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Safety and efficacy of a nonmyeloablative pretransplant conditioning regimen using total lymphoid irradiation with volumetric modulated arc therapy in healthy dogs: A pilot study

Sangho Kim[1] | Kenji Hosoya[2] | Natsuki Fukayama[2] | Tatsuya Deguchi[3] | Masahiro Okumura[1]

[1]Laboratory of Veterinary Surgery, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

[2]Laboratory of Advanced Veterinary Medicine, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan

[3]Graduate School of Veterinary Medicine, Veterinary Teaching Hospital, Hokkaido University, Sapporo, Japan

Correspondence
Kenji Hosoya, Laboratory of Advanced Veterinary Medicine, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Hokkaido, Japan.
Email: whitelittlesnow@aol.com

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Abstract

Allogeneic hematopoietic cell transplantation (HCT) has been an effective treatment for human patients with haematological malignancies (Baron & Storb, 2006; Bair et al., 2020; Copelan et al., 2019). However, the optimal pretransplant conditioning treatment is unclear in canine allogeneic HCT. This pilot study aimed to evaluate the safety and efficacy of total lymphoid irradiation (TLI) with volumetric modulated arc therapy (VMAT) for a nonmyeloablative HCT conditioning. Six healthy dogs were treated with 8 or 12 Gy TLI using VMAT. Haematological and physical changes were recorded over 8 weeks. To assess the effect of peripheral lymphocyte condition, lymphocyte subset and proliferative ability were examined. At the end of the experiment, necropsy was performed. All dogs showed mild-to-moderate neutropenia and thrombocytopenia, and these haematological changes resolved spontaneously. One dog treated with 8 Gy TLI developed transient cutaneous infection. No major complication was seen in the other seven dogs. Myelocytes and erythroblast cytopenia of bone marrow were detected in two dogs treated with 12 Gy TLI. This study is the first report of TLI using VMAT in dogs, and results suggest that this regimen is a feasible nonmyeloablative treatment.

KEYWORDS

dog, hematopoietic cell transplantation, pretransplant conditioning regimen, total lymphoid irradiation

1 | INTRODUCTION

In human medicine, allogeneic hematopoietic cell transplantation (HCT) is a critical therapy for haematological malignancies, such as lymphoma or leukemia (Bair et al., 2020; Baron & Storb, 2006; Copelan et al., 2019). In the veterinary field, there are few reports on allogeneic HCT for dogs (Lupu et al., 2006; Suter et al., 2015). Suter *et al.* reported an extended survival in canine case of acute lymphoblastic leukaemia treated with allogeneic HCT (Suter et al., 2015). Although allogeneic HCT has shown promise as a potential treatment for canine haematological malignancies, very few studies have investigated the application of this treatment in the veterinary clinical field.

Traditionally, supra-lethal doses of total body irradiation (TBI) and/or high-dose chemotherapy have been used for pretransplant

conditioning in allogeneic HCT in human medicine (Baron & Storb, 2006; Copelan et al., 2019). The main object in these treatments is induction of host immunosuppression, creation of marrow space for the graft and direct tumour destruction (Baron & Storb, 2006; Diaconescu et al., 2005). Radiation delivery to the tumour sites is not dependent on blood supply and is not influenced by variability in drug absorption, metabolism or distribution. Also, radiation therapy may exert a therapeutic effect on tumour cells that remain after the induction of chemotherapy (Wong et al., 2018). However, these aggressive treatments, which are referred to as myeloablative conditioning, have been associated with higher risk of transplant-related morbidity and mortality (Baron & Storb, 2006). For this reason, these treatments have limited their adaptation to non-comorbid and relatively young patients in human medicine (Baron & Storb, 2006). To expand the application range of allogeneic HCT to elderly patients or comorbid conditions, nonmyeloablative conditioning regimens have been extensively studied in both clinical and experimental settings (Baron & Sandmaier, 2006; Spinner et al., 2019; Storb et al., 1997, 1999). Previous research using dogs demonstrated that 2 Gy TBI combined with immunosuppressive drugs provided a stable allograft with a reduction in transplant-related toxicities compared with myeloablative conditioning (Storb et al., 1997, 1999). This conditioning regimen has been translated to human medicine, and is utilized widely in the clinical setting (Fatobene et al., 2020).

In human medicine, the use of a pretransplant conditioning regimen with TBI has declined for several possible reasons, including concerns about development of secondary cancer and an ongoing shift from viewing HCT as a cytotoxic therapy to more of an immunologic therapy (Wong et al., 2018). At the same time, more targeted irradiation techniques such as total lymphoid irradiation (TLI) or total marrow irradiation (TMI) are being evaluated in preclinical animal experiment and human clinical trials (Rosenthal et al., 2011; Schultheiss et al., 2007; Storb et al., 1999). For TLI, the gross target volume (GTV) mainly consists of major perivascular lymph nodes, spleen and thymus, excluding the bone. For TMI, GTV is marrow-containing bony skeleton. A combination of these two irradiation fields is called total marrow and lymphoid irradiation (TMLI). These conditioning treatments are used according to disease, stage or patient conditions (Lebeer et al., 2020; Schultheiss et al., 2007). TLI is one of the nonmyeloablative conditioning techniques in allogeneic hematopoietic cell or organ transplantation in human patients (McKay et al., 2014; Spinner et al., 2019). The main objective of TLI is to induce sufficient immunosuppression to prevent graft rejection. In the previous study, this conditioning regimen provided graft tolerance by changing the balance of T cells, such as natural killer (NK) T cells and $CD^{4+}CD^{25+}$ regulatory T (T-reg) cells (Nador et al., 2010). Because NK and T-reg cells, which were resistant to radiation, had the capacity to suppress the residual alloreactive T cells, increasing the ratio of these cells to other T cells in the recipients favours allogeneic transplantation. In human clinical studies, nonmyeloablative TLI and antithymocyte globulin (ATG) conditioning is a useful regimen not only for protection against graft-versus-host disease but also for improved prognosis by the graft-versus-leukaemia effect for human haematological malignancies (Kohrt et al., 2009; Rezvani et al., 2015). With TLI and TMLI, there is concern about the lack of direct antitumor effect outside the radiation fields. However, Baron et al. reported the 5-year overall survival rate using fludarabine plus 2 Gy TBI or 8 Gy TLI plus ATG was identical in human patients with haematological disorders who were treated with allogeneic HCT (Baron et al., 2015; Spinner et al., 2019). In this study, the radiation fields of TLI included major lymph nodes and spleen, but not the bone marrow to avoid severe myelosuppression.

Volumetric modulated arc therapy (VMAT), which is a type of intensity-modulated radiotherapy (IMRT) method, combines rotational dose delivery and multileaf collimator (MLC)-based IMRT. In VMAT, MLC shape, gantry speed and dose rate are continuously changed during irradiation. This technique allows a highly conformal dose at the targets while sparing normal tissues and decreasing delivery time compared with traditional IMRT or conformal radiation therapy. Based on these superior characteristics, VMAT has been applied to HCT conditioning treatment using radiotherapy to achieve dose escalation for target organs and dose reduction for organ at risk (OAR) in people (Ocanto et al., 2019; Paix et al., 2018; Springer et al., 2016). To our knowledge, no study has investigated the application of TLI in combination with VMAT in dogs; and the safety and feasibility of such treatment have not been elucidated. Here, we evaluated the feasibility and safety of two difference doses of TLI using VMAT and assessed the effects of TLI on peripheral lymphocyte properties in healthy dogs.

2 | MATERIALS AND METHODS

2.1 | Animals

Six laboratory intact male beagles were used in the evaluation of the safety and efficacy of TLI. The dogs weighed 9.9–13.1 kg (mean, 11.6 kg) and were aged 16–20 months (mean, 18.0 months). These dogs were divided into two groups according to their assigned TLI dose (8 or 12 Gy).

All animal procedures utilized in this study were reviewed and approved by the Institutional Animal Care and Use Committee of our institute (approval number: 17-0007). No abnormalities were observed on physical examination and blood laboratory tests (e.g., complete blood counts [CBCs] and general serum chemistry). CBCs and serum chemistry tests were conducted using a Procyte (IDEXX Laboratories, Tokyo, Japan) and Dri-Chem 7000V (FUJIFILM) respectively.

2.2 | TLI procedure

For the TLI and planning computed tomography (CT) procedure, all animals were anesthetized with propofol (6.0 mg/kg) administered intravenously. Subsequently, animals were intubated and maintained on a mixture of isoflurane or sevoflurane in oxygen. During planning

CT (1–2 weeks prior to TLI treatment), all dogs were positioned in sternal recumbency, and the entire body was immobilized using a vacuum deformable mattress (ESFORM, Engineering System). CT images were acquired from two directions: head first from the dog's head to the thorax (cranial half body) and feet first from the toes to the abdomen (caudal half body) with a slice thickness of 2 mm (Aquilion PRIME, TOSHIBA).

The Monaco 5.11.01 treatment planning system (Elekta) was used for contouring and planning. Heterogeneity corrections were applied in these treatment planning. Target lymphoid tissues were defined as parotid, mandibular, retropharyngeal, superficial cervical, axillary and sternal lymph nodes for the cranial half body CT scan, and mesenteric, medial iliac, sacral, hypogastric, popliteal, and superficial inguinal lymph nodes and spleen for the caudal half body CT scan. These lymphoid tissues were contoured as the GTV. The clinical target volume (CTV) was manually delineated on the adipose and soft tissue around each lymph node. The following organs were contoured for OAR: eyes (including lens), brain, larynx, lungs, heart, liver, kidneys, adrenal glands, stomach, small and large intestine, bladder, ribs, scapula, humerus, femur, vertebrae and spinal cord. Dose prescription to the CTV consisted of a 2 or 3 Gy single dose, delivered once a day for 4 consecutive days to a total dose of 8 or 12 Gy respectively. Due to concerns over excessive dosing of the gastrointestinal tissue, the prescribed dose for mesenteric lymph nodes was limited to 8 Gy. All TLI treatments were planned to use a VMAT technique, and all procedures were delivered using 6 MV photons from a linear accelerator (Elekta Synergy, Elekta). This treatment device was equipped with 160-leaf multileaf collimator, and this equipment that had leaf width of 5 mm and maximum field size of 40×40 cm^2 was used as the beam modification device. In this study, the TLI plan comprised three fields. The head to thorax (cranial half body) area was planned with one field (referred to as cranial field), and the abdomen to toes (caudal half body) area was planned using two fields (referred to as caudal I and caudal II fields, respectively) due to the size of the field. The isocentes were defined separately for each field. Dose constraints were set for target lymphoid tissues, bone marrows, lungs, heart, eyes, kidneys, liver and small and large intestines. Following dose calculations, the prescription doses to the target lymphoid tissues were adjusted and recalculated with reference to the dose volume histograms (DVH). Constraints on the conditioning of organs and target lymphoid tissues were standardized across all dogs. In this TLI plan, no bolus was used. Quality assurance (QA) included verification of the dose distribution using a helical diode array (ArcCheck, Sun Nuclear Co.) and measurements of the absolute central dose using a farmer-type ionization chamber (Type 30013; PTW) and a dosimeter (Ramtec Smart, TOYO MEDIC CO.) for each field. All measurements were analysed using the analysis software (SNC patient software version 6.7.3; Sun Nuclear Co.) using a global gamma analysis 3%/3 mm on relative dose. For the absolute central dose, the ratio of the difference between the theoretical values calculated on the TPS and the actual measured values was assessed. An ACVR board-certified radiation oncologist (K. H.) evaluated the uniformity of each of the TLI plans.

For all dogs, TLI treatments were delivered in one session. As the isocenters of each field were different, dogs were moved when finished at one field irradiation. An anesthetized dog's whole body was immobilized in sternal recumbency using a vacuum mattress. It was placed on the computer-controlled robotic couch-top (HexaPOD evo, Elekta) for the head-first scan. To correct for any set-up errors, CT images were acquired using a kV cone beam CT (CBCT) integrated with a linear accelerator. These images and the planning CT images were then overlaid on the imaging software (XVI, Elekta), and any misalignment of position was measured based on the bone, target lymphoid tissues and OAR. Following automated set-up error correction using the computer assisting six degrees of freedom couch-top, the first field was irradiated for the "cranial half body" scan. The dog was then repositioned for the feet-first scan, and the caudal I and caudal II positioning were corrected using a similar method to that described above. The couch was moved craniocaudally when irradiating or getting the CBCT images for each field. After repositioning, the caudal I and caudal II fields were irradiated sequentially for the "caudal half body" scans. These images using CBCT were acquired for every treatment sessions. An ACVR-boarded radiation oncologist (K. H.) corrected and approved the set-up errors in all fields.

For evaluation of TLI treatment, CBCs and serum biochemical analyses were performed over 8 weeks. Adverse effects in the blood/bone marrow were graded based on the Veterinary Cooperative Oncology Group—common terminology criteria for adverse events v1.1 (Veterinary Cooperative Oncology Group, 2016). The dogs received enrofloxacin (10 mg/kg, PO, q24h) in the event where neutropenia (<3,000 cells/μl) was observed. The necropsy was conducted to assess a histological change of the major organs and lymphoid tissues, including heart, liver, small and large intestines, kidneys, spleen, humeral and femoral bone marrow, and mandibular, mesenteric and popliteal lymph nodes, 9 weeks after completion of the TLI in all dogs.

2.3 | Peripheral blood lymphocyte subset analysis

Flow cytometric investigation of peripheral blood quantifying the percentage of T, B and T-reg cells was performed before, and at 1, 4 and 8 weeks after the TLI treatments. Peripheral blood samples were collected via the jugular vein and peripheral blood mononuclear cells (PBMC) separated using density gradient centrifugation (Lymphoprep, Abbott Diagnostics Technologies AS). The PBMC samples were incubated with fluorescein isothiocyanate (FITC)-conjugated rat anti-canine CD3 (diluted to 1:10 in 2% fetal bovine serum), phycoerythrin (PE)-conjugated mouse anti-canine CD21 (1:10), FITC-conjugated rat anti-canine CD4, allophycocyanin (APC)-conjugated rat anti-canine CD8, PE-conjugated mouse anti-canine CD25, APC-conjugated rat anti-canine FOXP3 or PE-conjugated mouse anti-human CD56 monoclonal antibodies, and were analysed using FACS Versa (BD Bioscience). The lymphocyte population was determined from forward scatter and side scatter plots. The gating strategies for these lymphocyte subsets were as follows:

T-cells ($CD^{3+}CD^{21-}$), B-cells ($CD^{3-}CD^{21-}$), T-helper cells ($CD^{4+}CD^{8-}$), T-cytotoxic cells ($CD^{4-}CD^{8+}$), T-reg cells ($CD^{4+}CD^{25+}FOXP^{3+}$) and NK cells ($CD^{3-}CD^{56+}$).

2.4 | Lymphocyte stimulation test

To evaluate lymphocyte proliferation capability, PBMC was separated from heparinized peripheral blood before, and at 1, 4 and 8 weeks after the TLI treatments as described above. PBMC was incubated with 0.5 μM carboxyfluorescein succinimidyl ester (CFSE) (Vybrant CFDA SE Cell Tracer Kit; Thermo Fisher) for 10 min in the dark at 37°C. After washing the cells, CFSE-labelled PMBC were cultured at 37°C in 5% CO_2 in Roswell Park Memorial Institute 1,640 medium (Gibco by Life Technologies), with 10% fetal bovine serum with or without 1.0 μg/ml concanavalin A (ConA) for 4 days before antibody staining and analysis by flow cytometry. To assess T-lymphocyte proliferation activity, cells were stained with APC-conjugated mouse anti-canine CD3 monoclonal antibody (1:50). The gating strategy for this analysis was that the mononuclear cell population was determined from forward scatter and side scatter plots. CD^{3+} cells were second gated within the mononuclear cells, and the border of the fluorescence intensity of the CFSE was set using a sample without ConA stimulation. The proliferating T-cell (CFSE low) population was defined as the lower fluorescence intensity cells of this border. The percentage of CFSE low population was determined based on the percentage of CD^{3+} cells.

2.5 | Statistical analysis

Statistical analysis was performed using a commercially available statistical program (JMP Pro version 12.1.0; SAS institute and Microsoft Excel 2013; Microsoft). The results are presented as mean ± standard deviation. To evaluate differences in apheresis product counts and characteristics between the three mobilization groups, the Tukey multiple-comparison test was used. p values < 0.05 were considered statistically significant.

2.6 | Cell line validation statement

No cell lines were utilized for this study.

3 | RESULTS

3.1 | TLI dose distribution

Figure 1 shows the dose distribution of each field. All dose information was obtained by planning DVH. Table 1 shows mean, maximum and minimum doses, the median organ dose (D_{50}) and the minimum dose required to cover 95% of each target volume (D_{95}) for lymphoid target tissues in each plan. Table 2 shows planning volume, D_{50} and D_{95} for OAR in each plan.

In the 8 Gy group, mean irradiation time at cranial, caudal I and caudal II field were 228.0 ± 56.5 (range 174–306 s), 207.0 ± 24.8 (range 172–226 s) and 229.3 ± 22.3 (range 198–248 s) respectively. In the 12 Gy group, mean irradiation time at cranial, caudal I and caudal II field were 216.0 ± 38.9 (range 180–270 s), 234.7 ± 25.5 (range 211–270 s) and 305.0 ± 35.7 (range 256–340 s) respectively.

In the 8 Gy group, the difference of mean absolute central doses and mean gamma pass rate at cranial half body (cranial felid) were 2.6 ± 1.5 (range 0.8%–4.6%) and 98.5 ± 0.8 (range 97.4%–99.3%) respectively. The difference of mean absolute central doses and mean gamma pass rate at caudal half body (combined caudal I and caudal II felid) were 2.7 ± 1.6 (range 0.9%–4.7%), 97.9 ± 2.0 (range 97.4%–99.8%) respectively. In the 12 Gy group, the difference of mean absolute central doses and mean gamma pass rate at cranial half body were 6.1 ± 4.0 (range 0.9%–10.4%) and 99.6 ± 0.2 (range 99.3%–99.9%) respectively. The difference of mean absolute central doses and mean gamma pass rate at caudal half body were 1.4 ± 1.1 (range 0.4%–2.9%) and 95.1 ± 3.6 (range 90.2%–98.5%) respectively.

3.2 | TLI-related toxicities

TLI treatment was successfully administered to all six animals according to the planned schedule. The kinetics of peripheral neutrophil counts

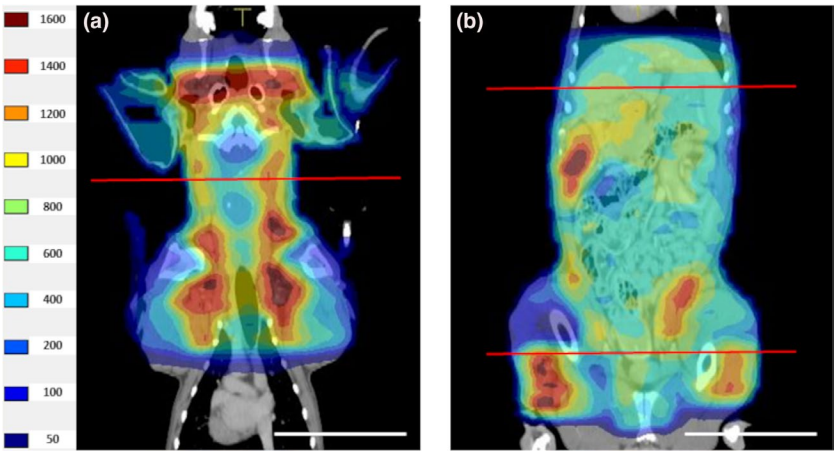

FIGURE 1 Total lymphoid irradiation with volumetric modulated arc therapy dose distribution (colour wash) on coronal view for cranial (a) and combined with caudal I and caudal II (b) field (1 of 12 Gy group dog). The doses (cGy) represented by each colour are shown on the left colour bar. The red and white lines represent each field isocenter and scale bar (10 cm) respectively

TABLE 1 Planned target organ doses

Prescription dose	Lymphoid tissues of the cranial half body	Lymphoid tissues of the caudal half body	Lymph nodes in the abdominal cavity	Spleen
Mean (cGy)				
8 Gy ($n = 3$)	1,164.4 ± 9.4	900.1 ± 25.7	693.4 ± 34.8	974.4 ± 14.8
12 Gy ($n = 3$)	1,550.9 ± 28.6	1,336.3 ± 31.5	741.6 ± 11.9	1,363.1 ± 27.1
Maximum (cGy)				
8 Gy ($n = 3$)	1,433.3 ± 8.0	1,289.6 ± 60.3	1,055.6 ± 124.7	1,706.1 ± 186.5
12 Gy ($n = 3$)	1,822.5 ± 29.3	2,125.6 ± 100.0	1,232.0 ± 17.5	2,387.4 ± 197.4
Minimum (cGy)				
8 Gy ($n = 3$)	895.1 ± 33.6	438.9 ± 63.6	242.9 ± 8.0	305.1 ± 73.5
12 Gy ($n = 3$)	1,142.3 ± 204.4	439.8 ± 152.1	203.3 ± 25.4	422.6 ± 144.9
D_{50} (cGy)				
8 Gy ($n = 3$)	1,092.1 ± 24.2	891.9 ± 29.8	687.3 ± 37.6	941.0 ± 26.0
12 Gy ($n = 3$)	1,467.2 ± 18.4	1,318.5 ± 22.8	733.1 ± 28.8	1,327.3 ± 34.6
D_{95} (cGy)				
8 Gy ($n = 3$)	883.0 ± 30.2	733.4 ± 56.0	543.5 ± 27.4	766.2 ± 43.0
12 Gy ($n = 3$)	1,201.5 ± 111.8	1,022.9 ± 61.0	504.1 ± 31.8	1,058.8 ± 15.2

Note: All doses are reported as means ± standard deviation (cGy).

Abbreviations: D_{50}, median organ dose; D_{95}, minimum dose required to cover 95% of each target volume.

varied erratically in each of the animals following TLI (Figure 2a). Two and three dogs experienced Grade 1 neutropenia in the 8 and 12 Gy groups respectively. One dog in the 8 Gy group developed infection symptoms, including anorexia, pyrexia and an increase in neutrophils, 26 days after TLI. Concurrently, drainage was observed from the previously blood collection site, including hind limb and neck. This dog was treated with enrofloxacin and ampicillin until 43 days following TLI when the signs of infection abated. Peripheral platelet (PLT) counts decreased in all dogs, five of the six dogs were Grade 2 and the remaining dog from the 12 Gy group was Grade 1 (Figure 2b). The median time for the PLT count nadir was 13 days (range 12–14 days) from the commencement of TLI procedures. The PLT counts gradually recovered to the normal range in all dogs. Serum biochemical analysis showed no notable abnormalities throughout the follow-up period.

In both groups, histopathological analysis of the major organs revealed no abnormalities that could be attributed to TLI. Similarly, lymphoid tissues did not show any histopathological changes in the 8 Gy group. Noticeable reduction in cell numbers of bone marrow was detected in two of the three dogs in the 12 Gy group. Especially, myelocyte and erythroblast cell numbers markedly declined in these bone marrows. In these two dogs, a slight decrease in cell numbers was also detected in the spleen and mandibular lymph node. Another dog in the 12 Gy group showed a slight decrease in myelocyte series cell numbers.

3.3 | Lymphocyte analyses

Peripheral lymphocyte counts were dramatically decreased in all dogs (Figure 3). The median time for the lymphocyte counts nadir was 5 days (range 5–9 days) after the start of TLI procedures. Although lymphocyte counts recovered gradually over time, the cell numbers remained lower than before TLI throughout the follow-up period in both group dogs.

In the peripheral lymphocyte subset analyses, no notable changes were observed in the percentage of CD^{3+} T-cells in lymphocytes (Figure 4a,b), although percentages of CD^{21+} B-cells decreased after TLI (Figure 4c,d). As shown in Figure 4d, the mean percentages of CD^{21+} B-cells in the 12 Gy group significantly decreased at 1 and 4 weeks compared with cell counts measured before TLI. Although significant differences could not be detected in both groups, percentages of CD^{4+} T-helper cells were lower than the pre-TLI levels (Figure 4e,f). In CD^{8+} T-cytotoxic cells, T-reg cells and NK cells, there was no significant difference in numbers before and after TLI at any time (Figure 4g–l). Mean values for CD^{4+}/CD^{8+} cell ratios obtained before TLI and 1, 4 and 8 weeks after TLI, respectively, were 2.1 ± 0.2/2.5 ± 0.2, 1.8 ± 0.6/3.1 ± 0.6, 1.1 ± 0.2/1.4 ± 0.6 and 1.5 ± 0.3/1.5 ± 0.3 (8 Gy/12 Gy). In the ratios, statistical significance was not obtained at each time point.

Following TLI procedures, CD^{3+} T-cell proliferation decreased at all time points compared with proliferation before TLI in both groups (Figure 5a,b). Particularly, the proliferation rate in the 12 Gy group showed a marked decrease compared with that of the 8 Gy group. As opposed to the proliferation rate in the 8 Gy group, which showed a gradual recovery trend at 8 weeks, the proliferation rate in the 12 Gy group continued to decrease until the end of the observation period.

4 | DISCUSSION

This pilot study evaluated the effect and safety of TLI using VMAT as a conditioning treatment for canine allogeneic HCT. No severe

TABLE 2 Planned organ at risk volumes and doses

Prescription dose	Bone marrow of the cranial half body	Bone marrow of the caudal half body	Heart	Lungs	Eyes	Kidneys	Liver
Planning volume (cm^3)							
8 Gy ($n = 3$)	350.3 ± 43.7	351.9 ± 54.2	181.8 ± 20.7	764.0 ± 96.5	11.7 ± 1.1	139.1 ± 22.8	367.4 ± 21.6
12 Gy ($n = 3$)	288.5 ± 36.1	319.6 ± 32.5	173.2 ± 24.4	547.1 ± 190.3	12.4 ± 0.7	105.1 ± 14.6	345.1 ± 44.7
D_{50} (cGy)							
8 Gy ($n = 3$)	74.9 ± 33.4	143.8 ± 15.8	10.1 ± 2.8	4.2 ± 0.1	10.2 ± 3.7	267.7 ± 10.8	717.6 ± 27.4
12 Gy ($n = 3$)	122.6 ± 17.6	165.7 ± 11.0	12.8 ± 2.2	6.6 ± 0.8	20.0 ± 2.5	262.4 ± 9.2	709.8 ± 25.5
D_{95} (cGy)							
8 Gy ($n = 3$)	1.0 ± 0.1	14.4 ± 4.2	3.5 ± 0.0	1.8 ± 0.0	8.5 ± 1.3	126.8 ± 3.0	566.3 ± 41.0
12 Gy ($n = 3$)	1.8 ± 0.0	15.1 ± 1.6	5.5 ± 0.7	2.9 ± 0.2	14.2 ± 1.9	127.4 ± 8.0	540.3 ± 4.7

Note: All volumes (cm^3) and doses (cGy) are reported as means ± standard deviation.

Abbreviations: D_{50}, median organ dose; D_{95}, minimum dose required to cover 95% of each target volume.

FIGURE 2 Changes in peripheral neutrophil (a) and platelet (b) counts after 8 or 12 Gy total lymphoid irradiation (TLI) in six healthy dogs. Each coloured line represents variation for each individual dog in each group. Peripheral neutrophil counts reference range, 2,950–11,640 cells/μl. Peripheral platelet counts reference range, 148–484 × 10^3cells/μl

adverse effects were observed in 8 and 12 Gy TLI, and these procedures can therefore be performed as nonmyeloablative conditioning regimens.

In this study, targeted irradiation for the lymphoid tissues involving dose reduction to OAR was achieved using the VMAT technique. Nonmyeloablative conditioning, such as 2 Gy TBI or 8 Gy TLI, for allogeneic HCT or organ transplantation affords stable mixed donor/host chimerism or engraftment in human medicine (Lange et al., 2009; McKay et al., 2014; Spinner et al., 2019). In addition, dose escalation regimen of pretransplant conditioning has been attempted in human patients based on advances in radiation therapy equipment and prevailing IMRT techniques (Patel et al., 2014; Somlo et al., 2011; Wong et al., 2013). It is expected that treatment

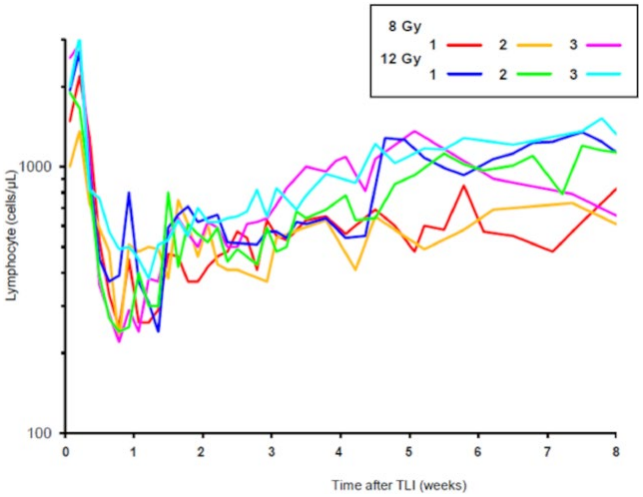

FIGURE 3 Changes in peripheral lymphocyte counts after 8 or 12 Gy total lymphoid irradiation (TLI) in six healthy dogs. Each coloured line represents variation for each individual dog in each group

outcomes will be improved by enhancing radiation therapy techniques for pretransplant conditioning. Higher TBI doses have, in fact, been found to be effective in reducing the relapse rate in human chronic and acute myeloid leukemia (Clift et al., 1991, 1998). Many canine multicentric lymphoma cases achieve clinical complete remission when treated with chemotherapy, but most of the patients eventually relapse. In a previous study, minimal residual diseases in lymph nodes were correlated with time to relapse in canine large B-cell lymphoma (Chalfon et al., 2019). In a clinical setting, relapse of multicentric lymphomas is observed as re-enlarged peripheral lymph nodes. Therefore, intensive treatment of lymphoid tissues with higher dose radiotherapy may not only have an immunosuppression effect but also a direct antitumor effect from the TLI itself.

Rosenthal *et al*. demonstrated the feasibility and safety of 12 Gy TMLI using IMRT with Helical Tomotherapy as a conditioning treatment for HCT (Rosenthal et al., 2011). Normal organ doses (D_{50}) with 12 Gy TMLI/TLI were recorded as follows: lung, 3.5–5.8 Gy; heart, 4.0–6.6 Gy; eyes, 0.2–2.5 Gy; kidneys, 4.6–6.8 Gy; and liver, 3.9–5.8 Gy (Rosenthal et al., 2011; Schultheiss et al., 2007). In TMI/TMLI treatment using Helical Tomotherapy, there was a better conformation of dose delivery to the targeted organ with sparing OARs compared with conventional procedures (Paix et al., 2018). In the present study, calculated doses for OARs were comparable or much lower than these doses at 8 Gy prescription for the abdominal lymph nodes. Additionally, no serious side effects were observed during the follow-up period, and notable histological changes in the major organs were not detect at autopsy; accordingly, we conclude that the sparing of normal tissues was sufficiently achieved using VMAT for TLI procedure planning in this study. Consideration for higher-dose prescription for targeted abdominal tissue will be needed in future studies.

In this study, the CTV was manually delineated to encompass soft tissue surrounding the lymph nodes. In general, the CTV is thought to be absent in the lymph nodes, but we considered that tumour cells could expand not only to lymph nodes but also to the surrounding soft tissue, including lymphatic or adipose tissue. For the spleen, priority was given to narrowing the irradiation field due to concern about radiological exposure to gastrointestinal tissue and the kidney, and the planning target volume (PTV) was not created.

To the best of our knowledge, there have been no reports of the TLI with VMAT in dogs, thus the acceptable pass rate is unknown. A previous human study suggested that the tolerance for TBI with VMAT QA required a pass rate of 95% at absolute dose (Symons et al., 2018). In the present study, the pass rates for absolute dose were >96% in all fields (data not shown), and the pass rates for relative dose were >95% except for caudal half body in one case. These results suggest that the dose congruency predicted by the TPS and the irradiation with treatment machine were acceptable.

In the current study, peripheral lymphocyte counts decreased in all dogs regardless of TLI dose. Specifically, the proliferation of CD^{4+} and CD^{21+} cells in the subset analyses declined in both 8 and 12 Gy TLI procedures. An experimental canine study demonstrated that the number of lymphocytes in the peripheral blood decreases after TBI or TLI procedures (Storb et al., 1999). In a previous clinical study, a subset analysis of peripheral blood lymphocytes in human patients after radiation therapy was performed, and the authors reported that the levels of B- and T-helper lymphocytes significantly decreased compared with those of other lymphocytes (Zhao et al., 2020). The human and veterinary clinical studies described a reduction in the levels of peripheral blood lymphocytes with localized radiotherapy (Belka et al., 1999; Kent et al., 2020). Consistent with our results, the previous study indicated that B-cells are the most radiosensitive of all leukocytes and suffer a long-lasting depletion (Belka et al., 1999). In addition, Sorror *et al*. reported a difference in the nadir of peripheral lymphocyte counts depending on the dose, in dogs irradiated at four different doses (0.5, 1.0, 2.0 and 3.0 Gy) of TBI (Sorror et al., 2008). Lymphocyte depletion was more intense at 2.0 (372 cells/μl) and 3.0 Gy (284 cells/μl) TBI compared to 0.5 (994 cells/μl) and 1.0 Gy (753 cells/μl) TBI. In the present study, lymphocyte count nadirs in 8 (220–250 cells/μl) and 12 Gy (240–380 cells/μl) TLI were equivalent to those at 2 Gy TBI, which is commonly used in nonmyeloablative conditioning. Although the relationship between the absolute number of peripheral lymphocytes and sustained engraftment has not been fully elucidated, our data suggest that this TLI regimen may be effective in obtaining sufficient peripheral lymphocyte depletion.

The significant changed balance of T-reg cells was not observed after both TLI conditions in the present study. In the previous experimental transplantation study using mice, the increased percentage in T-reg and NK cells was detected after conditioning procedures composed of TLI and anti-thymocyte serum (ATS). Additionally, this study reported that TLI and ATS have been shown to have different effects on lymphocyte subsets. TLI alone does not significantly affect the balance of the host T-reg cells (Nador et al., 2010). On the other hand, the increase in NK cells and depletion of CD^{8+} cells following TLI were detected in previous studies (Nador et al., 2010;

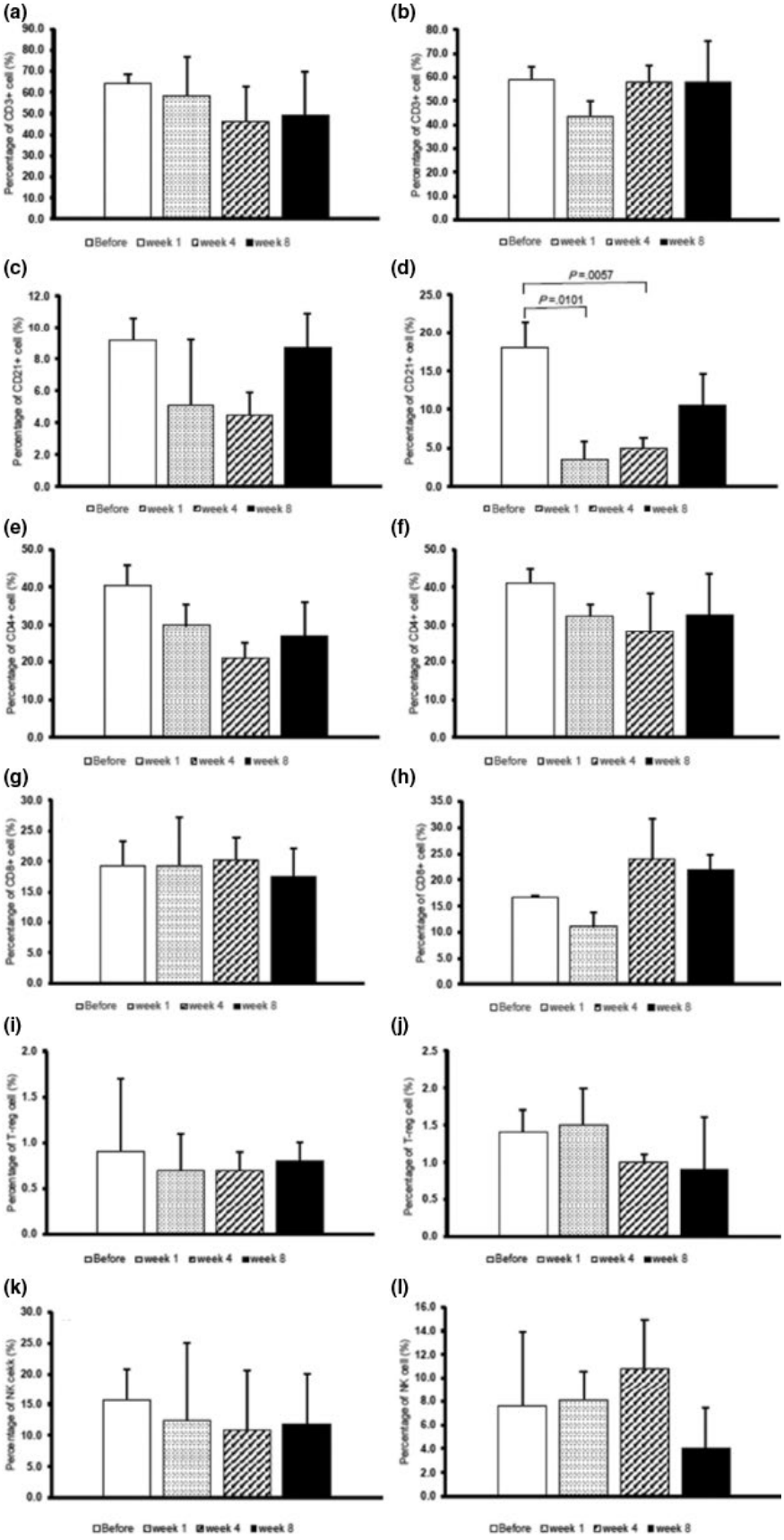

FIGURE 4 Changes in peripheral lymphocyte subset following each total lymphoid irradiation (TLI) condition. Mean ± standard deviation of each peripheral lymphocyte percentage. (a) CD^{3+} cell after 8 Gy TLI, (b) CD^{3+} cell after 12 Gy TLI, (c) CD^{21+} cell after 8 Gy TLI, (d) CD^{21+} cell after 12 Gy TLI, (e) CD^{4+} cell after 8 Gy, (f) CD^{4+} cell after 12 Gy TLI, (g) CD^{8+} cell after 8 Gy, (h) CD^{8+} cell after 12 Gy, (i) regulatory T cell (T-reg) after 8 Gy TLI, (j) T-reg after 12 Gy TLI, (k) natural killer (NK) cell after 8 Gy TLI and (l) NK cell after 12 Gy TLI

Zhang et al., 2012). In the current study, similar changes were not observed with our experimental conditions. The cause of the difference in these changes was not clear. However, it is possible that the animal species or TLI condition affected the difference in changed balance of lymphocyte subsets. Additionally, peripheral CD^{4+}/CD^{8+} T cells did not show any significant difference in any TLI condition

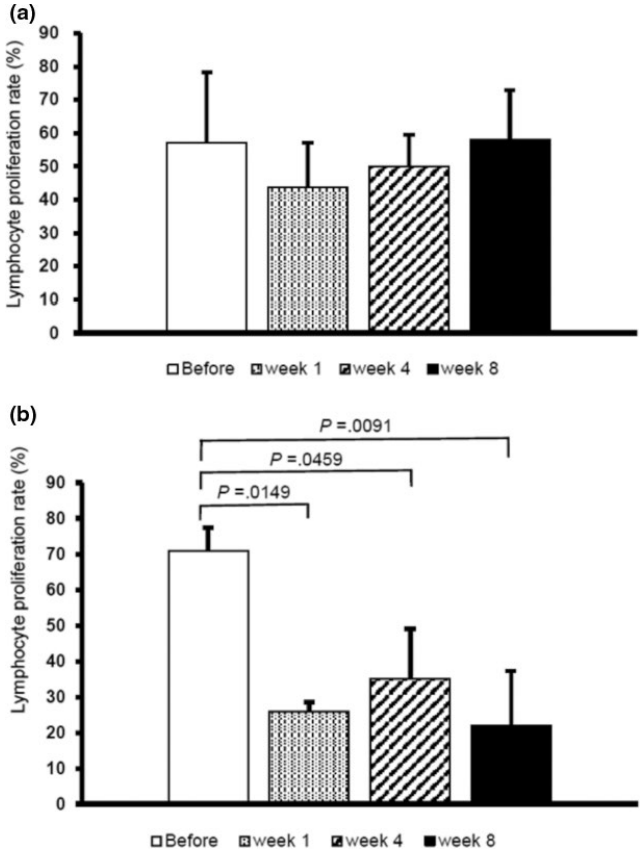

FIGURE 5 Changes in peripheral lymphocyte proliferation rate following (a) 8 ($n = 3$) and (b) 12 Gy ($n = 3$) TLI condition. Mean ± standard deviation of each proliferation rate

and time point. A previous study showed that these cell ratios increased on days 2 and 5 following TLI in the mouse model, but no significant difference was observed since the first week after TLI procedures (Field & Rouse, 2002). It was possible that the time point of measurement affected the current results.

Peripheral lymphocytes proliferative capacity was markedly inhibited by 12 Gy TLI. A previous study found no difference in lymphocytes proliferative response between 1 and 2 Gy TBI (Sorror et al., 2008). The relationships between the proliferative capacity of lymphocytes and stable allografts remain poorly understood, and few reports are available on lymphocytes proliferative response to TLI alone in human or veterinary medicine. Results of the current study indicated that lymphocytes proliferation activity may change in a dose-dependent manner in TLI.

In this study, most dogs experienced neutropenia and thrombocytopenia after TLI procedures. These changes, however, were transient and recovered spontaneously over time. Consistent with our results, previous canine studies described a decreased in these peripheral blood components after nonmyeloablative conditioning using radiation. In the 18 dogs treated with 2 Gy TBI without HCT, neutrophil counts reached a nadir in about 20 days and the mean nadir neutrophil count was 750 cells/μl (Storb et al., 1997). In the same report, thrombocytopenia nadirs were recorded at 15–24 days after TBI treatment, and both counts were 7,500 cells/μl. Neutropenia and thrombocytopenia persisted for 40 and 50 days respectively. Storb *et al.* reported that two dogs experienced a decrease in neutrophil counts after receiving 4.5 Gy TLI without HCT (Storb et al., 1999). The nadir neutrophil counts were 2,500 and 3,500 cells/μl, respectively, and these nadirs were recorded 8 days after TLI. Thrombocytopenia nadirs were recorded at 10 and 11 days after TLI, respectively, and both counts were 100,000 cells/μl. In these two reports, the authors concluded that the two procedures were safe based on most animals not exhibiting lasting signs or symptoms associated with neutropenia and thrombocytopenia. In our study, decreases in neutrophil and platelet counts were comparable or less severe than those of the previous studies, therefore we concluded that myelosuppression with either dose of TLI is well-tolerated in dogs.

In the 8 Gy group, one of the three dogs was confirmed to have developed an infection and experienced drainage from the blood sampling site at 26 days after TLI. This infection was deemed to have been a result not only of the immunosuppressive effect of TLI but also of the frequent blood sampling required for experimental data collection. Because of this event, sample collection sites were carefully disinfected to prevent infection resulting from experimental manipulations. Because of these measures there were no subsequent events related to infection in this study.

Our study had several limitations. First, the sample size of this study was small, and the fact that some difference, including post-TLI haematological changes and lymphocyte subset analysis, between the two groups did not achieve statistical significance may reflect a lack of power. Additionally, this study used only male dogs due to small sample size and efforts to reduce the difference between individuals. Thus, an additional extensive study is required to confirm the results obtained in the pilot study. Second, the observation period following the TLI is short, and it is not possible to evaluate the late toxicity of the radiation treatment. Several years of additional study is required to assess the late toxicity for this treatment. Third, this study did not perform in vivo dosimetry, and not include a physicist. These deficiencies may affect the optimal radiation planning and the implementation of accurate irradiation due to set-up error. Finally, the PTV was not created in the present study. This pilot study showed the safety for these TLI plans at 8 and 12 Gy. Thus, it was necessary to improve the plan by providing a uniform PTV for the lymph nodes and spleen in a future study.

In conclusion, to the best of our knowledge, this is the first study to evaluate the safety and feasibility of TLI using VMAT as pretransplant conditioning treatment in canine studies. Our results indicated that 8 and 12 Gy TLI using VMAT did not cause serious side effects and was feasible as a nonmyeloablative technique. Further studies with a large sample size and a practice of allogeneic HCT are warranted to fully demonstrate the efficacy of TLI as a nonmyeloablative conditioning regimen for HCT.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Sangho Kim: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Writing-original draft. **Kenji Hosoya:** Conceptualization; Project administration. **Natsuki Fukayama:** Formal analysis; Investigation; Methodology. **Tatsuya Deguchi:** Methodology. **Masahiro Okumura:** Supervision.

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DATA AVAILABILITY STATEMENT

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ORCID

Sangho Kim https://orcid.org/0000-0001-8451-4479

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