduces the possibility of mice developing GVHD, thereby reducing transplant-related morbidity and mortality. It is important that the depletion of donor T cells obtained *in vivo* is as close to 100% as possible. This will minimize the risk of GVHD due to the residual donor T cells, which play a fundamental role in the immunological attack on host tissues in both acute and chronic GVHD. While the cytokine production pattern of acute GVHD is mostly characterized by TH1 type, TH2 cytokines predominate in chronic GVHD.

REFINING THE PROTOCOL

For mice used in bone marrow ablation and reconstitution protocols, harms associated with procedures include discomfort and stress associated with the ablation procedure, the subsequent adverse effects of ablation (e.g. nausea, lethargy, off-target cell death caused by irradiation or chemical toxicity), discomfort and stress within reconstitution techniques, failures of engraftment, and GVHD or HVGD. These impacts may be exacerbated in the case of genetically altered animals, if the alteration renders them less able to cope with the protocol.

Apart from experimental procedures and their impacts, each animal also experiences many other events such as transport, marking for identification, capture, handling, restraint, laboratory housing and husbandry, and humane killing. These non-procedural or contingent harms can be anxiety-inducing, painful or distressing, and may affect the animal's ability to cope with experimental procedures.

The term "cumulative severity" is often used to describe how both procedural and non-procedural effects may accumulate over time and affect how the animal experiences both experimental and husbandry procedures. Cumulative effects may combine to increase severity to "severe", as defined by UK and European Union legislation. However, if each potential harm is recognized and refined, these discomfort, pain, anxiety or stress mitigations can collectively lead to an aggregation of marginal gains, significantly reducing severity. In procedures such as the ablation and reconstitution of hematopoietic tissues where several steps must be conducted in a sequence, there is huge potential to reduce suffering and improve welfare by reviewing every event experienced by the animal, identifying potential harms and considering how each one might be optimally refined. This approach applies to donor mice as well as to recipient animals (Box 2). Table 1 lists procedure-related adverse effects, with suggestions as to how each can be refined.

In some cases, the creation of a bone marrow chimaera may be an endpoint in itself, with animals humanely killed and their tissues used for studies. But in many others, creating a stable graft is just the beginning. Bone marrow ablation and reconstitution may sit within a complex protocol including multiple optional steps, within which animals can still experience adverse effects even post-reconstitution. Examples include surgical models, the administration of infectious agents, induction of diabetes and the induction of cancers (with or without therapies). Tumor induction can include invasive procedures such as intraosseous injections or hormone pellet implantation; the administration of therapeutic agents, e.g. using implanted osmotic pumps; tissue labeling; imaging, which requires general anesthesia; and blood sampling.

Mice used in these protocols may also be genetically altered (both donors and recipients); if the phenotype depends on inducible genes being switched on or off, there may also be adverse events associated with this process (e.g. tamoxifen administration). Protocols may

Table 1. Potential adverse effects and refinements in bone marrow ablation and reconstitution studies.

Potential adverse effect and welfare impact	How this may be refined
Bone marrow ablation	
Transfer into carousel for *irradiation* – stress due to unfamiliar environment (neophobia); noise and vibrations from apparatus; chamber may rotate. Some protocols involve separation from cage mates so that animals can be irradiated individually	• Irradiate animals in the home cage with nesting material *in situ* and cage mates if possible (removing any objects that could affect the beam, such as metal grids) • If animals need to be irradiated in a "pot"/pie, add nesting material • Monitor animal behavior and welfare with cameras • Limit each irradiation period as far as possible • If general anesthesia is required during irradiation (e.g. for partial body exposure) ensure that an appropriate agent is used and it is minimally aversive if gaseous
After-effects of *irradiation* – feelings of nausea and lethargy over the first 10 days post-ablation; animals may stop eating and lose weight and/or become dehydrated	• Consider splitting and/or reducing the doses, especially for immunocompromized animals • Optimize dose *rates* - this is critical • Review the source of radiation - subtle differences in energy level can have significant welfare consequences. Care should be taken as each individual source will produce slightly different energy and pilot studies should always be performed rather than simply replicating protocols carried out in different establishments • Sources of ionizing radiation will decay over time and need to be regularly calibrated (e.g. every 3 months) • Provide soft, palatable food/mash (without antibiotics, unless palatability is maintained), nutrient gels, treats such as oats or sunflower seed hearts (these can be also used as forage enrichment once animals have recovered sufficiently). Food can be provided on the floor for ease of access • Ensure that mice can reach sipper tubes or provide a solid water source • Consider additional nesting material, and/ or transferring the entire nest that was built pre-irradiation, and adjusting environmental temperature, to counter possible hypothermia
Capture, handling, restraint and administration of cytotoxic agent, for ablation via *chemotherapy*	• Capture mice using tunnels or cupped hands instead of by the tail; move into a restrainer for administration of substances • Ensure that administration of substances is fully refined
After-effects of *chemotherapy* – feelings of nausea and lethargy over the first 10 days post-ablation; animals may stop eating and lose weight	• Agent selection is key. Review chemotherapy agents and ensure that the drug of choice has the minimum side effects possible to achieve the objectives of the study • Soft, palatable food as above
Damage to gut epithelium, leading to infection risk post-ablation; potential sickness and gut pain, diarrhea and dehydration	• Approaches to avoid this include: • Providing good quality drinking water, e.g. autoclaved water • Administering antibiotics and/or acidified water, flavored to maintain palatability • If ablating via irradiation, prevent gut damage by fractionating radiation, i.e. splitting the dose • If diarrhea occurs, replacement of electrolytes and fluids lost through palatable additives to drinking water, or active injectable fluid therapy
Reconstitution	
Capture, handling, restraint and administration of hematopoietic cells	• Capture mice using tunnels or cupped hands instead of by the tail; move into a restrainer for administration of substances • Ensure that administration of substances is fully refined • Ensure that injected cells are viable and can contribute to all essential cell lineages

(*Continued*)

Table 1. Continued.

Potential adverse effect and welfare impact	How this may be refined
Failure of engraftment of donated bone marrow cells leading to hematopoetic failure. Anemia, clotting defects, immunodeficiency	• Ensure that injected cells are given intravenously and can reach and populate the bone marrow • Protect mice from adventitious infections until graft is established • Monitor carefully for anemia and other adverse events at critical period of engraftment and humanely kill those with failed engraftment
Host versus graft disease	• Humanely kill animals • Review ablation and reconstitution protocols to assess whether HVGD could be prevented in future
Graft versus host disease	• Humanely kill animals • Instigate full review of relevant processes, including quality controls, to prevent recurrence

well also involve aging animals, given that studies generally last for up to 60 weeks post-ablation. In these cases, there will also be issues with aging and frailty.[11]

It is clearly essential to strive to predict and understand what each animal will experience within complex protocols, involving multiple technical acts, often over an extended period of time. The refinement Roadmap, set out in the RSPCA's Focus on Severe Suffering website,[12] takes a practical, structured approach to itemizing both procedure- and non-procedure related adverse effects and identifying ways of mitigating these.

Optimize radiation dosing

The impact of radiation on animals can be significantly reduced by reviewing and optimizing dose rates, frequency and the number of doses. Essential factors to consider are the necessary total dose of radiation and the maximum dose rates per minute. More intense, higher doses per unit time will cause more tissue damage, so lower doses over longer time periods are to be preferred. This will mean longer in the chamber, which is likely to be a stressful environment, so animals should be well habituated to this prior to radiation.

Survival can be improved when doses are split, and so may chimaerism. Two split doses given 4 h apart is optimal for animal welfare and chimaerism. For senescent or immunocompromised animals, it may be better to administer between three and six lower doses. Whatever the protocol, doses should be approximately 4 h apart. This will maximize the opportunity that a fresh cohort of cells is actively proliferating, because cell cycles generally last about 4 h. When conducting any new protocol in any study, it is good practice to begin with a small number of animals and ensure that the radiation source is properly calibrated. It cannot be assumed, especially when ablating using irradiation, that following a routine protocol from a different laboratory will have the same effects on the animals in your facility. Examples of situations in which pilot, or dose ranging, studies may be appropriate include when using new strains of mice; genetically modified mice on a new or mixed background; or when reconstituting mice with Cre-loxP inducible gene constructs in relation to a particular component of the immune system. Pilot studies are also good practice if it is necessary to irradiate younger mice (< 8 weeks old), because their cells proliferate rapidly and the gut is immature and unstable. All pilot or dose-ranging studies require careful animal monitoring.

Refine housing, husbandry and care

Generic good practice for laboratory mouse housing, husbandry and care is set out in Box 3. These are basic provisions that aim to improve welfare by enabling mice to perform natural behaviors within the constraints of a laboratory setting. It is also widely accepted that better animal welfare leads to more valid and translatable results, because physiological responses to stress and distress are minimized. A recent systematic review has also found that better quality environments for laboratory mice significantly reduce morbidity and mortality.[13] Therefore, housing, husbandry and care for all mice used in bone marrow ablation and reconstitution studies, including donor and recipient animals, should accord with the provisions in Box 3 unless there are compelling scientific or animal health reasons to omit one or more elements.

Box 3. Good practice for laboratory mouse housing, husbandry and care includes

- Providing cages that are larger than the legal minimum.
- Housing in stable, compatible groups.
- Solid flooring.
- Appropriate litter/bedding.
- Adequate nesting material.
- A nest box, unless this causes aggression.
- Structures for climbing/resting, to make good use of available space and enable animals to retreat from one another.
- A varied diet, with the ability to forage.
- Minimal harmful sound and ultrasound.
- Appropriate light levels and regimes, including dawn/dusk ramping.
- Sympathetic cage change regimes, including "recovery" periods between cage change and procedures.
- Sympathetic handling, e.g. capture by hand or tunnel instead of by the tail, and training to cooperate with dosing.

Minimize infection risk

Infection risks can be mitigated by (1) maintaining colonies free from pathogens known to infect immunocompromised mice and (2) using appropriate barrier systems to shield recipients during the "at risk" phase, until reconstitution of all blood cell lineages is complete, which may take 21 days. It is important to carry out regular health screening to ensure that opportunistic organisms are absent, since clinical signs may not occur when animals are fully immunocompetent. Organisms of particular concern include *Staphylococcus* spp., murine *Helicobacter* spp., *Pseudomonas aeruginosa* and murine parvoviruses. As a minimum, it is recommended that colonies providing irradiated recipient mice are regularly screened.

Mitigate effects of ablation on the gut

Bone marrow ablation using either irradiation or chemical methods can affect all tissues, but has the greatest impact on cells that are rapidly proliferating, especially the gut epithelium. Juvenile animals are also more affected than mature individuals, because the former are still growing. Damage to the gut lining can cause diarrhea and dehydration. In some cases, the protection from gut dwelling microbes entering the host peritoneal cavity or bloodstream is lost, causing peritonitis and septicemia.

Approaches to limiting diarrhea following hematopoietic ablation include ensuring high quality drinking water and, in some cases, the administration of antibiotics and/or the acidification of drinking water. Other approaches can be found in the literature, e.g. modulating, or restoring, gut microbiota using fecal slurry collected from equivalent untreated animals and given by oral gavage.[14] These may have merit, and it is good practice to regularly review new approaches, but this guidance will be restricted to antibiotics and water quality because there is long term proof of successful use.

Antibiotics

Irradiation can encourage the translocation of bacteria across the gut wall, which increases the risk of generalized bacteremia and septicemia. Antibiotics have historically been clinically recommended to be added to the drinking water of irradiated mice as prophylaxis against overwhelming bacterial infection, especially by *Pseudomonas* spp., which often persist in biofilms. Commonly used antibiotics include enrofloxacin, ampicillin, trimethoprim sulphonamides and doxycycline. Prophylactic antibiotics may be given in food or water, or by injection, 1–3 days before ablation, and post ablation for 14–21 days. If the antibiotics are being administered via drinking water, flavored formulations or additives such as Ribena will improve palatability. However, it is important to consider carefully antibiotic use, as this may not be necessary in many circumstances. Routine prescribing to compensate for poor hygiene or husbandry is not acceptable, and prophylactic use should be reserved exclusively for cases of absolute necessity. Consult the attending veterinarian on a case-by-case basis for guidance on antibiotic use.

It is likely that most mice will experience neutropenia once irradiated, and antimicrobial therapy is recommended for irradiated human patients once they become neutropenic.[15] However, screening for neutropenia necessitates blood sampling, which means performing a procedure on a potentially debilitated animal, increasing the overall impact (the sampling procedure may require licensing under some regulations). Early antimicrobial intervention has been shown to provide support for irradiated mice, so it could be considered reasonable to provide such therapy without further data to quantify these effects. However, some antimicrobials can wipe out gut flora for long periods, which is detrimental to health and welfare. The need to treat irradiated mice can be regarded as debatable if the donor bone marrow is sterile (which it should be) and all animals are in a suitably clean environment. Antibiotics should not be administered solely for historical reasons (which could relate to previously poor hygiene levels in conventional housing) or because humans may routinely receive them in medical procedures. Decisions around antimicrobials should be made for each protocol,

including a critical review of husbandry practices, agents, doses, the potential to check therapeutic levels and administration routes.

If antimicrobials are necessary, either as prophylaxis or post-irradiation, administration in drinking water prevents handling stress associated with either oral or parenteral dosing. However, there may be a trade-off if the antibiotics do not reach a high enough plasma concentration to have a therapeutic effect.[16] There is also an increased risk of antibiotic resistance if the dosing methods result in sub-therapeutic levels. Surplus drinking water containing antibiotics should not be released into the environment.

Some institutions use antibiotics and acidified water, but this is not appropriate for all agents. For example, enrofloxacin is stable in water and in acidified water, but not in hyper chlorinated water; pharmaceutical grade doxycycline remains as a suspension when mixed with non-acidified water.[14]

Water quality

It is essential to ensure good water quality for all animals. This is important, as poor water quality may contain bacteria or biofilms which pose a health risk to the animal. To prevent contamination of the water, all watering equipment and water should be sterilized prior to use in animal facilities. Water can be treated by reverse osmosis, ultraviolet light or autoclaving.

Some establishments continue to use acidified water to reduce gut microbial counts, but this should not be regarded as an alternative to providing safe and potable drinking water free from contaminants, e.g. by autoclaving. Acidified water is sometimes used to reduce the risk of bacterial infections in irradiated mice with compromised immune systems, with or without concurrent antibiotic therapy. For example, acidified water can be given for about 48 h before ablation, switching to antibiotics on ablation and for the subsequent 10 days, then reverting to acidified water. Acidified water is especially protective against septicemia caused by *Pseudomonas aeruginosa*.[17] However, there is a lack of evidence that solely providing acidified water can fully protect the gut, but it can be used safely and provide benefits if it is properly formulated.

Like antimicrobials, acidified water can affect the gut microbiota and other physiological functions. Both the acid used, and the dose, will be critically important. For example, water with a pH of 2.5–3 should have a rapid bactericidal effect,[18] although a pH below 2.0 has been shown to cause a decline in water intake and reduced weight gain in male mice.[19] The acid used can also affect both neurological function and gut microbiota. Drinking water acidified with H_2SO_4 and HCl appears to affect behavior and gut microbiome in different ways: H_2SO_4 reduces gut microflora diversity more significantly, while HCl causes more significant behavioral changes.[20] It is necessary to check that the water is palatable, stable and stays acidified. Weakly acidified chlorous acid water ($HClO_2$) appears to be stable and maintains antimicrobial activity for 28 days.[21] The attending veterinarian should provide guidance on the appropriate use of acidified water.

Welfare assessment

Welfare assessment protocols should always be tailored to the individual species, strain and project. In the case of studies involving bone marrow ablation and reconstitution, in addition to the issues already discussed, a major cause of experimental failure and poor welfare including severe clinical signs and death, is failure of the donated graft to reconstitute the recipient's bone marrow. It is vital that failed grafts can be detected at an early stage – there is no ethical or scientific justification for keeping an animal alive in such a condition. Highly experienced and competent animal technologists are needed to advise on, and implement, welfare assessment schemes for protocols involving reconstitution.

Table 2 lists some useful welfare assessment indicators, using a format set out by a European Commission working group on severity assessment.[22] These include both objective and subjective criteria. For example, pale eyes and/or extremities compared with control animals indicate anemia, but this can be subjective unless quantified by blood sampling for white cell counts which is an additional, invasive procedure for the animal. Such signs of bone marrow reconstitution failure usually occur at predictable times following engraftment, allowing for scheduled clinical examinations, use of score sheets with predetermined endpoints and rapid action (Table 3).

Body weight should be monitored regularly, especially if animals are group housed. There is generally a "U-shaped" graph of body weight change over time (Figure 2), and animals should regain weight and color after 14–21 days. If the lost weight is not regained, the graft will have failed and the animal should be humanely killed.

Food and drink should also be regularly weighed and measured; although this will obviously provide an average value for group housed animals, good record keeping will indicate when one or more individuals may have a problem. Automated cage monitoring systems, that permit food and water consumption to be recorded from group housed mice, are also available.

Table 2. Useful indicators for welfare assessment of mice and designing clinical score sheets in bone marrow ablation and reconstitution studies.

High level category	Areas to focus on when observing animals	Specific indicators to monitor
Appearance	Body condition	Weight loss
	Coat and skin condition	Pale skin
		Piloerection
		Poor coat condition
		Lack of grooming
		Hair loss around face
		Jaundice
		Skin tenting
		Dry, flaky skin around eyes, and/or on ears, eyes and tail
	Eyes	Pale
	Mouth	Oral mucositis
	Other	Swollen head
		Diarrhea
Body functions	Respiration	Hyperventilation tachypnea
	Food/water intake	Decrease
	Body temperature	Decrease
Environment	Abnormalities in the housing environment	Litter, e.g. evidence of diarrhea or stereotypic behaviour
		Nesting materials, e.g. poor nest construction
		Enrichment items, e.g. may be unused
Behaviors	Social interaction	Isolation from group
	Posture and mobility	Lethargic
		Unresponsive
		Hunched
Procedure-specific indicators	Identified on the basis of the individual project, its potential adverse effects and expected indicators of these	Frail
		Blood clotting abnormalities
		Petechial bleeds under skin
		Red rash around head
Free observations	A severity assessment scheme should always include a facility to note any observations of unexpected indicators of suffering	

Body temperature is a valid indicator of health and welfare. Thermal cameras, non-contact thermometry or RFID temperature transponders can be used for objective measurements. RFID can detect body temperature to 0.5°C and mice tend not to recover if a decrease of 0.5°C is sustained. Some systems may be difficult to calibrate with absolute temperatures, but are consistent within themselves; it is the change that is important. FLIR thermal imaging cameras which plug into mobile phones are another option, although less accurate. Cameras can be used to monitor behavior, both in cages and during irradiation. Although mice should be unaware that they are being irradiated, the chamber may be a stressful environment so animals should be well habituated to this prior to radiation.

If mice have undergone bone marrow ablation and reconstitution, with no other regulated procedures, no adverse effects should be detectable after 3 months. Animals should still be weighed at least once or twice per week in the absence of any observable clinical signs and observed for procedure-specific indicators such as frailty, poor coat condition and pallor.[23]

Humane endpoints

A humane endpoint can be defined as a predetermined limit to the level of suffering an animal can experience within a scientific procedure. This is a defined point at which an experimental animal's pain and/or distress is either ended or reduced, within the context of the scientific endpoints to be met. See www.humane-endpoints.info for more definitions, further information and guidance.

Table 3 sets out some examples of humane endpoints within bone marrow ablation and reconstitution studies.

Table 3. Examples of humane endpoints in bone marrow ablation and reconstitution studies.

Weight loss	Weight loss of 20% as an absolute maximum (15% wherever possible). If a juvenile animal is still growing, this should be measured against a control growth chart (ideally from animals of the same strain, within the same institution) as the comparison weight
	Failure to put on weight after 14 days. The initial weight loss can be steep (for example up to 15% over 72 h) but this should stabilize, with evidence of improvement by day 14. Further weight loss should not occur after day 14 and normal weight should be regained by day 21
	Local conditions may influence this humane end point and individual establishments may have additional local controls. Some will choose to monitor weight loss over a period of time, for example, if 10% of the body weight is lost within 7 days, they would weigh daily for the next 3 days and implement an endpoint if the animal does not begin to regain weight
	A body condition score of $< \frac{2}{5}$[20]
Anemia	Graft reconstitution failure is indicated by clotting abnormalities, petechial bleeds under the skin and especially by anemia. Pallor is typically observed between days 5 and 10. In dark skinned strains of mice the nail beds and mucous membranes should be monitored; in white mice the eyes and ears become pale
	Host Versus Graft Disease may lead to aplastic anemia, indicated by pallor, lethargy, weight loss, hunching, decreased temperature and decline in overall health
Graft Versus Host Disease	Weight loss, diarrhea, skin lesions, frailty and anemia beginning from 14 to 21 days and persisting
General sickness	A combination of indicators such as hunched posture, lethargy, dehydration and lack of grooming
	Weak, or no, response to external stimuli, or swollen oral mucosa, are signs of severe suffering
Body temperature	A fall of > 0.5°C, with non-recovery or a further fall in temperature
Respiration	Hyperventilation, with pale extremities and piloerection, indicates failure to reconstitute (likely severe anemia)
Effects of radiation	Mucosal damage, cutaneous lesions, radiation sickness

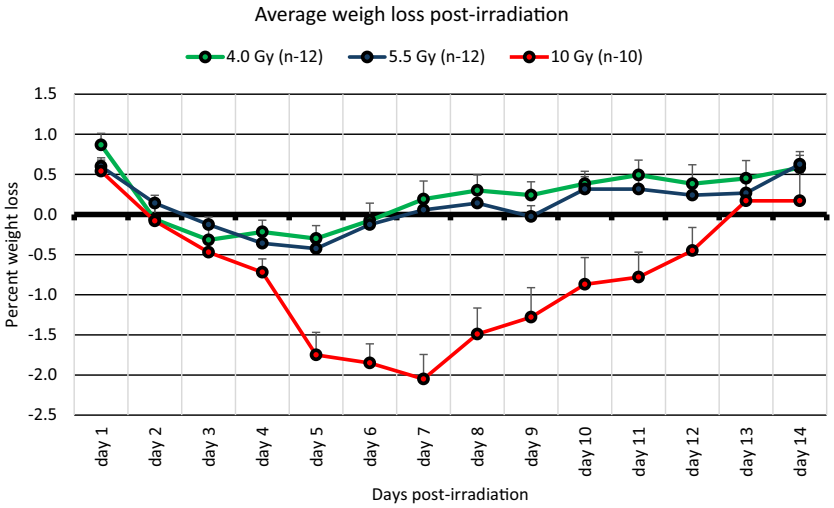

Figure 2. The average percentage weight loss observed post-gamma-irradiation in mice at different doses. This was done using an equal number of males and females for each condition. The study was performed at the Babraham Institute, with mice housed under specific-pathogen-free conditions.

Animals should usually start to recover a functional immune system within 2–3 weeks if a graft has been successful. Weight loss should be the only adverse effect; animals should not become significantly sick.

Avoiding mortality

Under some regulations (e.g. the UK Animals (Scientific Procedures) Act 1986, and EU Directive 2010/63), it is assumed that severity was "severe" if the animals die, unless there is evidence otherwise. Exceptions can be made in some cases, for example if death is rapid and observed, without signs of suffering, or if knowledge of the model indicates that suffering was unlikely. In the case of animals used in procedures involving bone marrow ablation and reconstitution, mortality is likely to be associated with severe suffering and stringent measures should be taken to avoid this.[24]

Mortality as a result of irradiation should not occur if dosing is appropriately optimized. It has also been reported that intraperitoneal administration of ascorbic acid, an antioxidant and free radical scavenger, is a radioprotective therapy which reduces mortality in C57BL/6 mice.[25] However, this is no substitute for optimizing radiation doses unless there is a justifiable scientific reason why this is not feasible.

Deaths due to infection post-reconstitution may occur if sorted stem cells are not sterile, for example, if a cell sorting machine has become contaminated. This may introduce bacteria, fungi, viruses or other pathogens that can lead to severe infections, compromising the health of the recipient and triggering an inflammatory response. Some immediate effects may occur within hours to a few days after the injection, such as changes in behavior, lethargy or signs of infection. Subacute effects, such as the development of localized or systemic infections, may manifest over the course of days to weeks. Infection may be preventable by testing aliquots of cells for bacteria after sorting, but it is usually necessary to implant them immediately to conserve viability.

Duran-Struuck and Dysko[2] sets out the following checklist for use if recipient mortality increases with suspected failure of engraftment:

- Evaluate the experience of personnel delivering the graft
- Reassess the number of cells in the inoculum
- Investigate whether the donor strain has any known molecular deficiencies that could affect homing of hematopoietic stem cells to the recipient marrow
- Request analyses for the variability of the grafted cells and the possibility of HVG disease
- Determine the origin (donor or recipient) of the cells

Operator training

The risks of adverse effects, mortality and failed engraftment can be significantly reduced by ensuring that personnel delivering the grafts have been properly trained and are sufficiently experienced and empathetic. Online training resources and realistic, synthetic mouse simulators with injectable veins are available for trainees to gain competence and confidence before progressing to living animals.[26] These are suitable for both initial and refresher courses.

Once trained and assessed as competent, technicians should regularly self-assess their capability in intravenous injections, both through transparent self-reporting and retrospective analysis of engraftment success. Such programs serve to maintain high standards, where 100% engraftment success rates are unlikely, the proportion of failure ascribed to failed intravenous injections needs to be known. This is particularly the case with lateral tail vein injections in pigmented mice such as C57BL/6, where the vein can be hard to see and easy to miss. Promoting vasodilation using a warming box or by dipping the tail in warm water[24] before attempting venepuncture can help to improve success rates. Continuous refresher training and assessment of staff on a "no-fault" basis is recommended.

High levels of technical skill and care are especially important in procedures with multiple steps, as the risk of cumulative severity applies to contingent harms from poor care and technique just as much as procedural harms themselves. Although we have primarily discussed intravenous administration of substances here, other techniques such as blood sampling require equivalent levels of competence.

GOOD EXPERIMENTAL DESIGN AND REPORTING PRACTICE

Literature reviews have identified serious issues with the design, analysis and reporting of animal use in a significant number of papers. Despite efforts to address this issue, such as the ARRIVE guidelines produced by the UK National Centre for the Replacement, Refinement and Reduction of animals in research (NC3Rs), important information on randomization, blinding, sample size justification, and animal characteristics is still missing from many publications.[27] Without these details, experimental reports lose value when planning future studies and contribute to the reproducibility crisis within science.

We strongly encourage authors to ensure that manuscripts adhere to the ARRIVE guidelines. This is important not only to achieve greater transparency and reproducibility, but also to share good practice about refinement, which is an essential component of the scientific method. Now that many journals publish online, space is no longer an issue and supplementary materials can easily be added if necessary. It is also good practice to include brief information on refinement in posters and presentations, and to incorporate supplementary information into flyers to accompany poster presentations.

We also recommend the PREPARE guidelines, from the Norwegian National Consensus Platform for the 3Rs (Norecopa), which promote good practice with respect to planning animal experiments, including the 3Rs, experimental design and many other practical factors.[28] PREPARE is a valuable checklist with respect to achieving good quality, refined science, and it also helps to ensure

that the information required by ARRIVE will be available when the experiment is written up.

THE ROLE OF THE LOCAL ETHICS COMMITTEE

Local ethics or animal care and use committees (e.g. IACUCs, AECs, AWBs and AWERBs) should be able to advise on applying all 3Rs to projects involving bone marrow ablation and reconstitution. Many such bodies have tasks relating to advising on projects and protocols, and project monitoring and follow-up. Good relations, engagement and two-way communication between researchers and local committees are all necessary to enable effective implementation of these tasks.

In the case of protocols involving bone marrow ablation and reconstitution, committee members will need to be provided with information to enable them to understand the animals' lifetime experiences. This is especially important in relation to discussing humane endpoints, how these were decided, and how they will be implemented. Explaining how animals are likely to be feeling (e.g. lethargic, nauseous, anxious), rather than just stating what will be done to them (e.g. placed into a restraint device and irradiated), will enable good communication between the researcher and committee.

With respect to complex protocols, it is particularly important to ensure that project or protocol applications (or amendments) clearly state steps that are mandatory and which are optional. They should also detail what a typical animal's experience will be, and the maximum number of steps that any animal will experience. Regular retrospective feedback on project progress, including both predicted and unforeseen adverse events, in a supportive environment, will also help to understand and refine complex protocols.

It can be helpful for committees to review annual animal usage statistics, for example, taken from an online animal management system, including actual severity and mortality data. This gives members with different expertise opportunities to review practice and ask questions.

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AUTHOR CONTRIBUTIONS

Penny Hawkins: Conceptualization; investigation; methodology; resources; supervision; visualization; writing – original draft; writing – review and editing. **James Dooley:** Investigation; resources; supervision; writing – original draft; writing – review and editing. **Jessica Rodda:** Writing – review and editing. **Colin Gilbert:** Investigation; resources; supervision; visualization; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST

The authors declare that no conflicts of interest exist.

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