

Technical Note

A Facile and High-Throughput Immobilization Method for Fractionated Radiotherapy of Unanesthetized Mice Bearing Subcutaneous Tumors Using a 6 MV LINAC Clinical Facility

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Simple Summary

Here we describe a facile, cost-effective and high-throughput immobilization technique which enhances animal welfare and enables conducting preclinical studies using clinical facilities. The method eliminates administration of anesthetics which can potentially induce excess stress in animals and influence disease progression. The procedure can be employed for any intervention where animal immobilization is crucial. By applying this protocol, mice can safely be introduced to clinical environments for preclinical studies, leading to more translatable data.

Abstract

Anesthesia is the gold standard for immobilization of tumor-bearing mice before radiotherapy which potentially induces stress and distorts disease progression. Irradiation of preclinical cancer models with clinical MV linear accelerator (LINAC) beams can benefit the translation of new strategies in radiation oncology. However, logistical constraints prohibit widespread use of clinical facilities. Currently, there is no detailed protocol on how to safely introduce mice to a clinical environment to be intervened on using hospital equipment. Here, a facile and high-throughput handling method is described that eliminates anesthesia and enables fractionated radiotherapy of multiple mice simultaneously for high-throughput studies. Subcutaneous breast tumor-bearing BALB/c mice were restrained in plastic restraint cones within a containment tray and received four fractions of 4 Gy X-rays from a 6 MV LINAC source over two weeks (two fractions/week). Both short- and long-term follow-up revealed no identifiable health issues or complications associated with the restraint procedure or radiation exposure in terms of body weight loss, skin burns or body condition scores. This method not only benefits animal welfare but also data quality by reducing stress/discomfort levels and confounding effects of anesthetics. It can be applied to a broader range of studies where mice need to be immobilized before intervention.

Keywords: mice; anesthetics; immobilization; radiotherapy; LINAC; tumor



Academic Editor: Michael I. Koukourakis

Received: 26 September 2025

Revised: 31 January 2026

Accepted: 1 February 2026

Published: 4 February 2026

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1. Introduction

Immobilization of animals is critical to experimental interventions and often requires anesthesia and/or analgesia to obtain adequate restraint and to reduce stress or pain [1]. Anesthesia of mice is complicated because of the animals' size, metabolic rate, and the high risk of hypothermia and hypoglycemia [1]. Preclinical radiation oncology research is one area where immobilization is crucial.

Even light anesthesia before radiotherapy can not only influence stress but tumor progression, leading to distorted results. For example, Jayasundar et al. demonstrated that light anesthesia with pentobarbitals for magnetic resonance spectroscopy studies influences tumor temperatures and intracellular and extracellular tumor pH, whereas these parameters remained constant for mice physically restrained [2]. They recommended that, wherever possible, physical restraint in a suitably designed holder should be practiced for immobilizing animals [2]. In another study, Bezu et al. reported that locally injected anesthetics reduced tumor growth and prolonged survival in several models of tumors established in immunocompetent mice [3].

Radiation therapy (radiotherapy) is recommended for management of almost two-thirds of all solid human tumors [4]. Strategies to enhance efficacy of radiotherapy are being sought to potentiate radiobiological responses and/or reduce side effects to improve patient outcomes. The translation of preclinical studies, however, is slow, partly because preclinical studies often do not resemble clinical practice. Preclinical research with small animal irradiators using low-energy X-rays does not always well represent the high energies from linear accelerator (LINAC) sources used clinically. For example, one area of research where X-ray energy plays a pivotal role is radiosensitization using high-atomic number metallic nanoparticles such as gold (Au), gadolinium (Gd) and hafnium (Hf), where disparate results can result from low-energy X-rays.

The use of such nanoparticles originally aimed to increase local X-ray absorption cross sections of tumor tissue and has a high dependence on X-ray energy [5]. Because this enhancement depends strongly on photon energy, preclinical studies performed at kV energies, where photoelectric interactions dominate, may overestimate the enhancement achievable with MV clinical beams where physical interactions are dominated by Compton scattering [5]. Therefore, extrapolating kV results to clinical practice requires caution [5]. More translatable data and insight can be achieved using high-energy (MV) X-rays from a clinical LINAC source.

However, logistics and animal welfare considerations in preclinical studies constrain methods and study scopes. The majority of studies using clinical beam radiation anesthetize and irradiate mice individually, which is a laborious process requiring a specialized skill set and equipment, and the irradiation of a statistically relevant number of animals can potentially take hours. Not only can this exert excess stress/discomfort on animals, it is often logistically impossible in clinical environments due to the commitment of resources that fundamentally exist for patient care.

Because of these difficulties, most preclinical studies are limited to single-dose radiation treatment, which does not represent clinical practice. A search of the literature in Scopus identified nine papers published since 1975 describing fractionated radiotherapy of mice using clinical LINAC facilities, eight of which were conducted under anesthesia [6–13], while only one was not [14]. An approach enabling immobilization of multiple mice simultaneously for irradiation with a LINAC source, with a thorough description of irradiation planning and dosimetry, has been presented by Karagounis et al. [15]. We present an alternative approach here with additional consideration of institutional logistics and measures of animal welfare.

The described handling method is simple, facile, cost-effective and high-throughput; it safely introduces mice to a clinical facility, eliminates administration of anesthetics and enables fractionated radiotherapy of multiple mice in a reasonably short time (i.e., 28 mice in approximately 20 min). The approach is suitable for any scenario requiring full-body or partial-body irradiation with any radiation source. Animal monitoring, in terms of standard visual criteria, indicated no method-induced distress, which can enhance animal welfare and data quality by reducing stress/discomfort and confounding effects of anesthetics. To demonstrate the utility and conduct an assessment of the method, a common setup of a partial irradiation of the hind quarters of mice bearing subcutaneous tumor was conducted. This is presented for demonstrative purposes and various experimental parameters and limitations, especially with respect to normal tissue toxicities and biological outcomes, need to be considered and are included in the discussion. Mice bearing subcutaneous breast tumors were successfully administered four fractions of 4 Gy X-rays from a medical 6 MV LINAC located in the Royal Adelaide Hospital (RAH) over two weeks using this restraint procedure. No complications associated with either radiation therapy or the procedure were observed. This method facilitates access to clinical environments and can be adapted for any intervention where immobilization is required.

2. Materials and Methods

A total of 28 female BALB/c mice (6–8 weeks old, weight: 17.75 ± 1.07 g) were procured from the Animal Resources Centre in Perth, Western Australia, and housed in the University of South Australia (UniSA) (now Adelaide University) Core Animal Facility, Adelaide, South Australia. The animals were acclimatized to their surroundings and food for a minimum of 5 days and were regularly examined to ensure health prior to any intervention. Animal experiments were performed in accordance with the animal experimentation and ethics guidelines of UniSA (Ethics No: U34-21). A maximum of 4 mice were kept in each ventilated cage with access to food and water *ad libitum*. The mouse 4T1 breast cancer model was induced based on the well-established protocol by subcutaneous injection of 5×10^5 4T1 cells suspended in 100 μ L of PBS (vehicle) into the right flank [16]. The tumor volume at the site was measured with a precision caliper and calculated using the equation: $V = 0.5 \times L \times W^2$ (V: volume; L: length; W: width). Once tumors reached 5 mm in diameter or on day 10, the animals were randomized into two equal groups representing untreated (sham) or irradiated (treatment) conditions, comprising fourteen mice each ($n = 14$). Half of the mice in each group were allocated to a short-term follow-up study, and the other half was allocated to survival monitoring (Figure 1). The animals showing signs of suffering, defined as body weight loss $\geq 10\%$ over 24 h, labored breathing, a body condition score (BCS) $< 2/5$ (based on the commonly used scoring on a scale of 1 to 5 described by Ullman-Culleré et al. [17]), loss of the righting reflex, or inability to eat or drink, were humanely killed. For the survival study, the endpoint decision was made based on the following criteria: tumor volume: ≥ 1688 mm³, tumor diameter > 15 mm in any direction, or bleeding from tumors and deep ulceration, and advice from the animal facility vets with data recorded on clinical record sheets.

Animals were monitored daily according to the criteria described in Table 1, and tumor size and body weight were measured every third day. All details were recorded in clinical record sheets. If a score of 2 or greater was recorded for an individual mouse, the university veterinarian was consulted immediately, and a decision would be made on humanely killing the mouse or to proceed with escalated monitoring frequency (twice daily).

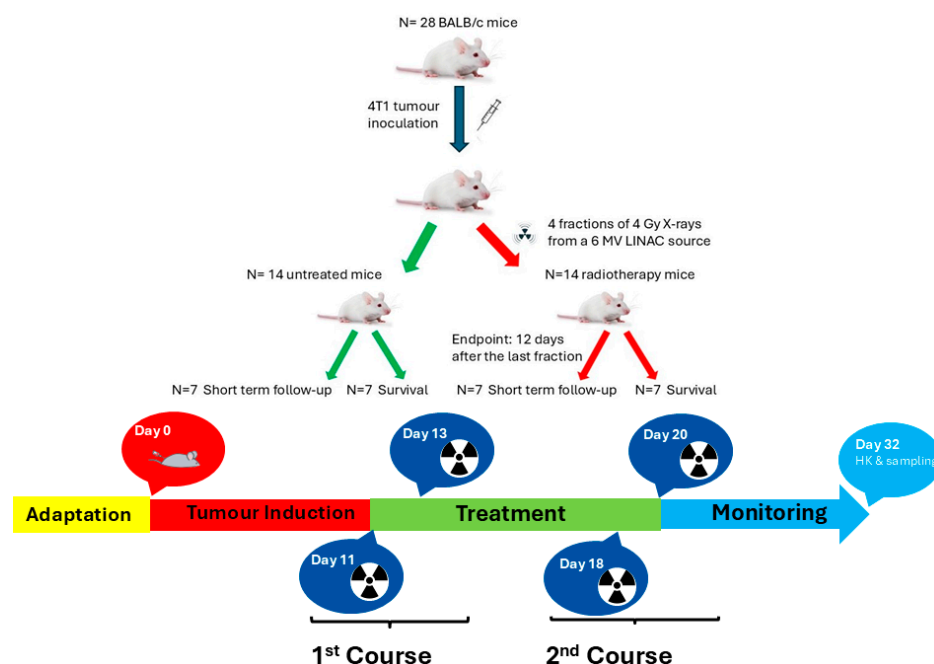


Figure 1. The schematic of the study design. Twelve days after the last fraction of radiotherapy, the animals were humanely killed (HK) for tissue sampling.

Table 1. Monitoring criteria implemented during the study.

		Normal	Moderate Abnormality	Significant/Sustained Abnormality
Score		0	1	2
1	Movement	Active, spontaneous free movement	Lack of spontaneous movement/hyperactivity/occasional ataxia/circling/dragging legs	Reduced movement on stimulation/frequent ataxia, circling, locomotion defects
2	Hunching	Extended back, normal movement and gait	Slight arching on movement	Arched on movement
3	Ruffled coat	Smooth coat, very mild ruffling (for NSG/NRG)	Mild–moderate ruffling	Significant ruffling over entire coat
4	Abnormal breathing	Normal breathing in character and frequency	Increased respiratory rate or slight increased effort	Marked increase in respiratory rate with marked effort
5	Skin tenting/eyes sunken	Soft, pliable, elastic, bright eyes (>75% open)	Skin crinkled, slight tenting when skin lifted	Skin tenting and sunken eyes
		NO (not present—leave blank)		YES (present—score of 1)
6	Tumor inflammation/ulceration	Unbroken/clean, dry skin covering tumor		Wet tumor surface, redness, swelling, scabbing,
7	Dermatitis	Clean, dry, normal skin appearance		Redness, scaly appearance, inflammation, irritation

As the transport distance between the animal facility and the RAH is less than a kilometer, mice were transported in the original cages placed inside a transport crate (two cages per crate) and secured with soft padding material between the cages and the crate. A fine net was placed over the transport crate for an additional level of containment and to aid in discretion while not inhibiting airflow. A risk assessment was performed for four key risks foreseen by the hospital team, including potential risks to the animals during transport or during the irradiation procedures, risks to personnel, risk to the hospital

equipment, and any associated risks to patient treatment schedules or patient health due to unforeseen circumstances.

Within the hospital, crates were kept in a closed room where lighting could be controlled and dimmed. “L”-shaped cross-section lengths of plastic were used for sealing underneath doors for additional containment. Individual cages were transferred into a larger containment crate, and the mice were then individually restrained in conical plastic AIMSTM Rodent Restraint Bags (Figure 2). The size of the opening was found to be highly variable, such that mice could climb through some openings. Thus, a hot iron was used to seal the plastic to have uniform sized holes sufficiently small to prevent mice from climbing out. Meanwhile, a fine (≈ 5 mm) bird net along with plastic and cardboard tubes were on hand to assist in capturing any mouse that could have escaped.



Figure 2. The setup used for restraining mice and their arrangement for irradiation. (A) The original cage of mice was double-contained in a large crate to reduce risk of escape. (B) A small opening at the end of the cone allowed for breathing; the mice were positioned using the bolus material.

For some mice, having a light placed at the end of the restraint cone encouraged them to enter the cone. Once inside the restraint cone, the cone was fastened with a thin wire tie, with the tail protruding. Restrained mice were arranged within a containment tray custom-made from 6 mm thick Perspex with a countersunk lid. Mice were interspersed with dental putty as bolus material (Figure 2).

The bolus material assists in positioning mice across the radiation field and increases X-ray scattering to provide a more uniform dose distribution in the tumor volumes. A red plastic film was used on top of the containment tray to improve the animals' perception of being under cover as they are less sensitive to red light [18]. Next, the containment tray was carefully positioned on the LINAC couch (Figure 3).

Radiation treatment was planned on the RayStation treatment planning system (RaySearch Laboratories, v10b), utilizing a planning CT image set and a LINAC 6 MV beam model. Two rectangular fields were planned of 3×30 cm (indicated by the illuminated field in Figure 3A), defined by multi-leaf collimators, at gantry angles of 0 and 180 degrees (with dose evenly split between each geometry), which increased dose uniformity within the region of interest, compared to the use of one beam. The field led to irradiating 7 mice simultaneously. The predicted dose was confirmed using EBT3 Gafchromic film in mouse phantoms.

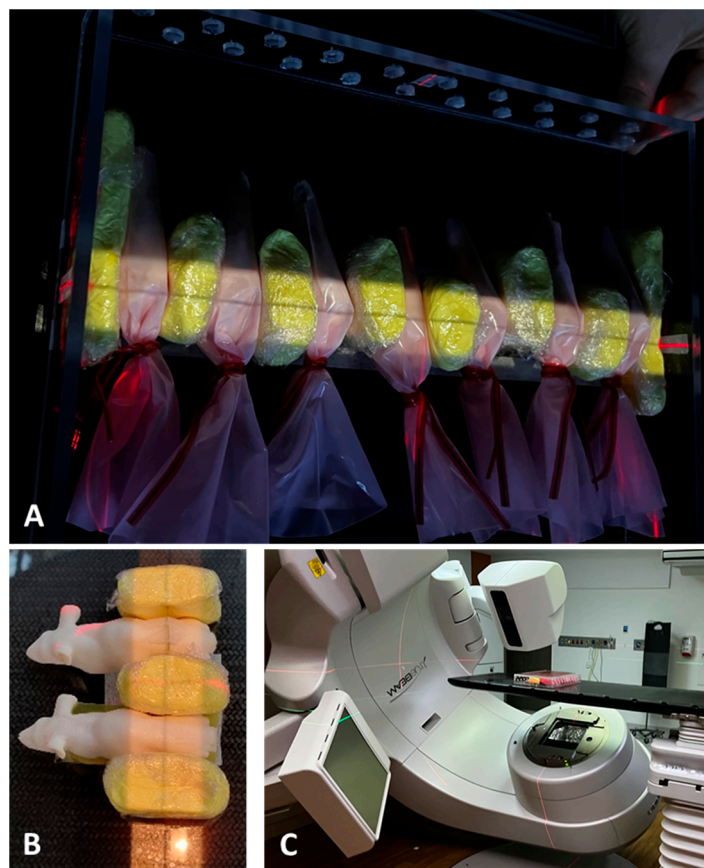


Figure 3. The restraint setup used to irradiate mice in the RAH. (A) Mice were positioned so tumors were in the illuminated radiation field. (B) To verify the deposited dose in the tumors and precision of the setup, dosimetry was performed using 3D-printed phantoms. (C) The LINAC irradiation of mice.

The planning system calculated acceptable dose uniformity in all directions within the phantoms ($\pm 5\%$) and at all possible animal positions (Lt/Rt and Ant/Post) within the rectangular field in which an animal may be positioned, excluding the beam penumbra in the animal Sup/Inf directions.

Each mouse received a dose of 4 Gy ($\pm 5\%$) per fraction at approximately 6 Gy per minute dose rate from a 6 MV LINAC (TrueBeam-Varian Medical Systems, Inc., Palo Alto, CA, USA). A total dose of 16 Gy was administered over two weeks to the mice, on Wednesdays and Fridays after patient treatments had been completed. A licensed Radiation Therapist operated the LINAC (in “QA mode”), while the Medical Physicist assisted university staff with the tray and LINAC gantry and couch setup within the bunker, ensuring that the Perspex tray aligned with lasers at the isocenter.

The animal facility is equipped with an automatic 12:12 h light–dark cycle, starting with red light at 7 pm. Radiotherapy courses were completed before approximately 6:30 pm, so the animals could almost immediately start their dark cycle after their return. Following each course, the animals were provided with soaked food to enhance rehydration and improve feeding under the assumption that radiation could irritate the gastrointestinal tract. After the last dose fraction of radiation treatment, the animals were monitored for another 12 days and then humanely killed. The follow-up included measuring body weight, tumor size, food intake, and overall health, as defined in Table 1.

3. Results

During the transportation and animal handling in the hospital, no signs of excess stress/discomfort were noticed except urination and defecation by some of the mice due

to minor transportation/handling stress. Additionally, over the course of a test run, the animals were observed in the containment tray for nearly 10 min with no visible signs of stress/discomfort. Meanwhile, the temperature inside the tray increased from 22 °C to 23 °C, sitting within an acceptable range. Each course of radiation treatment took approximately 10–12 min for 2×7 mice, including restraining the mice, irradiating them, and liberating them back into the original boxes. Doses across tumor volumes in mouse phantoms were within 5% of the prescribed 4 Gy (Figure 4).

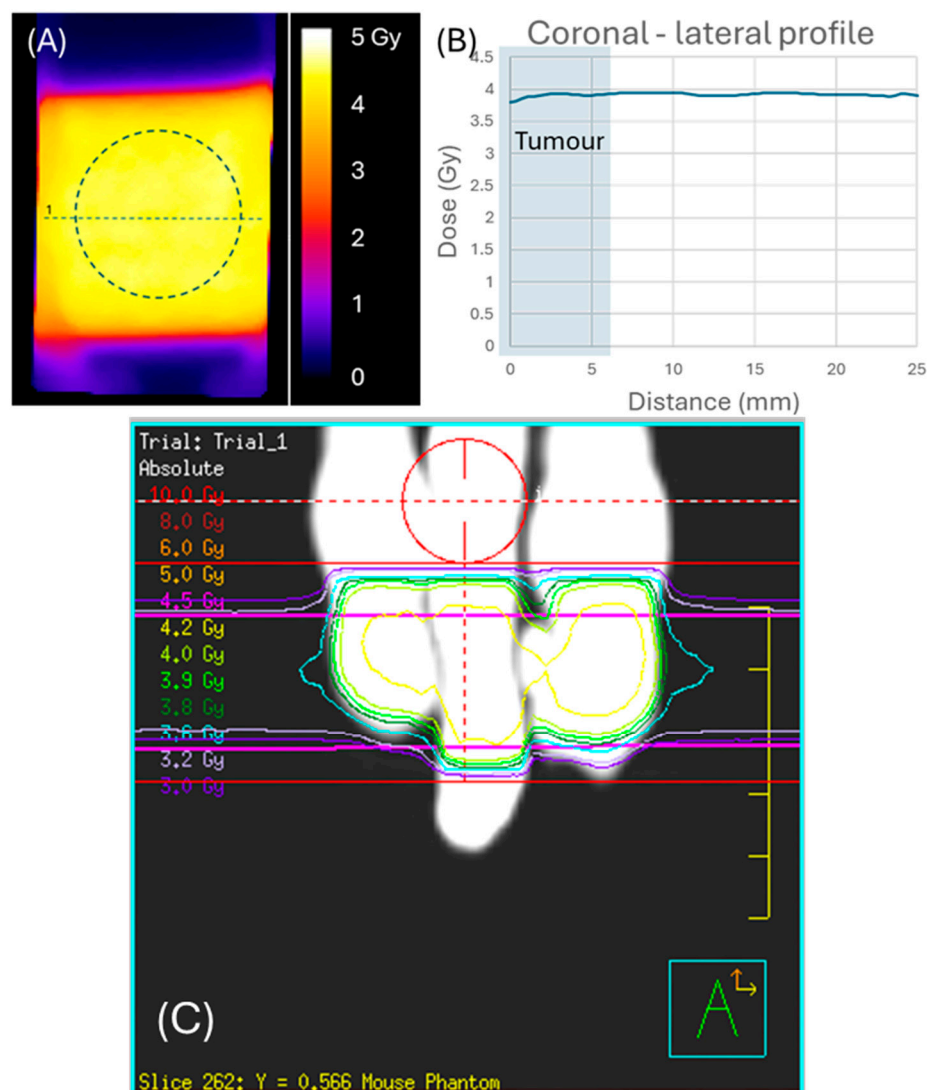


Figure 4. Mouse phantom dosimetry. (A) Gafchromic film image of radiation dose distribution in the coronal plane. The irradiation field across the film is clearly visible. (B) Dose distribution in a lateral profile (indicated by the dashed line in (A)) through the tumor volume. (C) CT image of 2 phantom mice (dorsal view) with bolus in the middle and isodose lines indicated by their respective colors. The tumors received ~3.9–4.2 Gy in the radiation field.

No skin burns or adverse reactions inside or outside the radiation field were observed. No rapid weight change (Figure 5A), sudden death, complications or variation in monitored BCS were observed for the mice that experienced immobilization. Mice were alternatively used for survival studies to determine the therapeutic effects of the interventions. The endpoint in the survival arms was reached because of respiratory distress due to tumor metastasis to lungs and poor animal condition resulting from the breast cancer progression rather than radiation treatment complications (Figure 5B). None of the animals exhibited

diarrhea or dermatitis. Tumor ulceration shape and severity were indistinguishable between arms. Deep cavitations in the center of tumors resulted in wet ulcers, but they were not bleeding or infected. Radiotherapy was found to delay onset of ulceration compared to untreated animals (Supplementary Table S1). On average, tumor ulceration was observed to commence at day 21.50 (± 3.51) in untreated animals compared to day 25.50 (± 3.15) in treated animals ($p = 0.0643$, Student's *t*-test).

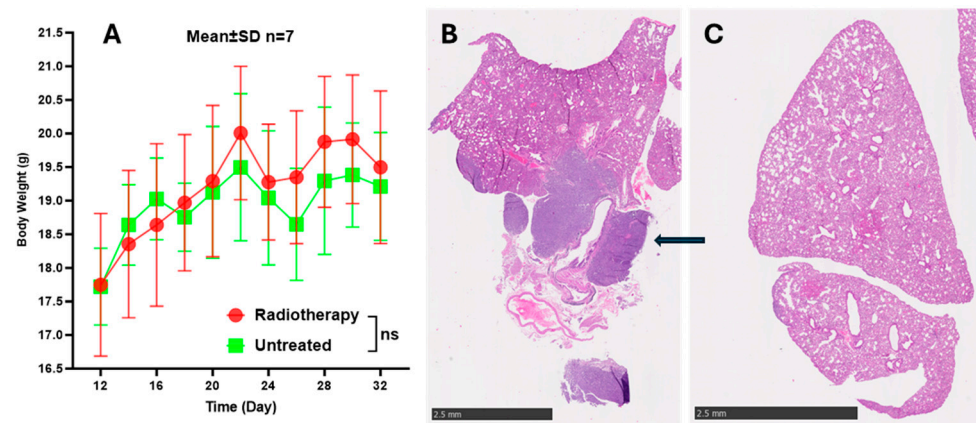


Figure 5. Effect of regimen on body weight and lung metastasis burden. **(A)** There was no significant difference (ns) in body weight between untreated and radiotherapy-receiving mice. **(B)** Histopathological examination of lung sections revealed large diffuse metastatic foci in the untreated mice (arrow); **(C)** whereas the foci were much smaller and sporadic in the treated animals (the right photomicrograph). H&E, scale bar = 2.5 μ m.

4. Discussion

Here we have provided detailed guidelines on introducing multiple mice to a clinical environment to be irradiated by a LINAC facility. Animal welfare and the safety of hospital personnel, patients and equipment were prioritized. Animals received intense monitoring (including daily checks and measurements every third day) against rigorous criteria, as described in Table 1. The entire procedure was overseen by experienced vets to ensure the highest possible animal welfare standards. The regimen was carefully designed and delivered to avoid severe toxicity or mortality. X-ray doses ≥ 6 Gy can induce intestinal injury and subsequently reduced food intake following total body irradiation of mice [19], and thus was avoided in the procedure described here. While irradiation of organs can be expected to induce side effects, no sign of radiation toxicity was observed over the period of this study in terms of body weight loss, skin burns or body condition scores.

This study is significant from several aspects. First, the method eliminates administration of anesthetics, which undermines both animal welfare and data quality. Second, the method is facile and high-throughput, which enables radiotherapy of several mice simultaneously. And third, the technique can be applied to a broader range of interventions where mice immobilization is of crucial importance.

While this report details a procedure to restrain mice with the principal aim of supporting preclinical radiotherapy research, there are many other technical aspects that demand consideration with respect to delivery of radiation. We fully support the considerations for minimal dosimetry reporting put forward by Marcu et al. [20] in response to the review of Draeger et al. [21]. Radiobiology and radiotherapy studies inextricably involve multiple professions and the input of (medical) physicists to ensure appropriate dosimetry must be conducted for accuracy and reproducibility of studies. This further ensures animal well-being and a reduction in animal usage through minimizing experimental variability. The delivered dose, dose distribution and radiobiological response depend on several factors,

including beam parameters and system configurations (energy and energy spectrum, field size, geometry, and source-to-sample distance), scattering/bolus material, the biological tissues irradiated, beam collimation and conformation. Furthermore, there should be dosimetric verification and dose calculation through treatment planning and consideration of positioning accuracy.

Preclinical radiotherapy studies using mouse models are a critical bridge between in vitro research and clinical application. To support this stage, specialized small animal irradiators have been developed, enabling the evaluation of novel radiation-based cancer therapies. Some of these systems feature advanced capabilities such as high-resolution imaging, precise positioning and conformal irradiation. An alternative strategy involves repurposing LINACs, commonly available in healthcare settings, for small animal research [13]. This capacity has been employed in several studies, as cited above. However, the main drawback of these studies is the use of anesthetics and irradiation of animals individually over prolonged procedures. Anesthetics can not only affect tumor growth rate and metastatic burden, they can also skew immune responses, resulting in distorted findings in radioimmunotherapy studies [21,22]. The stress due to long handling times confounds the results further. Tackling these challenges, we detailed a high-throughput handling method with acceptable precision for radiation treatment of unanesthetized mice using a clinical LINAC which minimizes animal stress/discomfort, labor, and handling time. A limitation though of this approach is that radiation delivery is not conformal to a specific treatment volume as is desirable in cancer treatment studies and as is achieved with modern conformal small animal systems. This will induce radiation toxicity in normal tissues dependent on the irradiation field and necessitate careful consideration of the tissue-specific side effects that can result. In other respects, however, this procedure can improve animal welfare and make radiation treatment of mice using clinical facilities logistically feasible and efficient, which in turn could enhance translational radiation oncology research.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/radiation6010006/s1>: Table S1: Clinical record sheet (CRS) score data for individual mice over the experimental course. Radiotherapy delayed tumor ulceration.

Author Contributions: Conceptualization, I.K. and W.P.; methodology, T.T., Q.V.-C.L., W.P., C.B., H.L. and I.K.; software, W.P.; validation, W.P., I.K. and H.L.; formal analysis, A.N., Q.V.-C.L. and W.P.; resources, I.K., W.P. and H.L.; data curation, A.N. and Q.V.-C.L.; writing—original draft preparation, A.N. and I.K.; writing—review and editing, C.B. and W.P.; visualization, A.N.; supervision, I.K., C.B., H.L. and W.P.; project administration, C.B., H.L., W.P. and I.K.; funding acquisition, I.K. and A.N. All authors have read and agreed to the published version of the manuscript.

Funding: The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by Tour de Cure grants RSP-253-18/19 and RSP-607-FY2023.

Institutional Review Board Statement: This work was performed in accordance with the animal experimentation and ethics guidelines of the University of South Australia and approved by the Animal Ethics Committee (No.: U34-21).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is available from the corresponding author upon reasonable request.

Acknowledgments: The authors wish to thank Daniel Ramm, previously at the Royal Adelaide Hospital, now at the ICON Cancer Centre, for film analysis assistance.

Conflicts of Interest: The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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