Allogeneic peripheral blood stem cell transplantation using nonmyeloablative pretransplant conditioning regimen in dogs

犬における骨髄非破壊的前処置を用いた
同種末梢血幹細胞移植に関する研究

Sangho Kim

Contents

Abbreviations

ACD-A	anticoagulant citrate dextrose formula-A
APC	allophycocyanin
CBCs	complete blood counts
CFSE	carboxyfluorescein succinimidyl ester
CMNC	continuous mononuclear cell collection
ConA	concanavalin A
CRI	continuous rate infusion
CsA	cyclosporine A
CT	computed tomography
CTV	clinical target volume
CXCR4	CXC chemokine receptor 4
DLA	dog leukocyte antigen
DVH	dose volume histogram
D_{50}	median organ dose
D_{95}	minimum dose required to cover 95% of each target volume
FITC	fluorescein isothiocyanate
G-CSF	granulocyte-colony stimulating factor
GTV	gross target volume
GVHD	graft-versus-host disease
GVL	graft-versus-luekemia

H/h	hours
HSCT	hematopoietic stem cell transplantation
HSC	hematopoietic stem cell
IMRT	intensity-modulated radiation therapy
Lym	lymphocyte
min	minutes
MMF	mycophenolate mofetil
MNC	mononuclear cell
Mono	monocyte
OAR	organ at risk
PBMCs	peripheral blood mononuclear cells
PBSC	peripheral blood $CD34^+$ stem cell
PE	phycoerythrin
PF	packing factor
Plt	platelet
PO	oral administration
QA	quality assurance
rh	recombinant human
SC	subcutaneous administration
SD	standard deviation
SDF-1α	stromal cell-derived factor-1α
TBI	total body irradiation

TLI	total lymphoid irradiation
TMLI	total marrow and lymphoid irradiation
TPS	treatment planning system
VMAT	volumetric modulated arc therapy
WBC	white blood cell

Preface

Hematopoietic stem cell (HSC) transplantation has achieved widespread use for the clinical treatment of both malignant and nonmalignant hematologic disorders in humans [Baron and Storb, 2006; Copelan *et al*., 2019]. Hematopoietic stem cell transplantation (HSCT) therapy is categorized as autologous and allogeneic transplantation, depending on the origin of the transplanted cells. In autologous HSCT, HSCs are collected from the patient's own blood before conditioning therapy, such as high-dose chemotherapy and total body irradiation (TBI). Autologous HSCT is not a concern with immune-mediated graft rejection, but the patient's own graft may be contaminated by residual tumor cells, which results in the possibility of recurrence in cancer patients [DiPersio *et al*., 2011]. On the other hand, allogeneic HSCT uses HSCs that are obtained from a genetically matched donor. The antitumor effect of allogeneic HSCT has been attributed not only to conditioning therapies but also to graft-versus-leukemia effects, which are related to the immune-mediated reaction of the donor immune cells to the remaining tumor cells [Copelan *et al*., 2019]. In previous studies in veterinary medicine, dogs with B- or T-cell lymphoma were treated with autologous peripheral HSC transplantation combined with high-dose chemotherapy and TBI [Warry *et al*., 2014; Willcox *et al*., 2012]. The findings of these studies suggested that peripheral blood $CD34^+$ stem cell (PBSC) transplantation

can cure a subpopulation of dogs with B- and T-cell lymphoma. In addition, Suter *et al* (2015) reported long-term stable full donor chimerism and favorable prognosis in a canine case of acute lymphoblastic leukemia treated with allogeneic HSCT. Although HSCT has shown promise as a highly effective treatment for canine hematologic malignancies, transplantation therapy is still an unfamiliar treatment in the veterinary clinical field.

In human medicine, PBSCs collected using apheresis machines are the most common type of HSCs used for transplantation [Körbling and Freireich, 2011]. Granulocyte-colony stimulating factor (G-CSF) is the most frequently used agent for facilitating PBSC mobilization in both human and veterinary medicine [Burroughs *et al*., 2005; Warry *et al*., 2014; Willcox *et al*., 2012]. However, 10 to 29% of human patients, such as the elderly, heavily pretreated patients, or patients with significant myelophthisis, fail to obtain optimal PBSC yields (>2.0 × 10^6 $CD34^+$ cells/kg) with a regimen of G-CSF and high-dose chemotherapy mobilization to proceed with autologous HSC transplantation [Sancho *et al*., 2012; Jantunen and Kvalheim, 2010]. In addition, higher PBSC yields, such as >4-5 × 10^6 $CD34^+$ cells/kg has been associated with faster neutrophil and platelet recovery, reduced hospitalization, decreased blood transfusion requirement, and reduced need for antibiotic therapy [Giralt *et al*., 2014; Gunn *et al*., 2003; Stiff *et al*., 2011], although the eventual rate of successful marrow reconstruction is not affected, as long as the minimum target $CD34^+$ cells are transplanted [Mohty *et al*., 2018]. Therefore, to make

this treatment more feasible, determining a mobilization protocol that can yield higher PBSC counts for transplantation is essential.

The Spectra Optia® apheresis system (TerumoBCT, Lakewood, CO, USA) was introduced in 2014 to replace the COBE® Spectra apheresis system, which had been the most popular apheresis machine used in humans for several decades. The Spectra Optia® apheresis system has an advanced automated interface management system that automatically detects and maintains a buffy coat interface for PBSC collection [Even-Or *et al*., 2017]. An earlier version of the Spectra Optia® supported only a mononuclear cell (MNC) collection procedure, and had a secondary collection chamber that separated the MNCs from platelets. The continuous mononuclear cell collection (CMNC) procedure can be used on the newer version of the Spectra Optia®. In contrast to the MNC collection procedure, the CMNC procedure eliminates the secondary collection chamber and allows for MNCs to be transferred continuously from the buffy coat into the collection bag [Pandey and Cottler-Fox, 2018]. Although the COBE® Spectra machine is safe and efficient at collecting PBSCs from veterinary species, including dogs [Warry *et al*., 2014; Willcox *et al*., 2012], to my knowledge no published study has examined the safety and feasibility of the use of the Spectra Optia® machine in dogs.

Pretransplant conditioning treatments were designed to eliminate residual malignant cells and eradicate the recipient's immune system. Traditionally, supralethal doses of TBI

and/or high-dose chemotherapy have been used for pretransplant conditioning, which is also referred to as myeloablative conditioning, in human allogeneic HSCT [Baron and Storb, 2006]. These aggressive conditioning regimens have been associated with treatment-related toxicity and mortality; therefore, they are not used in patients with advanced aged or comorbidities [Baron and Storb, 2006]. Nonmyeloablative conditioning has attracted a lot of interest for its use in extending the application of allogeneic HSCT to patients with a variety of conditions, and various regimens have yielded practical applications in human medicine [Baron and Sandmaier, 2006; Storb *et al*., 1997; Storb *et al*., 1999; Spinner *et al*., 2019]. Storb *et al* (2001) proposed the following criteria for nonmyeloablative conditioning: (1) no eradication of host hematopoiesis, (2) prompt hematologic recovery (<4 weeks) without HSCT, and (3) the presence of mixed chimerism upon engraftment. Although there have been no reports of allogeneic HSCT using nonmyeloablative conditioning for clinical cases in veterinary medicine, previous preclinical research using dogs showed that the use of 2 Gy TBI combined with immunosuppressive drugs provided a stable allograft with a reduction in transplant-related toxicities [Storb *et al*., 1997; Storb *et al*., 1999]. This regimen has been translated to human medicine as nonmyeloablative conditioning [Fatobene *et al*., 2020]. Nonmyeloablative conditioning likely enables safe and secure allogeneic HSCT therapy in veterinary medicine and is considered one of the important factors in the spread of

canine transplantation therapy, as it is for human medicine.

The objectives of the present study were to establish allogeneic PBSC transplantation using nonmyeloablative conditioning with the goal of practical application in veterinary medicine. In chapter I, the effective mobilization protocols were investigated for efficiently collecting the required number of PBSCs from the recipient. In chapter II, to establish a safe, and effective pretransplant conditioning treatment for the donor dog, the feasibility of total lymphoid irradiation (TLI) as a nonmyeloablative HSCT conditioning method was evaluated. The present study demonstrates that PBSC apheresis and TLI are efficacious in achieving a safe HSCT treatment regimen for use in the veterinary field.

Chapter I

Comparison of three mobilization protocols for peripheral blood stem cell apheresis in healthy dogs

Introduction

Peripheral blood $CD34^+$ stem cell (PBSC) sourced by apheresis is the most common type of hematopoietic stem cell (HSC) used for transplantation in human medicine [Körbling and Freireich, 2011]. Autologous stem cell transplantation combined with high-dose chemotherapy is the part of standard therapy in human patients with aggressive hematologic malignancies [Schmitz *et al*., 2002]. In previous studies in veterinary medicine, dogs with B- or T-cell lymphoma were treated with autologous peripheral hematopoietic stem cell transplantation (HSCT) combined with high-dose chemotherapy and total body irradiation (TBI) [Warry *et al*., 2014; Willcox *et al*., 2012]. The findings of these studies suggested PBSC transplantation as an effective treatment option for dogs with lymphoma.

In general, the peripheral blood of various animals, such as humans, rhesus macaques, and dogs, contains a small number of HSCs [Burroughs *et al*., 2005; Haynes *et al*., 2017]. In both human and veterinary medicine, granulocyte colony-stimulating factor (G-CSF) is the most commonly used agent for facilitating PBSC mobilization [Burroughs *et al*., 2005; Warry *et al*., 2014; Willcox *et al*., 2012]. Apheresis is typically initiated 4-5 days after beginning G-CSF mobilization. The accepted minimal cell dose needed for a successful transplant in humans is $>2.0 \times 10^6$ cells/kg. However, 10-29% of

human patients fail to obtain the adequate PBSC yields with the G-CSF and chemotherapy mobilization regimen to proceed with autologous HSCT [Jantunen and Kvalheim, 2010; Sancho *et al*., 2012]. In addition, higher PBSC yields, such as >4–5 × 10^6 cells/kg has been associated with faster neutrophil and platelet (Plt) recovery, reduced hospitalization, need for blood transfusion, and antibiotic therapy [Giralt *et al*., 2014; Gunn *et al*., 2003; Stiff *et al*., 2011], although eventual rate of successful marrow reconstruction is not affected as long as the minimum target $CD34^+$ cells are transplanted [Mohty *et al*., 2018]; therefore, the determination of a mobilization protocol that can yield higher PBSC counts for transplantation is essential to make this treatment successful.

Plerixafor reversibly inhibits the binding of chemokine stromal cell-derived factor-1α (SDF-1α) to its cognate receptor CXC chemokine receptor 4 (CXCR4) [Kessans *et al*., 2010]. Bone marrow stromal cells and HSCs express SDF-1α and CXCR4, respectively. Plerixafor disrupts the binding of CXCR4 and SDF-1α; as a result, HSCs in the bone marrow mobilize into the bloodstream. In general, plerixafor is used in the combination with G-CSF. In current human clinical studies, the HSC mobilization regimen, comprising G-CSF plus low-dose plerixafor (0.24 mg/kg), has been found to be more efficient than G-CSF alone for PBSC mobilization [DiPersio *et al*., 2009; Teusink *et al*., 2016]. In these studies, patients were treated with G-CSF for 4-5 days and were given plerixafor several hours prior to the apheresis. A study using a canine transplantation

model has shown that a single administration of high-dose plerixafor (4 mg/kg) without G-CSF results in a mobilization of PBSCs sufficient for transplantation [Burroughs *et al*., 2005]. To the best of my knowledge, no study has evaluated the effectiveness of a regime comprising G-CSF and low-dose plerixafor for mobilization of PBSCs in veterinary small animal practice.

The Spectra Optia® (TERUMO BCT, Tokyo, Japan) continuous mononuclear cell collection (CMNC) protocol is a recently introduced apheresis system. This protocol allows for the white blood cells to be continually collected from buffy coat into the collection bag. The efficiency and safety of this novel system has been reported in several human clinical studies [Cancelas *et al*., 2016; Even-Or *et al*., 2017; Punzel *et al*., 2017]. Especially, this system enhances automation and allows reproducibility and flexibility for the operator performing the apheresis procedure. There is no published study, to my knowledge, that has examined the safety and feasibility of using the Spectra Optia® machine in dogs. Therefore, in this study, there were two goals; the primary objective was to evaluate the feasibility and safety of the Spectra Optia® apheresis system and the CMNC procedure in dogs; the secondary objective was to compare three mobilization regimens in healthy dog (G-CSF alone, high-dose plerixafor alone, G-CSF plus low-dose plerixafor combination), to determine which regime yielded the highest number of harvested PBSCs when collections were performed using Spectra Optia® apheresis

system and the CMNC protocol.

Materials and methods

Animals

Nine healthy laboratory beagles (one intact male and eight intact females) were used in this study. The dogs weighed 9.7-11.4 kg (mean 10.3 kg) and were aged 12-19 months (mean 14.3 months). These dogs were divided into three groups according to the assigned mobilization protocol (plerixafor, G-CSF, and combination regimen). Each dog underwent mobilization and apheresis once. All animal procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Hokkaido University (approval number: 15-0102). Prior to the experiments, blood laboratory tests [e.g., complete blood counts (CBCs) and general blood chemistry components] revealed that blood parameters of each animal were within the normal ranges. These tests were conducted using a Procyte™ (IDEXX Laboratories, Tokyo, Japan) and Dri-Chem 7000V (FUJIFILM, Tokyo, Japan).

Stem Cell Mobilization Regimens

Bone marrow-derived stem cells were mobilized to peripheral blood using one of the three regimens. In plerixafor group (Dogs A, B, and C), the dogs were administered high-dose plerixafor [4 mg/kg subcutaneous administration (SC)] (Sigma Aldrich, Tokyo,

Japan) 5 hours (h) before starting the apheresis [Warry *et al*., 2014]. In recombinant human (rh) G-CSF (Chugai Pharmaceutical, Tokyo, Japan) group (Dogs D, E, and F), the dogs were administered rhG-CSF (5 μg/kg SC q12h) starting 5 days before apheresis [Suter, 2011]. Apheresis was performed at 9 h after the last dose of rhG-CSF. In the combination group (Dogs G, H, and I), the dogs were administered the same protocol as in the rhG-CSF group, and low-dose plerixafor (0.24 mg/kg SC) was administered 4 h after the last rhG-CSF injection [Dipersio *et al*., 2009]. Apheresis was then performed at 5 h after plerixafor administration.

During the mobilization treatment, CBCs were performed on Days 1, 2, 3, and 4 after rhG-CSF administration and 1, 2, 3, and 4 h following plerixafor administration. At the same time, peripheral blood samples were collected for PBSC analysis using flow cytometry.

Apheresis Procedure

For the apheresis procedure, all animals were anesthetized with propofol (6.0 mg/kg) (DS Pharma Animal Health, Osaka, Japan) administered intravenously. Subsequently, the animals were intubated and maintained on a mixture of isoflurane (DS Pharma Animal Health) in oxygen. An 18-gauge, 5.1-cm catheter was placed percutaneously in a jugular vein. This catheter served to obtain blood samples and as an apheresis line. In addition, a

20-gauge, 5.1-cm catheter was placed in the saphenous vein as a blood transfusion line. All apheresis procedures were performed on the Spectra Optia® CMNC protocol using intermediate density layer disposable tubing set (TERUMO BCT). The initial priming of the tubing kit used stored autologous whole blood. This blood (200 mL) was extracted 2-3 weeks before apheresis; it was stored in a sterile blood-collection bag containing an anticoagulant citrate dextrose formula-A (ACD-A) (TERUMO, Tokyo, Japan) at 4°C until the day of apheresis [Lupu *et al*., 2008]. The target blood volume to be processed was three times the total canine blood volume (90 mL/kg × 3). The ACD-A solution (TERUMO) infusion rate was set at 1.2 mL/min/L of total blood volume, and the ACD-A ratio was set at 1:12. The collection preference ranged from 50-70, and the inlet flow rate was set at 14-16 mL/min.

Animal Monitoring during Apheresis

During apheresis, animals were monitored every 5 minutes (min) by continuous electrocardiogram, oxygen saturation via pulse oximetry, end-tidal carbon dioxide, indirect blood pressure measurement, and rectal temperature. Air-heating pads were used to maintain the body temperature of the dogs within the physiological range. In the event where the mean arterial blood pressure decreased below 60 mmHg, a continuous rate infusion (CRI) of dopamine hydrochloride (Kyowa Hakko Kirin, Tokyo, Japan) at 3-7

μg/kg/min was used to increase the blood pressure. Blood samples were collected every 20 min during apheresis to assess CBCs, sodium, potassium, chloride, and serum calcium. To avoid anticoagulant induced hypocalcemia, 0.85% calcium gluconate solution (Nichi-Iko, Toyama, Japan) was administered as a CRI throughout apheresis. The infusion rate ranged from 8.5 to 42.5 mg Ca/kg/h, depending on the serum calcium concentration.

Apheresis Product (PBSC) Analysis

A sample of the apheresis product was subjected to PBSC count using FACS Versa™ (BD Bioscience, San Jose, CA, USA) and CBCs analysis. The phycoerythrin (PE)-conjugated mouse anti-canine CD34 [clone IH-6 (BD Bioscience), diluted to 1:40 in 2% fetal bovine serum (BD Bioscience)] and fluorescein isothiocyanate (FITC)-conjugated rat anti-canine CD45 (1:20, eBioscience, Santa Clara, CA, USA) monoclonal antibodies were used for flow cytometry. A PBSC was defined as the cell that was CD34-positive and CD45 weakly-positive, and the PBSC count was calculated from the following formulae:

Total white blood cell (WBC) count yield (count) = [product volume (mL)] × (WBC count/μL) × 1,000,

PBSC yield (cells/kg) = (total WBC yield) × [(CD34-positive and CD45 weakly-positive cell count)/(CD45-positive cell count)]/body weight (kg).

Plt loss in the dogs was calculated as follows:

Plt loss = [1 – (post-apheresis Plt count/pre-apheresis Plt count)].

Statistical analysis

Statistical analysis was performed using Microsoft® Excel 2013 (Microsoft, Redmond, WA, USA). The results are presented as mean ± standard deviation (SD). To detect differences in apheresis product counts and characteristics between the three mobilization groups, Tukey multiple comparison test was used. P values < 0.05 were considered statistically significant.

Results

Mobilization

Figure 1 shows the kinetics of WBC and monocyte counts before and after the administration of rhG-CSF and/or plerixafor in peripheral blood. In the plerixafor group (Dogs A, B, and C), the absolute WBC counts increased 1.2-, 1.8-, and 2.2-fold, respectively, at 4 h after plerixafor administration. The absolute monocyte counts in Dogs A, B, and C increased by 1.9-, 4.9-, and 2.0-fold, respectively, 4 h after plerixafor administration. In the rhG-CSF group (Dogs D, E, and F), the absolute WBC counts increased by 3.6-, 3.7-, and 3.4-fold, respectively, at 4 days after the start of mobilization. The absolute monocyte counts in Dogs D, E, and F increased 2.1-, 7.1-, and 3.9-fold, respectively, at 4 days after rhG-CSF administration. In the combination group (Dogs G, H, and I), the absolute WBC counts increased by 2.4-, 2.5-, and 3.6-fold, respectively, at 4 days after rhG-CSF administration. The absolute monocyte counts in Dogs G, H, and I increased by 3.5-, 4.1-, and 3.1-fold, respectively, at 4 days after rhG-CSF administration. The WBC counts in these three dogs further increased by 3.3-, 3.3-, and 4.3-fold, respectively, after plerixafor administration compared with the WBC count before mobilization. The monocyte counts in these three dogs reached the highest level at 2 h after plerixafor administration and increased by 4.8-, 7.9-, and 4.4-fold, respectively, over

the pre-mobilization values.

Flow cytometry was performed before and during each mobilization protocol to measure PBSCs, except for Dog B, in which the flow cytometric analysis could not be performed due to technical error. In general, the degree of mobilization varied significantly among the dogs in all mobilization protocols, although there was a consistent trend that the number of PBSCs increased with the procedure. The PBSC count in Dogs A and C increased by 1.5- and 1.9-fold, respectively, at 4 h after plerixafor administration compared with the PBSC count before the start of mobilization (Figure 2). The percentage of the PBSCs in total WBC before and 4 h after mobilization ranged 0.011-0.056% and 0.070-0.098%, respectively. In the rhG-CSF group (Dogs D, E, and F), the PBSC count increased by 4.3-, 4.3-, and 5.6-fold, respectively, 4 days after the start of mobilization (Figure 2). The percentage of the PBSCs in total WBC before and 4 days after mobilization ranged 0.051-0.067% and 0.062-0.11%, respectively. In the combination group (Dogs G, H, and I), the PBSC count increased by 3.9-, 8.9-, and 3.8-fold, respectively 4 days after rhG-CSF administration (Figure 2). The percentage of the PBSCs in total WBC before and 4 days after mobilization ranged 0.020-0.057% and 0.039-0.069%, respectively. In this group, plerixafor administration further enhanced the PBSC mobilization. The PBSC count in Dogs G and I reached a maximum value (increases of 18.3- and 5.8-fold compared with the PBSC count before the start of

mobilization, respectively) at 2 h after plerixafor administration. The PBSC count in Dog H reached a maximum value (28.3-fold) at 4 h after plerixafor administration. The percentage of the PBSCs in total WBC at 4 h after plerixafor ranged 0.052-0.17%.

In one of G-CSF-mobilized dogs (Dog E) and two of the G-CSF/Plerixafor combination group (Dogs G and I), peripheral Plt counts decreased by 26-45% at day 4 (Figures 3). In two of the plerixafor mobilized dogs (Dogs A and C) and one of the G-CSF/Plerixafor combination group (Dog H), hematocrit decreased by 15-25% at 4 h after plerixafor administration (Figures 3). These changes, however, remained within the reference range (37.3-61.7 %), and no clinical signs associated with the mobilization were observed in any of the dogs.

Table 1 shows the collection parameters in the three mobilization groups. There were no significant differences among the three groups in any apheresis parameter. The mean blood volume processed, ACD volume, collection length, product volume, and ACD volume in product in all nine dogs were 2.8 ± 0.2 L, 255.8 ± 12.7 mL, 229.3 ± 11.0 min, 116.9 ± 29.6 mL, and 13.3 ± 3.9 mL, respectively.

Apheresis Product Composition

The apheresis products in the three groups contained a variable PBSC count (Figure 4A). The total PBSC count in Dogs A, B, and C (the plerixafor group) were 1.6, 1.6, and

0.7 × 10^6 cells/kg, respectively; in Dogs D, E, and F (the rhG-CSF group), 5.3, 3.3, and 4.1 × 10^6 cells/kg, respectively; and in Dogs G, H, and I (the combination group), 8.6, 6.3, and 4.4 × 10^6 cells/kg, respectively. The mean PBSC counts of the apheresis products in plerixafor, rhG-CSF, and the combination groups was 1.3 ± 0.24, 4.2 ± 0.47, and 6.4 ± 0.9 × 10^6 cells/kg, respectively. As shown in Figures 4A and B, the mean PBSC (P = 0.033) and monocyte (P = 0.022) counts of the apheresis product in the combination group was significantly higher than those in the plerixafor group.

Adverse events

Peripheral Plt counts decreased during the apheresis procedure in all nine dogs (Figures 5). The mean peripheral Plt count was 263 ± 43.2 × 10^3 cells/μL at the beginning of apheresis and 134 ± 31.1 × 10^3 cells/μL at the end of it. In five of the nine dogs, Plt counts decreased below the reference range (148-484 × 10^3 cells/μL); however, no clinical signs related with thrombocytopenia were observed. The mean time for the Plt count to recover to the normal range was 2.8 ± 1.3 days after apheresis. The mean serum calcium value was 10.5 ± 0.4 mg/dL at the beginning of apheresis and 8.2 ± 0.5 mg/dL at the end of it. In eight of the nine dogs, serum calcium concentration was lower after apheresis than the reference value. Serum calcium values returned to the normal range by 24 h after apheresis in all nine dogs. No other clinical complication was observed.

Table 1. Apheresis parameters of the nine dogs that underwent cell mobilization with each regimen.

	Plerixafor	**G-CSF**	**Combination**
Blood volume processed (L)			
Mean ± SD	2.97 ± 0.1	2.76 ± 0.1	2.66 ± 0.0
ACD volume (mL)			
Mean ± SD	270 ± 0.0	251 ± 8.5	246 ± 9.7
Collection duration (minute)			
Mean ± SD	227 ± 2.6	222 ± 5.9	239 ± 12.8
Product volume (mL)			
Mean ± SD	106 ± 4.3	138 ± 42.8	107 ± 11.2

Abbreviations: Combination, G-CSF + plerixafor mobilization; ACD, anticoagulant citrate dextrose; SD, standard deviation.

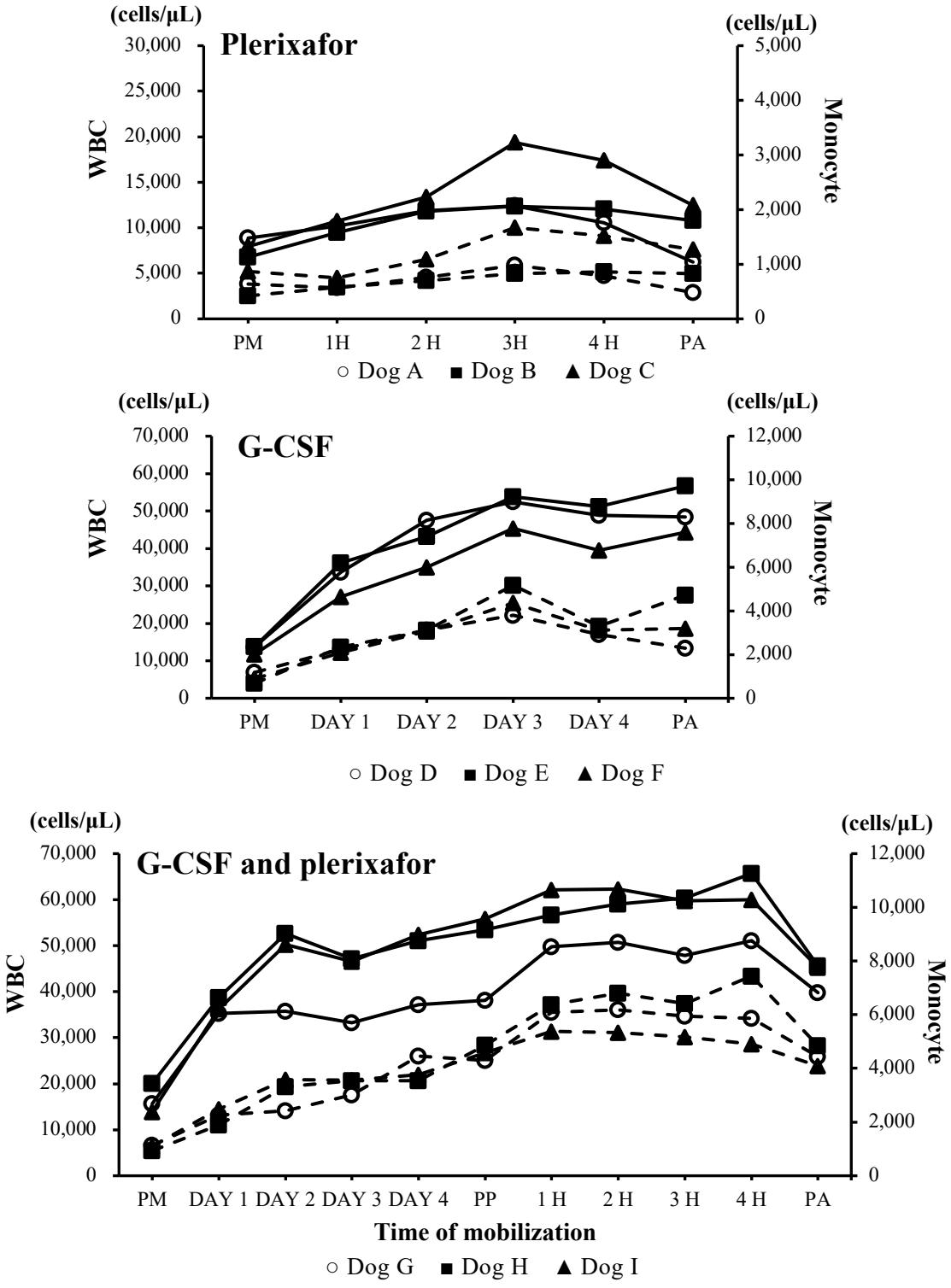

Figure 1. Changes in peripheral WBC and monocyte counts pre-mobilization (PM), 1 (1 H), 2 (2 H), 3 (3H) and 4 (4 H) hours after plerixafor administration, after 1 (DAY 1), 2 (DAY 2), 3 (DAY 3) and 4 (DAY 4) days of G-CSF administration, pre-plerixafor administration (PP), and pre-apheresis (PA).

WBC
Monocyte

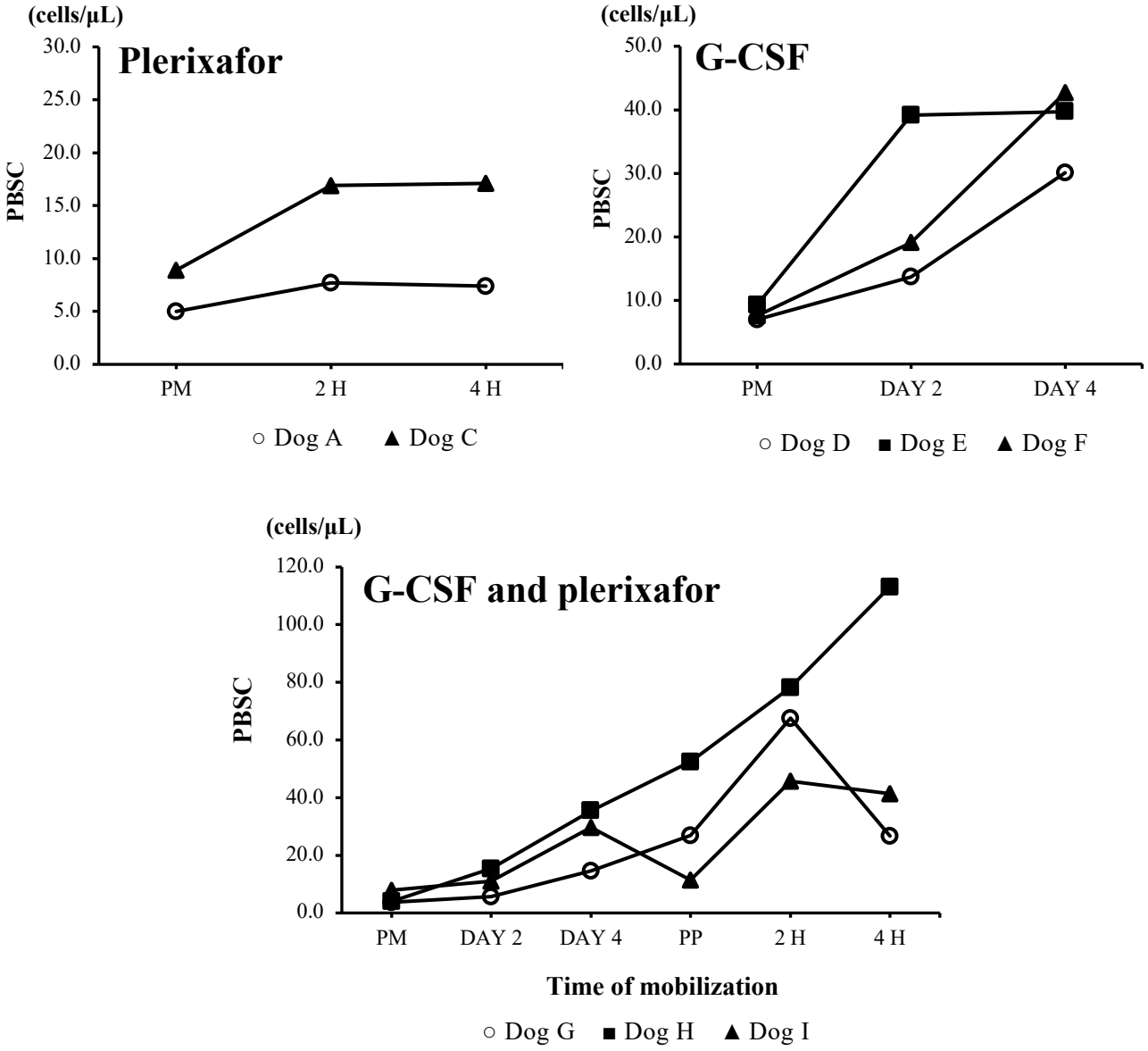

Figure 2. Changes in PBSC counts pre-mobilization (PM), 2 (2 H) and 4 (4 H) h after plerixafor administration, after 2 (DAY 2) and 4 (DAY 4) days of G-CSF administration, and pre-plerixafor administration (PP).

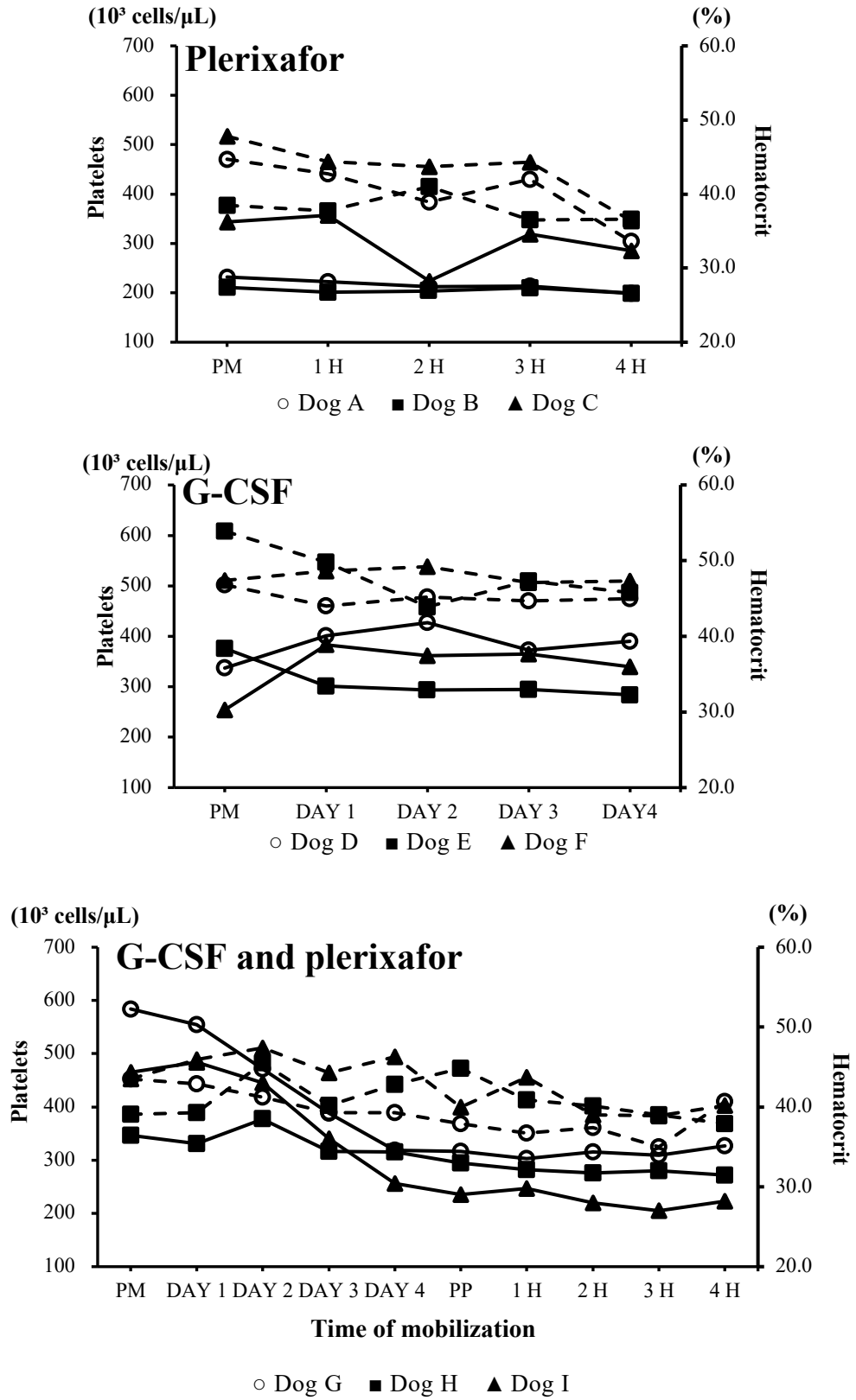

Figure 3. Changes in peripheral Plts and hematocrit pre-mobilization (PM), 1 (1 H), 2 (2 H), 3 (3 H), and 4 (4 H) hours after plerixafor administration, after 1 (DAY 1), 2 (DAY 2), 3 (DAY 3), and 4 (DAY 4) days of G-CSF administration, and pre-plerixafor administration (PP).

Platelets
Hematocrit

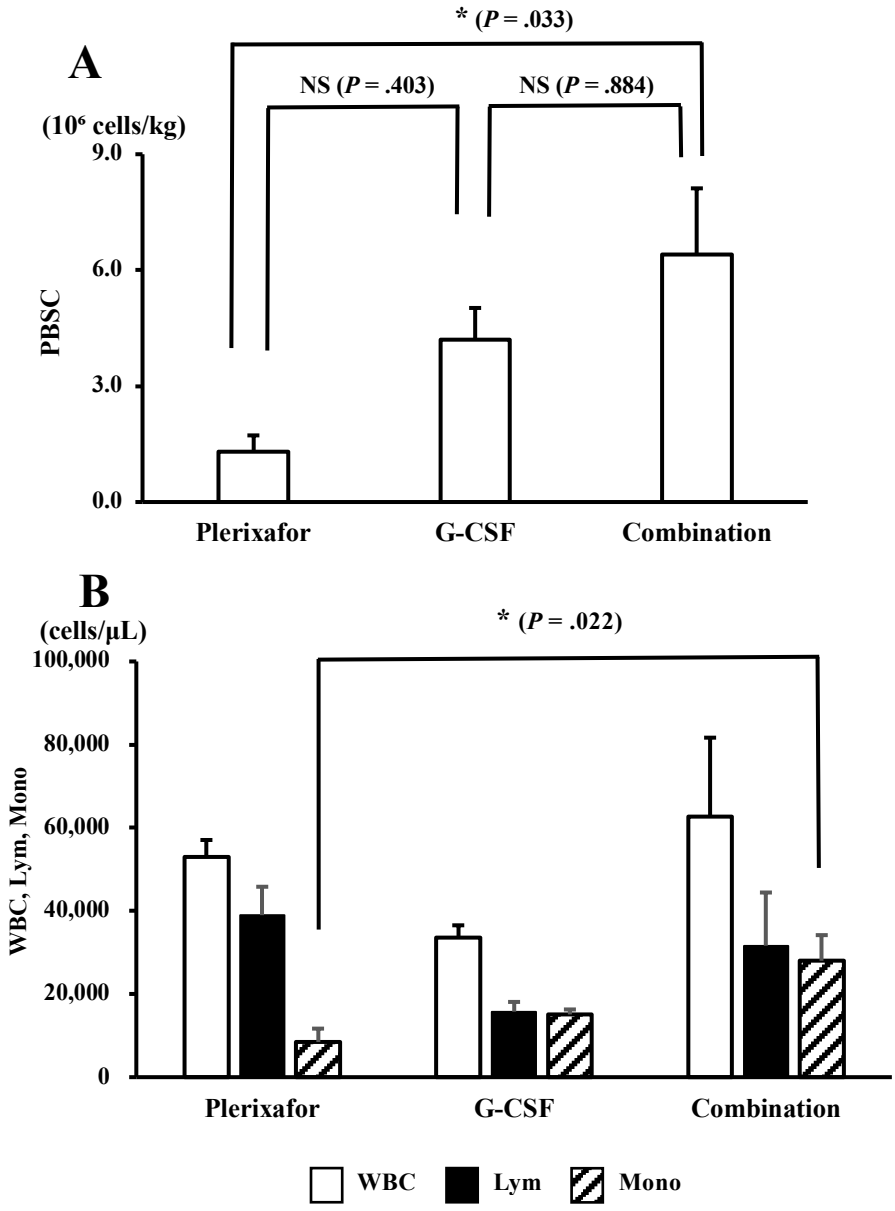

Figure 4. Mean ± SD of PBSC, WBC, lymphocyte (Lym), and monocyte (Mono) of the apheresis products in the three groups. PBSC (A) counts were quantified by flow cytometry. WBC, Lym, and Mono (B) were measured by hematology analyzer. Abbreviation: SD, standard deviation, NS, no statistical difference. *$P < 0.05$.

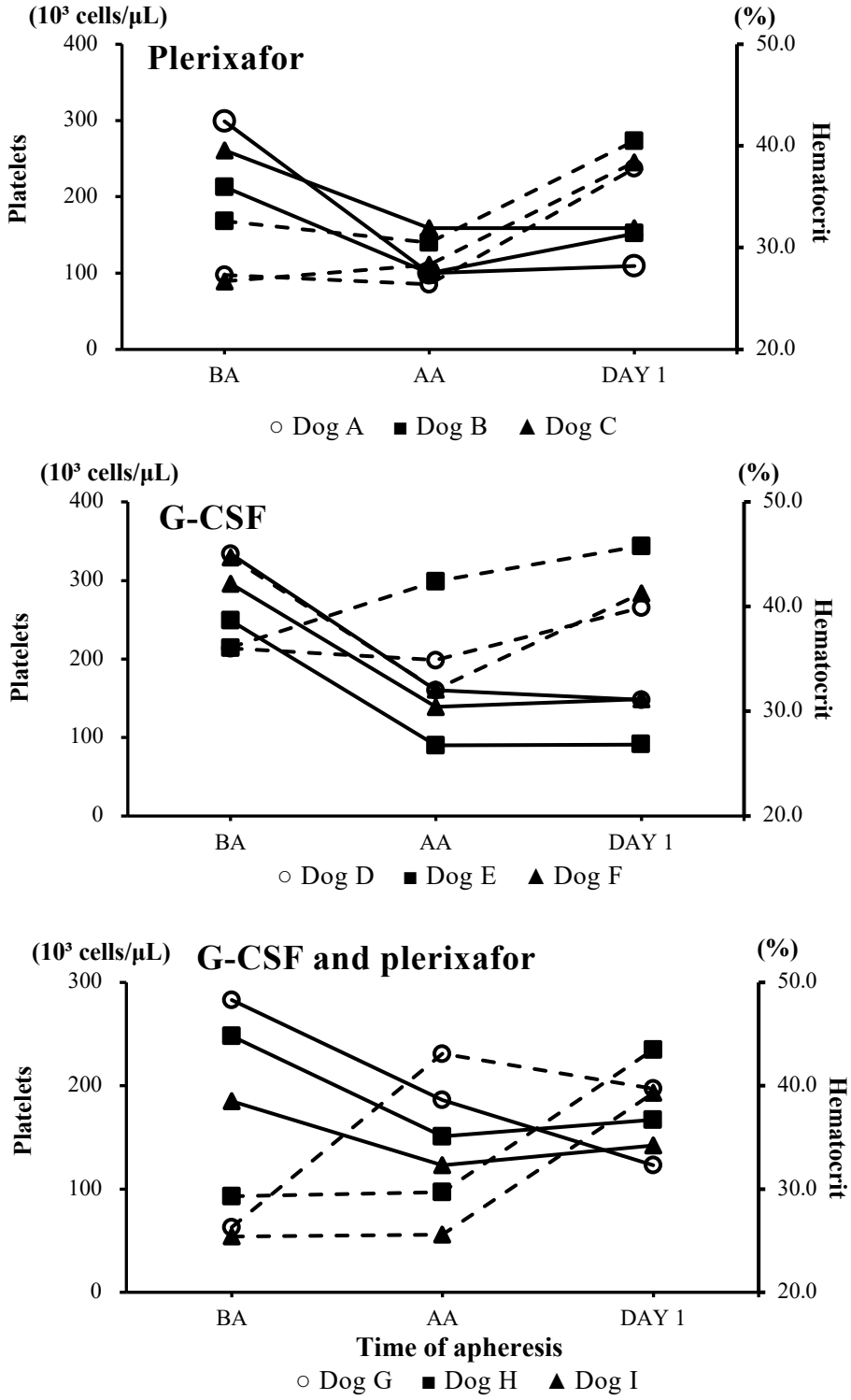

Figure 5. Changes in peripheral Plts and hematocrit before the start of apheresis (BA), after apheresis (AA), and the day after apheresis (DAY 1).

Platelets
Hematocrit

Discussion

This study compared three mobilization protocols for canine PBSC apheresis using the Spectra Optia® CMNC protocol. It was shown that the mobilization protocol using G-CSF or G-CSF + low-dose plerixafor in healthy beagle dogs can facilitate the collection of the necessary and sufficient amount of PBSCs for a HSCT. In addition, it was demonstrated that apheresis using the Spectra Optia® CMNC protocol in dogs weighing <12 kg is safe and feasible as with conventional machines.

The apheresis products contained >4.0 × 10^6 PBSCs/kg in all three dogs of the G-CSF + low-dose plerixafor combination protocol group. According to human and canine studies, the minimum cell dose needed for successful HSCT is 2.0 × 10^6 PBSCs/kg. However, a higher number of infused PBSCs, such as >4-5 × 10^6 PBSCs/kg, is targeted for the treatment in humans as it reportedly leads to earlier Plt and neutrophil recovery and better clinical outcome [Leung and Kwong, 2010; Mohty *et al*., 2018; Willcox *et al*., 2012]. Non-Hodgkin's lymphoma data of human autologous PBSC transplantation suggest that with the G-CSF and plerixafor mobilization protocol, a significantly higher proportion of patients (59%) can achieve the target PBSC count (>5.0 × 10^6 PBSCs/kg) in fewer days of apheresis compared with that by G-CSF alone (20%) [Dipersio *et al*., 2009]. In human tumor cases of poor mobilization in response to G-CSF alone, plerixafor

may rescue patients from mobilization failure. In a previous study, 75% (42/56 cases) of patients who had failed to achieve a minimal cell dose or did not proceed to apheresis due to a low PBSC count with conventional mobilization protocol (without plerixafor) successfully reached the target PBSC count (>2.0 × 10^6 PBSCs/kg) after undergoing a G-CSF + plerixafor protocol [Duarte *et al*., 2011]. To the best of my knowledge, this combination protocol has not been evaluated in terms of efficacy and feasibility of apheresis in experimental or clinical canine studies. In veterinary medicine, as general anesthesia is necessary for apheresis, it is desirable to collect the target PBSC count using a single procedure. Moreover, this indicates that if a set PBSC count is to be extracted, using this mobilization protocol would enable reduction in the volume of blood processed, and therefore, a shorter duration of anesthesia would be needed for the apheresis procedure. Accordingly, it is important to increase the number of extracted PBSCs by enhancing the mobilization effect. This data suggested that a G-CSF + low-dose plerixafor protocol is a more effective mobilization regimen for canine apheresis than G-CSF or high-dose plerixafor alone.

In the plerixafor group, all three dogs failed to obtain sufficient PBSC yields (i.e., <2.0 × 10^6 PBSCs/kg). In a previous study, a single administration of high-dose plerixafor resulted in efficient mobilization of PBSC for HSCT in dogs (median PBSC count contained in the apheresis product, 3.9 × 10^6/kg) [Burroughs *et al*., 2005]. Conversely,

the elevation of peripheral blood WBC and HSC counts up to 4 h after plerixafor administration in the previous study was comparable to that in the present study. Variations in apheresis parameters and/or the timing of the collection of cells may have contributed to the failure to obtain a sufficient number of PBSCs in this study. In a similar study using a rhesus macaque model, the apheresis procedures were initiated 3-6 h after plerixafor administration [Haynes *et al*., 2017]. In the canine transplantation model study, the number of circulating PBSCs peaked at 8 h following plerixafor administration in three of four dogs [Burroughs *et al*., 2005]. Based on these animal model studies, the mobilization protocol consisted of approximately a 4-h apheresis initiated 5 h after plerixafor administration. In human medicine, plerixafor is recommended to be given 10-11 h before apheresis [Stewart *et al*., 2009]. It is suggested that the response time to plerixafor varies depending on the individual or species. By delaying the start of apheresis by 1-2 h, it is possible to increase the number of PBSCs collected by apheresis. Furthermore, as the present study aimed at comparing the three mobilization regimens, apheresis parameters were standardized based on the characteristics described above. These apheresis parameters may also have played a major role in the outcome of PBSC collection by apheresis in this study. Therefore, further consideration will be needed in future studies.

In the current study, peripheral Plt counts and hematocrit decreased after plerixafor

administration in some dogs. In the rhesus macaque model study, subcutaneous injection of plerixafor resulted in a decrease in peripheral Plt counts [Haynes *et al*., 2017]. Furthermore, plerixafor influences red blood cells and Plts; it reportedly causes anemia or thrombocytopenia in 3-6% of human patients [Cashen *et al*., 2008; Kessans *et al*., 2010]. However, few studies on plerixafor mention on the severities of these adverse events or the occurrence of any other serious adverse events. In the present study, clinical symptoms related to thrombocytopenia or anemia were not observed after plerixafor administration; therefore, it was shown that plerixafor and the mobilization protocols used in this study can be safely performed in dogs.

Overall, the mean Plt counts in peripheral blood pre- and post-apheresis were 263 ± 43.2 and 134 ± 31.1 × 10^3 cells/μL, respectively. The mean Plt attrition rate associated with apheresis using the Spectra Optia® CMNC protocol was 48% (33.5-66.5%). Consistent with this results, previous human and canine apheresis studies described a decrease in Plt count (27.0-44.7%) after apheresis compared with the count before apheresis [Lee *et al*., 2017; Punzel *et al*., 2017; Putensen *et al*., 2018; Suter, 2011]. Loss of Plts is an adverse effect commonly associated with apheresis. Punzel et al. evaluated the impact of a lower packing factor (PF) for the CMNC protocol on decreased product Plt yield [Punzel *et al*., 2017]. Although the PF was set uniformly by 4.5 (machine default value) in the present study, it is possible to preserve Plts if a lower PF, such as 4.0, is used.

In this study, eight of the nine dogs experienced mild anemia before blood was collected to the apheresis machine. Although no abnormal hematological changes were identified on the day of apheresis in these dogs (data not shown), anemia was confirmed after the induction of anesthesia. This hematological change in all eight dogs was resolved, and hemoglobin values were within the normal range by 24 h after apheresis. The cause of anemia in these eight dogs was unclear. However, it is possible that the mobilization regimens and apheresis procedure were not the direct cause of this phenomenon. Apheresis in pediatric patients and dogs (i.e., low-weight patients) has raised similar concerns regarding a larger machine and tubing kit priming volume (extracorporeal volume) compared with the patient's blood volume [Even-Or *et al*., 2017]. Therefore, in these low-weight patients, the apheresis machine was primed with saline, albumin solution, packed red blood cells, or whole blood. In a previous study, three dogs weighing <14.0 kg had mild-to-moderate anemia (packed cell volume; 17-35%) after apheresis [Posner *et al*., 2013]. In that study, a combination of colloid and crystalloid solutions, saline, and autologous whole blood were used as priming solutions. In the present study, the machine priming was performed using stored autologous whole blood, which resulted in the maintenance of the hematocrit during apheresis. Thus, this technique is considered to be effective for a more secure and safer apheresis for small-to-middle-sized dogs.

In the present study, eight of the nine dogs developed hypocalcemia during

apheresis. The cause was likely the insufficient administration of calcium gluconate. The citrate contained within the anticoagulant has been utilized for apheresis; therefore, citrate-induced hypocalcemia is well known as an apheresis-related complication. In a human study, the serum calcium concentration decreases by 33-39% after apheresis compared with the concentration before apheresis, and this decrease is associated with the citrate dose [Bolan *et al*., 2001]. Therefore, a CRI of calcium gluconate solution was used to prevent citrate-induced hypocalcemia throughout apheresis. In a previous study, the concentration of the calcium gluconate solution used in apheresis was approximately 10 times higher than that used in the present study [Suter, 2011]. Thus, if the solution was changed to a higher concentration, a decrease in serum calcium concentration could be prevented during apheresis.

The limitations of the present study include the small sample size and lack of the confirmation regarding engraftment ability of the collected stem cells. First, the sample size was small, and the fact that some differences between the groups did not reach statistical significance may reflect a lack of power. Second, potential factors affecting the PBSC yield could not be analyzed. Third, only evaluated the number of the collected PBSCs was evaluated. Accordingly, further studies are required to consider the differences in the engraftment rate or recovery period and the hematopoietic efficacy of the mobilization regimen or transplanted cell number.

In conclusion, these results indicate that the apheresis procedure using Spectra Optia® CMNC protocol allows safe and adequate collection of PBSCs in healthy dogs after mobilization with a combination regimen comprising G-CSF and low-dose plerixafor. In addition, this mobilization protocol may contribute to a decrease in the percentage of collection failure associated with conventional mobilization protocols in dogs. Adverse effects from this procedure were transient and mild without any clinical signs. A further study in a clinical setting to evaluate the safety and efficacy of this mobilization protocol and apheresis procedure is warranted.

Summary

In veterinary medicine, hematopoietic stem cells are generally mobilized into peripheral circulation using a granulocyte-colony stimulating factor (G-CSF). This study aimed to evaluate the peripheral blood stem cell (PBSC) mobilization effect of three different regimens for PBSC apheresis with Spectra Optia® continuous mononuclear cell collection (CMNC) protocol in healthy dogs. In this study, PBSC mobilization was performed using high-dose plerixafor alone, a G-CSF alone, or the combination of the low-dose plerixafor and G-CSF. Three dogs were assigned to each mobilization protocol. The total blood volume processed was uniformly set as 270 mL/kg and the number of PBSCs within the apheresis product were compared. Changes in complete blood count, PBSC counts, and blood chemistry analysis were monitored before, during, and after apheresis. All dogs tolerated the apheresis procedure using the Spectra Optia® system with minimal adverse effects. The mean PBSC counts of the apheresis products for plerixafor, G-CSF, and the combination group were 1.3 ± 0.24, 4.2 ± 0.47, and $6.4 \pm 0.9 \times 10^6$ cells/kg, respectively. These results indicate that PBSC mobilization with the combination of G-CSF and plerixafor appeared the most effective protocol than traditional mobilization regimens in dogs. Further, the apheresis procedure using Spectra

Optia® CMNC protocol in dogs is safe and feasible.

Chapter II

Nonmyeloablative pretransplant conditioning regimen using total lymphoid irradiation with volumetric arc therapy in healthy dogs

Introduction

Allogeneic HSCT is a promising treatment for human patients with hematological neoplastic or non-neoplastic diseases [Baron and Storb, 2006]. In human medicine, allogeneic HSCT is the critical therapy for hematologic malignancies, such as lymphoma or leukemia [Bair *et al*., 2020; Copelan *et al*., 2019]. The major anti-tumor effects of allogeneic HSCT are produced by pretransplant conditioning treatment, such as TBI and high-dose chemotherapy, and a posttransplant immune response called graft-versus-leukemia (GVL) effect [Copelan *et al*., 2019]. Transplant conditioning is an area of active research, and a number of preclinical studies with dogs have been performed to determine the optimal conditioning treatment for allogeneic HSCT [Storb *et al*., 1997; Storb *et al*., 1999]. In the veterinary field, there are few reports on allogeneic HSCT for dogs [Lupu *et al*., 2006; Suter *et al*., 2015]. Suter *et al* (2015) reported a favorable prognosis in canine case of acute lymphoblastic leukemia treated with allogeneic HSCT. Although allogeneic HSCT has shown promise as a potential treatment for canine hematological malignancies, very few studies have investigated the application of this treatment in the veterinary clinical field.

Traditionally, supralethal doses of TBI and/or high-dose chemotherapy have been used for pretransplant conditioning in allogeneic HSCT. The main object in these treatments is the induction of host immunosuppression, creation of marrow space for the graft, and direct tumor destruction [Diaconescu *et al*., 2005; Baron

and Storb, 2006]. However, these aggressive treatments, which are referred to as myeloablative conditioning, have been associated with higher risk of transplant-related morbidity and mortality. For this reason, these treatments have limited adaptation to non-comorbid and relatively young patients in human medicine [Baron and Storb, 2006]. To expand the application range of allogeneic HSCT to elderly patients or comorbid conditions, nonmyeloablative conditioning regimens have been extensively studied in both clinical and experimental settings [Baron and Sandmaier, 2006; Spinner *et al*., 2019; Storb *et al*., 1997; Storb *et al*., 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 [Storb *et al*., 1997; Storb *et al*., 1999]. This conditioning regimen has been translated to human medicine, and is utilized widely in the clinical setting [Fatobene *et al*., 2020].

Total lymphoid irradiation (TLI) is one of the non-myeloablative 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 human clinical studies, non-myeloablative TLI and antithymocyte globulin conditioning is a useful regimen not only for the protection against graft-versus-host disease (GVHD) but also for improved prognosis by the GVL effect for human hematological malignancies [Kohrt *et al*., 2009; Rezvani *et al*., 2015]. A study using a canine transplantation model has shown that 4.5 Gy TLI, targeted to cervical, thoracic, and

abdominal lymph nodes, in the combination with immunosuppression drugs, showed initial allogeneic marrow cell engraftment, and eventually, half of the dogs maintained long-term donor/host chimerism [Storb *et al*., 1999]. Volumetric modulated arc therapy (VMAT) is one of the radiation dose delivery techniques, which results in a highly conformal dose at target, while sparing normal tissues. Based on these superior characteristics, VMAT has been applied to HSCT conditioning treatment using radiotherapy, such as TBI and TLI, in order 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 my knowledge, no study has investigated the application of TLI in combination with VMAT in dogs; and the safety and feasibility of such treatment has not been elucidated. Here, the feasibility and safety of two difference doses of TLI using VMAT was evaluated, and assessed the effects of TLI on peripheral lymphocyte properties in healthy dogs. Moreover, dog leukocyte antigen (DLA)-matched canine allogeneic HSCT with TLI conditioning was investigated to verify the engraftment ability of this regimen.

Materials and methods

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 Hokkaido University (approval number: 17-0007). No abnormalities were observed on physical examination and blood laboratory tests [e.g., CBCs and general serum chemistry]. These tests were conducted using a Procyte™ and Dri-Chem 7000V.

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 (DS Pharma Animal Health) in oxygen. During planning CT (1-2 weeks prior to TLI treatment) all dogs were positioned in sternal recumbency, and the entire body immobilized using a vacuum deformable cushion (ESFORM, Engineering System, Nagano, Japan). Planning 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, Tokyo, Japan).

The Monaco® 5.11.01 treatment planning system (TPS) (Elekta, Tokyo, Japan) was used for contouring and 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 for the gross target volume (GTV). The clinical target volume (CTV) was delineated on the adipose 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 four 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 using a VMAT technique, and all procedures were delivered using 6 MV photons from a linear accelerator (Elekta Synergy®, Elekta, Tokyo, Japan). 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 histogram (DVH). Constraints on the conditioning of organs and target lymphoid tissues were standardized across all dogs. Quality assurance (QA) included verification of the dose distribution using a helical diode array (ArcCheck®, Sun Nuclear Co., Melbourne, FL, USA) and measurements of the absolute central dose using a farmer-type ionization chamber (Type 30013, PTW, Freiburg, Germany) and a dosimeter (Ramtec Smart, TOYO MEDIC CO., Tokyo, Japan) for each field. All measurements were analyzed 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.

For all dogs TLI treatments were delivered in one session. An anesthetized dog with its whole body immobilized using a cushion, was placed on the 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 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 set-up error

correction using a computer-controlled robotic 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. After repositioning, the second and third fields were irradiated sequentially for the “caudal half body” scans.

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 [VCOG-CTCAE following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1, 2016]. The dogs received enrofloxacin [10 mg/kg, oral administration (PO), q24h] (Bayer, Osaka, Japan) 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, nine weeks after completion of the TLI in all dogs.

Peripheral Blood Lymphocyte Subset analysis

Flow cytometric investigation of peripheral blood quantifying the percentage of T, B, and regulatory T (T-reg) cells, were 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 (PBMCs) were separated using

density gradient centrifugation (Lymphoprep™, Abbott Diagnostics Technologies AS, Oslo, Norway). The PBMCs samples were incubated with FITC-conjugated rat anti-canine CD3 (diluted to 1:10 in 2% fetal bovine serum), 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, or APC-conjugated rat anti-canine FOXP3 monoclonal antibodies, and were analyzed using FACS Versa™. The lymphocyte population was determined from forward scatter and side scatter plots. The gating strategies for these lymphocyte subsets were as follows: T-cells ($CD3^+CD21^-$), B-cells ($CD3^-CD21^+$), T-helper cells ($CD4^+CD8^-$), T-cytotoxic cells ($CD4^-CD8^+$), T-reg cells ($CD4^+CD25^+FOXP3^+$).

Lymphocyte Stimulation Test

To evaluate lymphocyte proliferation capability, PBMCs were separated from heparinized peripheral blood before, and at 1, 4, and 8 weeks after the TLI treatments as described above. PBMCs were incubated with 0.5 μM carboxyfluorescein succinimidyl ester (CFSE) (Vybrant™ CFDA SE Cell Tracer Kit, Thermo Fisher) for 10 minutes in the dark at 37°C. After washing the cells, CFSE-labeled PBMCs, were cultured at 37°C, in 5% CO_2 in Roswell Park Memorial Institute 1640 medium (Gibco by Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum with or without 1.0 μg/ml concanavalin A (ConA) (Sigma Aldrich) for four 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 as follows: the mononuclear cells population was determined from forward scatter and side scatter plots. CD3-positive 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 CD3-positive cells.

Allogeneic HSCT

In the allogeneic HSCT experiment, a pair of sibling intact male beagles was used as donor and recipient. Prior to this experiment, blood samples were sent to the Fred Hutchinson Cancer Research Center to check the DLA-types (DRB-1, DLA-88, DQA-1, and DQB-1) of four sibling dogs. In this study, the two dogs which had four identical DLA-type pairs were chosen as donor and recipient. These dogs were both aged 13 months, and weighed 10.1 and 11.0 kg, respectively.

Recipient conditioning for HSCT was performed by using the 12 Gy TLI with VMAT regimen from the first experiment. The day of commencement of TLI was defined as day 0. On the last day of the TLI (day 3), PBSCs were collected from the donor as chapter I described. Briefly, PBSCs were mobilized using the combination protocol, which comprised rhG-CSF (5 μg/kg, SC, q12h) and plerixafor (0.24 mg/kg, SC). Apheresis procedure was conducted using a Spectra

Optia® and the total blood volume to be processed was three times the whole-body blood volume. In this study, 7.2×10^6 CD34-positive cells/kg were collected, and all of these cells were infused intravenously into the recipient promptly after the completion of all TLI processes. An immunosuppression agent consisting of cyclosporine A (CsA) (Kyoritsu Pharmaceutical, Tokyo, Japan) and mycophenolate mofetil (MMF) (Chugai Pharmaceutical) was administered to the recipient dog. The immunosuppression regimen consisted of CsA at 10 mg/kg (PO, q24h) on days 2 to 38, 7.5 mg/kg on days 39 to 53, 5 mg/kg on days 54 to 78, and 3 mg/kg on days 79 to 103, and MMF at 10 mg/kg (PO, q12h) on days 3 to 30. The recipient also received antibiotic drugs as follows: polymyxin B (8,000 U/kg, PO, q8h) (Pfizer, Tokyo, Japan) on days 2 to 17, kanamycin (6 mg/kg, PO, q24h) (Meiji Seika Pharma, Tokyo, Japan) on days 2 to 17, enrofloxacin (10 mg/kg, PO, q24h) on days 3 to 17.

In order to evaluate adverse effects of allogeneic HSCT, CBCs and serum chemistry were performed until day 115 (16 weeks). To analyze engraftment and donor/recipient chimerism, peripheral blood of the recipient was collected before HSCT and every 1 to 2 weeks after HSCT. Collected blood samples were separated into PBMCs and granulocytes using a density gradient centrifugation method (Polymorphprep™, Abbott Diagnostics Technologies AS), and genomic DNA was extracted (High Pure PCR Template Preparation Kit, Roche Diagnostics, Basel, Swiss) from each cell fraction. Chimerism was assessed using tetranucleotide repeat-based markers (clone name, 2001) [Francisco *et al*., 1996], to distinguish donor and recipient, by fluorescent variable number tandem repeat polymerase

chain reaction (PCR) assays. The PCR products were analyzed by capillary electrophoresis, and these procedures were outsourced to a commercial laboratory (System Biotics, Kanagawa, Japan). The rate of donor chimerism was calculated using the following formula: Donor chimerism = [(a donor specific peak area)/ {(a donor specific peak area) + (a recipient specific peak area)}] × 100. In this study, HSCT engraftment was defined as detection of > 2% of donor-derived DNA in both cell fractions [Lange *et al*., 2009].

Statistical analysis

Statistical analysis was performed using a commercially available statistical program (JMP® Pro version 12.1.0; SAS institute, Cary, NC, USA and Microsoft® Excel 2013). The results are presented as mean ± SD. 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.

Results

TLI dose distribution

All dose information was obtained by planning DVH. Table 1 shows 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 plans. In the 8 Gy plan group, mean D_{50} and D_{95} for the target lymphoid tissues of the whole body were 731.5 ± 127.0 (range 522.7 to 900.2 cGy) and 903.1 ± 147.2 cGy (range 641.4 to 1109.2 cGy), respectively. In the 12 Gy plan group, mean D_{50} and D_{95} for the target lymphoid tissues of the whole body were 946.8 ± 270.9 (range 455.7 to 1242.1 cGy) and 1,211.5 ± 283.4 cGy (range 703.4 to 1484.2 cGy), respectively. Table 2 shows D_{50} and D_{95} for the OAR in each plan. In the 8 Gy plan group, mean D_{50} and D_{95} for the bone marrow of the whole body were 7.7 ± 7.2 (range 0.9 to 19.1 cGy) and 109.4 ± 41.2 cGy (range 34 to 155.8 cGy), respectively. In the 12 Gy plan group, mean D_{50} and D_{95} for the bone marrow of the whole body were 8.5 ± 6.7 (range 1.8 to 17.0 cGy) and 144.1 ± 25.0 cGy (range 103.4 to 177.2 cGy), respectively.

In the 8 Gy group, the difference of mean absolute central doses and mean gamma pass rate at cranial half body (cranial field) were 2.6 ± 1.5 (range 0.8-4.6 %), 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 field) 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 %), 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 %), 95.1 ± 3.6 (range 90.2-98.5 %), respectively.

TLI-related toxicities

TLI treatment was successfully administered to all six animals according to the planned schedule. The kinetics of peripheral neutrophil counts varied erratically in each of the animals following TLI (Figure 1). 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, at 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 Plt counts decreased in all dogs, 5 of the 6 dogs were Grade 2 and the remaining dog from the 12 Gy group was Grade 1 (Figure 2). The median time for the Plt count nadir was 13 days (range 12 to 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 2 of the 3 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.

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 to 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 all dogs.

In the peripheral lymphocyte subset analyses, no notable changes were observed in the percentage of CD3-positive T-cells in lymphocytes, although percentages of CD21-positive B-cells decreased after TLI (Figure 4). As shown in Figure 4, the mean percentages of CD21-positive 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 CD4-positive T-helper cells were lower than the pre-TLI levels (Figure 4). In CD8-positive T-cytotoxic cells and T-reg cells, there was no significant difference in numbers before and after TLI at any time (Figure 4).

Following TLI procedures, CD3-positive T-cell proliferation decreased at all time points compared to proliferation before TLI in both groups (Figure 5). 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.

Allogeneic PBSC engraftment

Figure 6 shows granulocyte, lymphocyte, and Plt count changes from before TLI (day -1) to 16 weeks after HSCT (day 115) in the recipient dog. The granulocyte and Plt counts transiently decreased after TLI. These bone marrow toxicities were recorded as Grade 1, and no other hematological and serum biochemical toxicities were observed. Vomiting was observed for four days after allogeneic HSCT, and maropitant (2.0 mg/kg, PO, q24h) (Zoetis, Tokyo, Japan) was administered for five days, although there were no other clinical signs suggesting acute GVHD during the follow-up period.

Figure 7 shows the donor chimerism in the PBMCs and granulocyte populations in the recipient dog. Donor-derived DNA was detected in PBMCs and granulocytes at one and two weeks after HSCT, respectively. After two weeks of HSCT, the allograft engraftment was deemed successful based on the detection of donor-derived DNA in both cellular populations. Engraftment was sustained during the follow-up period in this study.

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
D_{50} (cGy)	**8Gy (n = 3)**	1,092.1 ± 24.2	891.9 ± 29.8	687.3 ± 37.6	941.0 ± 26.0
	12Gy (n = 3)	1,467.2 ± 18.4	1,318.5 ± 22.8	733.1 ± 28.8	1,327.3 ± 34.6
D_{95} (cGy)	**8Gy (n = 3)**	883.0 ± 30.2	733.4 ± 56.0	543.5 ± 27.4	766.2 ± 43.0
	12Gy (n = 3)	1,201.5 ± 111.8	1,022.9 ± 61.0	504.1 ± 31.8	1,058.8 ± 15.2

All doses are reported as means ± standard deviation.

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

Table 2: Planned organ at risk doses

	Prescription dose	Bone marrow of the cranial half body	Bone marrow of the caudal half body	Heart	Lungs	Eyes	Kidneys	Liver
D_{50} (cGy)	**8Gy (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
	12Gy (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)	**8Gy (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
	12Gy (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

All doses are reported as means ± standard deviation.

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

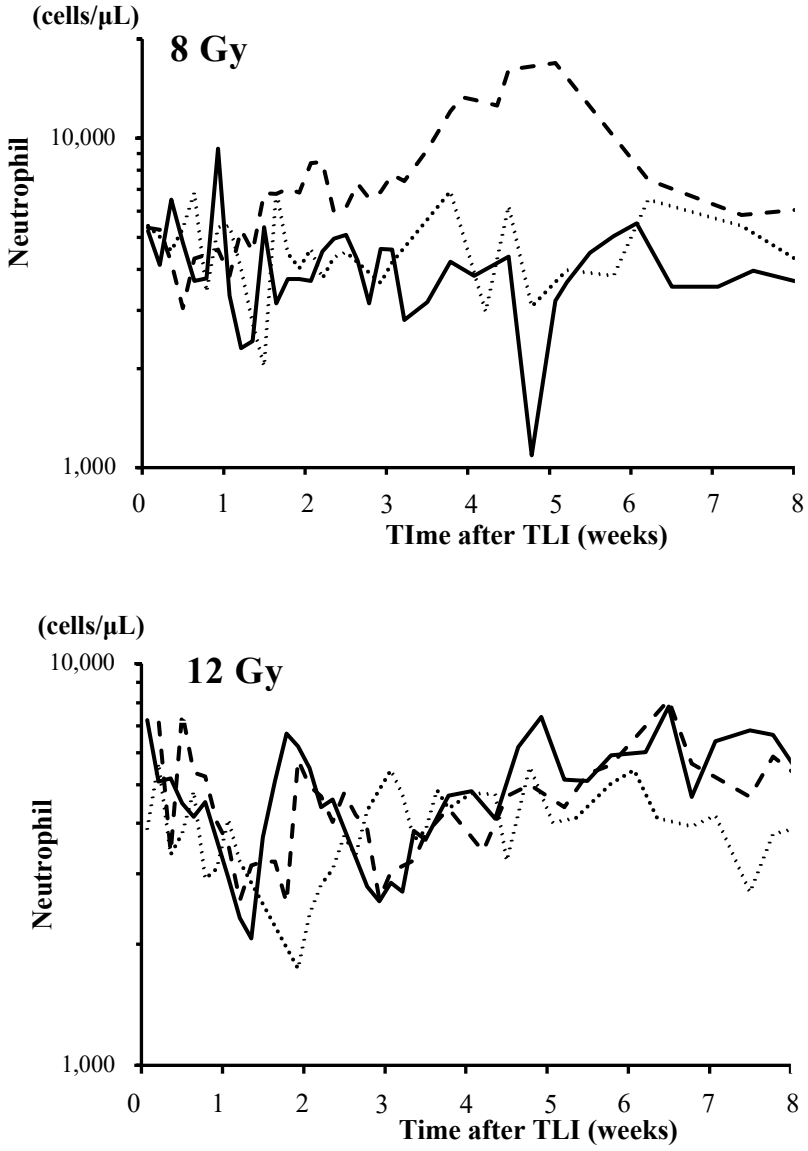

Figure 1. Changes in peripheral neutrophil counts after 8 or 12 Gy TLI in six healthy dogs. Each line (solid, dotted, and dashed line) represents the individual dog variation in each group.

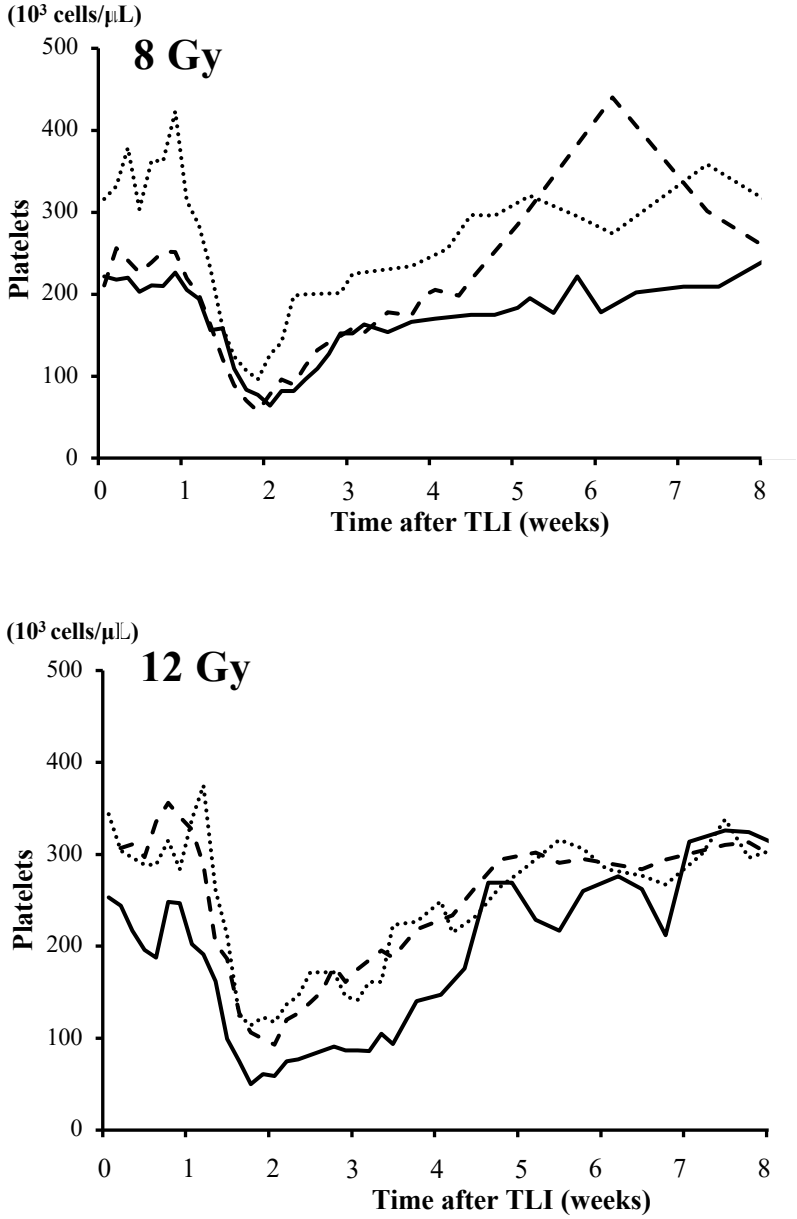

Figure 2. Changes in peripheral Plts after 8 and 12 Gy TLI in six healthy dogs. Each line (solid, dotted, and dashed line) represents the individual dog variation in each group.

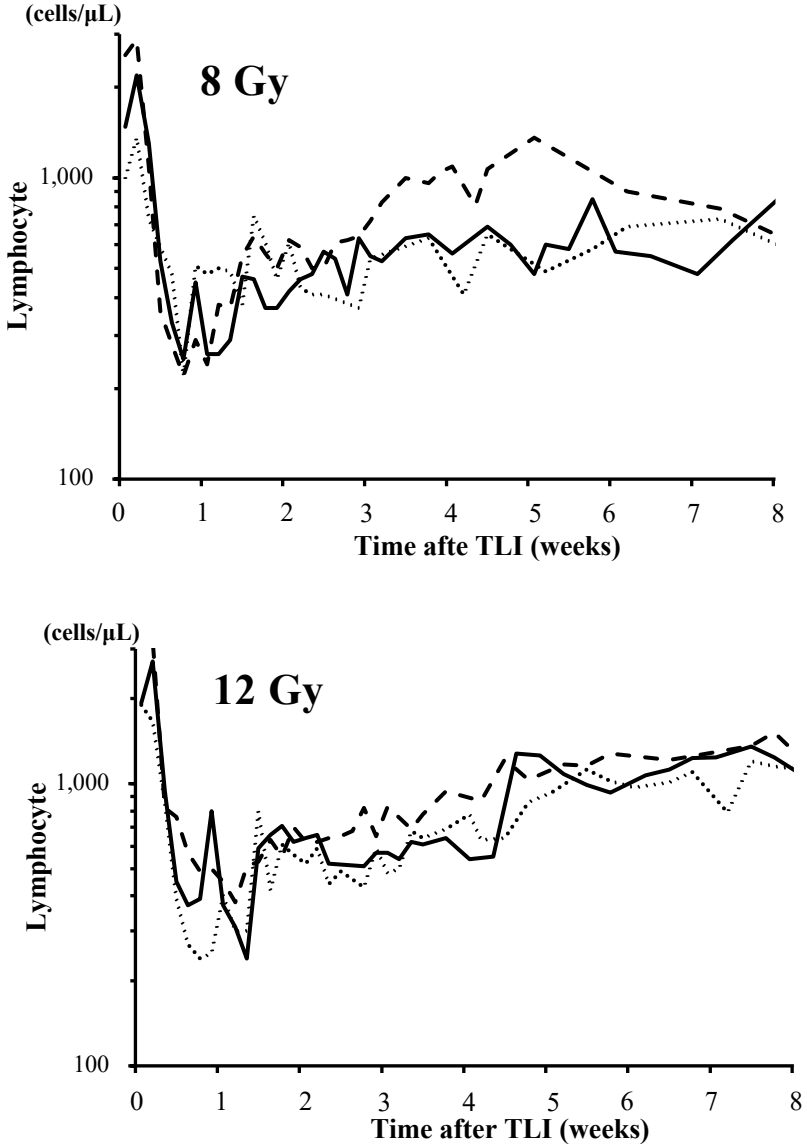

Figure 3. Changes in peripheral lymphocyte counts after 8 and 12 Gy TLI in six healthy dogs. Each line (solid, dotted, and dashed line) represents the individual dog variation in each group.

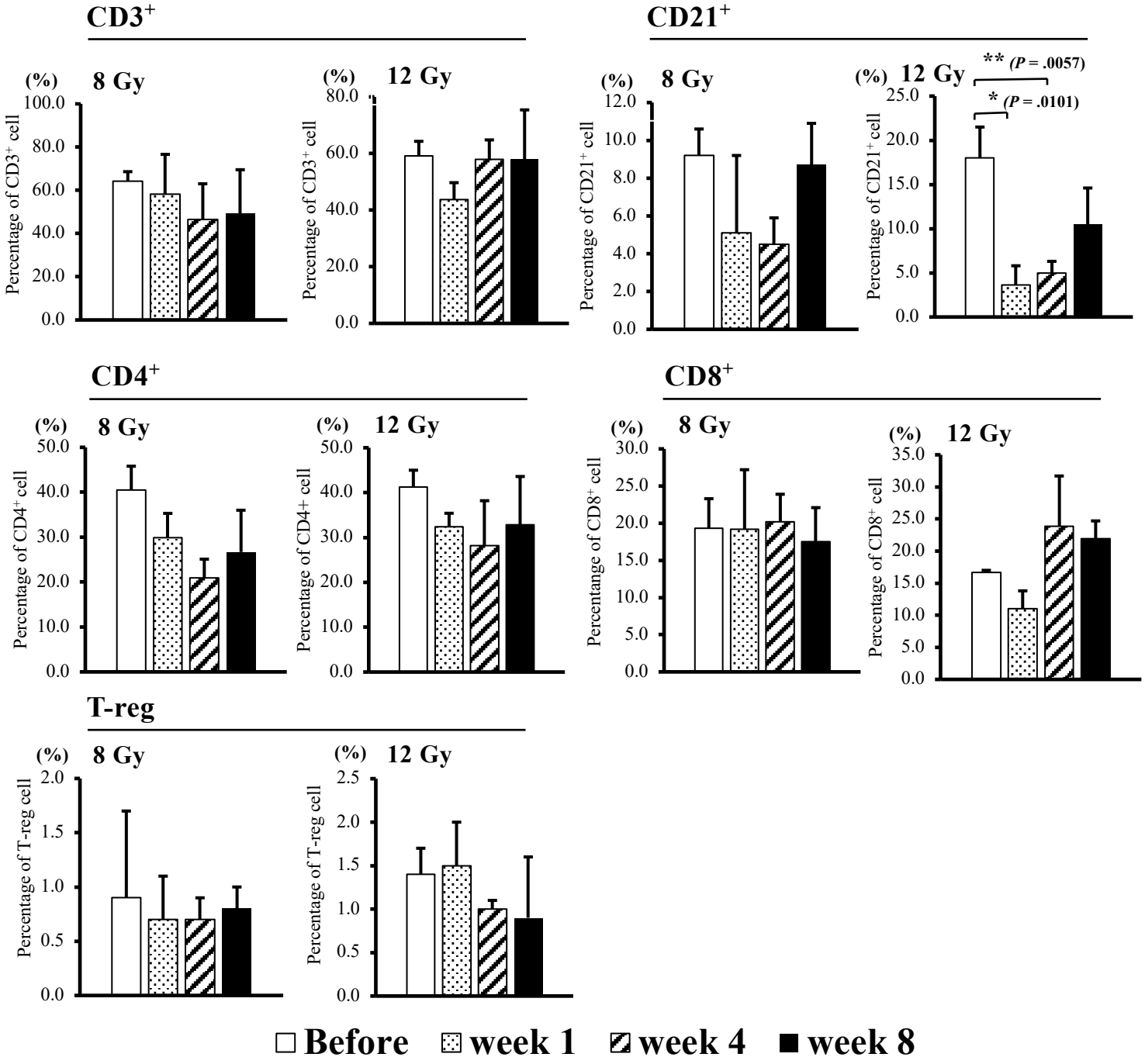

Figure 4. Changes in peripheral lymphocyte subset following each TLI condition. Mean ± standard deviation of each peripheral lymphocyte percentage. *$P < 0.05$, **$P < 0.01$.

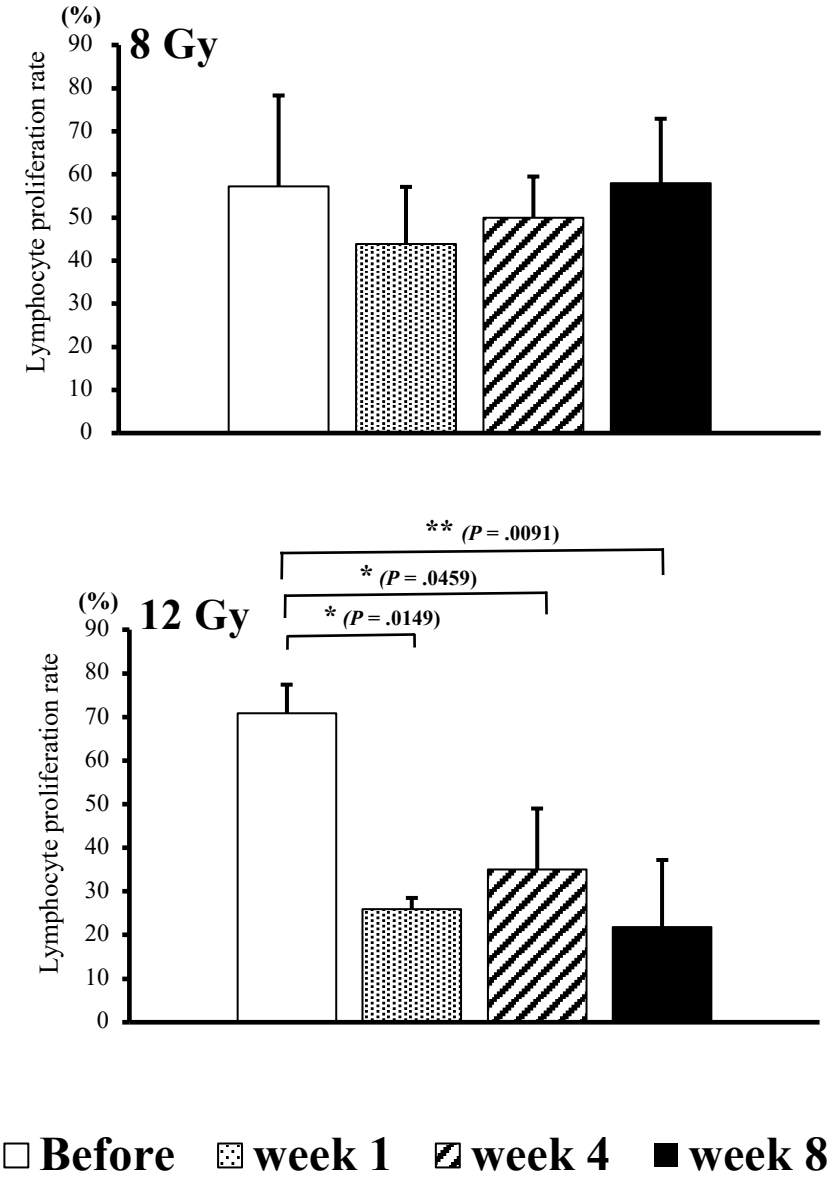

Figure 5. Changes in peripheral lymphocyte proliferation rate following 8 and 12 Gy TLI condition. Mean ± standard deviation of each proliferation rate. **P* < 0.05, ***P* < 0.01.

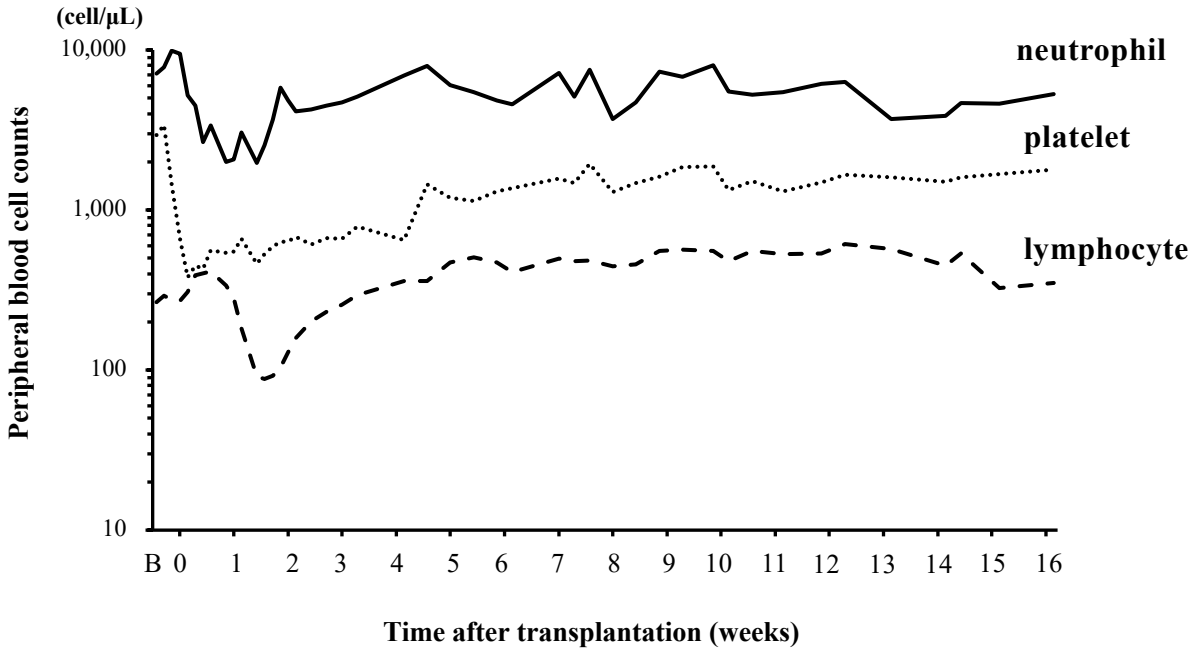

Figure 6. Changes in peripheral neutrophil (solid line), platelet (dashed line), and lymphocyte counts (dotted line) before (B) and after allogeneic HSCT.

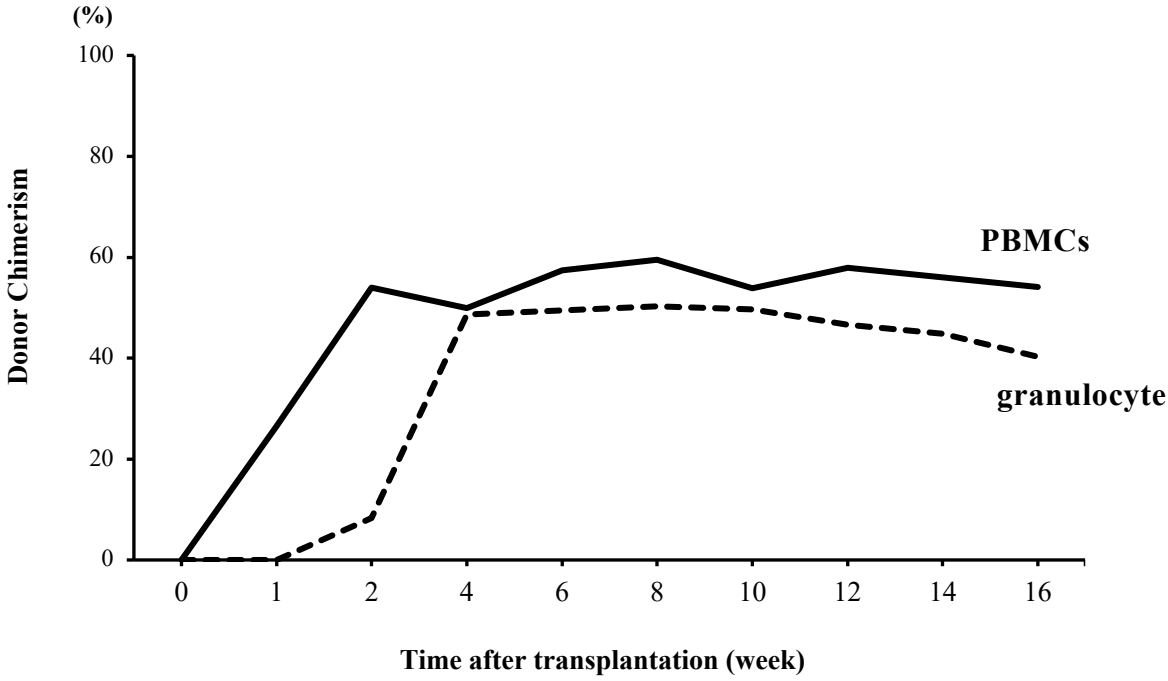

Figure 7. Donor chimerism in the PBMCs (solid line) and granulocyte populations (dashed line) in the recipient dog after allogeneic hematopoietic transplantation.

Discussion

This study evaluated the efficacy and safety of TLI using VMAT as a conditioning treatment for canine allogeneic HSCT. No severe adverse effects were observed in 8 and 12 Gy TLI, and these conditioning regimens can therefore be performed nonmyeloablatively in healthy dogs. In addition, 12 Gy TLI combined with CsA and MMF achieved sustained engraftment of DLA-identical littermate hematopoietic stem cells.

In this study, a favorable dose concentration for target organs and dose reduction to OAR in TLI plans was obtained by the use of VMAT technique. Nonmyeloablative conditioning, such as 2 Gy TBI or 8 Gy TLI, for allogeneic HSCT 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 intensity-modulated radiation therapy (IMRT) techniques [Patel *et al*., 2014; Somlo *et al*., 2011; Wong *et al*., 2013]. It is expected that treatment 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; Clift *et al*., 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 are often evidences 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 total marrow and lymph node irradiation (TMLI) using IMRT as a conditioning treatment for HSCT [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 the present study, calculated doses for OARs were comparable or much lower than these doses. 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, it was concluded that the sparing of normal tissues was sufficiently achieved using VMAT for TLI procedure planning in this study.

To the best of my 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 greater than 96% in all fields (data not shown), and the pass rates for

relative dose were greater than 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 CD4-positive and CD21-positive 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]. Belka *et al* (1999) described a reduction in the levels of peripheral blood lymphocytes with localized radiotherapy. Consistent with the present study results, this study indicated that B-cells are the most radiosensitive of all leukocytes and suffer a long-lasting depletion. 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, the data suggest that this TLI regimen may be effective in obtaining sufficient peripheral lymphocyte depletion.

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 this 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 HSCT, 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 to 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* (1999) reported that two dogs experienced a decrease in neutrophil counts after receiving 4.5 Gy TLI without HSCT. 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 on the basis of most animals not exhibiting lasting signs or symptoms associated with neutropenia and thrombocytopenia. In the present study, decreases in neutrophil and platelet counts were comparable or less severe than those of the previous studies, therefore it was 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 and CsA/MMF, but also of the frequent blood sampling required for experimental data collection. As a consequence of this event, sample collection sites were carefully disinfected to prevent infection resulting from experimental manipulations. As a consequence of these measures, there were no subsequent events related to infection in this study.

In the present study, allogeneic hematopoietic engraftment was achieved in DLA-identical littermate dogs given 12 Gy TLI and posttransplant immunosuppression with CsA/MMF over a 16 weeks period following HSCT. At four weeks after HSCT, the percentage of donor-derived genes reached approximately 50% in PBMCs and granulocytes, and remained higher than 40% at the end of this study. In a previous canine allogeneic HSCT study, the percentage

of chimerism following the 1 Gy TBI regime (PBMCs; 8-19%, granulocytes; 11-27%) was significantly lower than the 2 Gy TBI regimen (PBMCs; 5-44%, granulocytes; 10-74%) at four weeks after transplantation [Lange *et al*., 2009]. Moreover, the 1 Gy regimen showed eventual graft rejection in all dogs. Several previous studies of conditioning for transplantation showed that HSCT graft rejection often occurred by around 16 weeks after transplantation [Lange *et al*., 2009; Storb *et al*., 1997], and the follow-up period in the current study was determined in reference to these data. The TLI doses of the present allogeneic HSCT were more than twice those used in the previous study [Storb *et al*., 1999]. Additionally, the percentage of the donor chimerism was considered to be relatively high. Thus, it was suggested that pretransplantation conditioning using a combination of 12 Gy TLI and CsA/MMF may induce an immunosuppressive effect on DLA-identical donor-derived HSC engraftment similar to that of a nonmyeloablative regimen.

The previous canine HSCT study demonstrated that the combination of CsA and MMF was the most effective treatment for sustained engraftment [Storb *et al*., 1997]. In general, CsA is often used 35 days after transplantation. However, the previous study showed that prolonging CsA administration from 35 days to 100 days, when the pretransplantation conditioning was attenuated, significantly increased the success rate of engraftment for G-CSF mobilized PBSC transplantation [Zaucha *et al*., 2001]. The treatment regimen and posttransplant immunosuppressive drugs used in this study were determined with reference to

prior studies, and HSCT engraftment was successfully obtained.

In the current study, the recipient dog experienced vomiting for several days soon after transplantation, but no other clinical signs of suspected acute GVHD were observed during the follow-up period. The efficacy of this HSCT regimen for GVHD is still not completely evaluated, and further investigation into both acute and chronic GVHD is required.

This study had several limitations. First, the sample size of this study was small, and the fact that some difference, including post-TLI hematological changes and lymphocyte subset analysis, between the two groups did not achieve statistical significance may reflect a lack of power. Second, this study attempted HSCT on only one pair of dogs, and the observation period was relatively short. Thus, an additional extensive and long-term study is required in experimental and clinical settings to properly evaluate the success rate of HSCT and stable engraftment.

In conclusion, to the best of my knowledge, this is the first study to evaluate the safety and feasibility of TLI using VMAT as pretransplant conditioning treatment in canine studies. The results indicated that 8 and 12 Gy TLI using VMAT did not cause serious side effects and was feasible as a nonmyeloablative technique. In addition, the combination of 12 Gy TLI and immunosuppressive drugs may be useful as a nonmyeloablative conditioning regimen for canine allogeneic HSCT. A further study with a large sample size is warranted to fully demonstrate the efficacy of TLI as a nonmyeloablative conditioning regimen for HSCT.

Summary

The optimal pretransplant conditioning treatment is unclear in canine allogeneic HSCT. To evaluate whether total lymphoid irradiation (TLI) with volumetric modulated arc therapy (VMAT) is safe and effective as a nonmyeloablative HSCT conditioning treatment. In the first study, six healthy dogs were treated with 8 or 12 Gy TLI using VMAT. Hematological and physical changes were recorded. To assess the effect of peripheral lymphocyte condition, lymphocyte subset and proliferative ability were examined. In the second study, allogeneic HSCT was performed in dog-leukocyte-antigen-identical littermates using 12Gy TLI. Chimerism analysis performed at 16- weeks to confirm successful engraftment. In the first study, all dogs showed mild-to-moderate neutropenia and thrombocytopenia, and these hematological changes resolved spontaneously. One dog treated with 8Gy TLI developed transient cutaneous infection. In allogeneic HSCT experiment, recipient dog showed no major complication before and after transplantation. Donor/recipient chimerism was detected from the second week after transplantation, and maintained during the follow-up period. TLI with VMAT was a feasible nonmyeloablative treatment, and may be useful for canine allogeneic HSCT conditioning.

Conclusion

The aim of the present study was to establish allogeneic PBSC transplantation using nonmyeloablative conditioning with the goal of practical application in veterinary medicine. Therefore, this study focused on devising an effective PBSC mobilization protocol and safety pretransplant conditioning regimen in dogs.

In chapter I, to evaluate the effective PBSC mobilization protocol, this study performed PBSC apheresis using the Spectra Optia® CMNC using high-dose plerixafor alone, a G-CSF alone, or a combination of low-dose plerixafor and G-CSF in nine healthy dogs. Regardless of the mobilization protocol used, the total volume of blood processed was uniformly set as 270 mL/kg, and the numbers of PBSCs within the apheresis product were compared. All dogs tolerated the apheresis procedure using the Spectra Optia® CMNC protocol with minimal adverse effects. The mean PBSC counts of the apheresis products for plerixafor, G-CSF, and the combination groups were 1.3 ± 0.24, 4.2 ± 0.47, and 6.4 ± 0.9 × 10^6 cells/kg, respectively. These results showed that the apheresis procedure using the Spectra Optia® CMNC protocol in dogs is safe, and PBSC mobilization using the combination of G-CSF and plerixafor appears to be more effective than either compound alone in mobilizing PBSCs to the peripheral blood in dogs.

In chapter II, to evaluate the safety and effect of TLI with VMAT as a nonmyeloablative HSCT conditioning treatment, six healthy dogs were treated with

8 or 12 Gy TLI, and allogeneic HSCT was attempted in a pair of DLA-identical littermates. In the safety trial of TLI, hematologic and physical changes were recorded, and necropsy was performed at the end of the experiment. Peripheral Plt counts decreased in all dogs after the TLI procedures, but no uniform hematologic kinetics were observed in the other parameters. One dog irradiated with 8 Gy TLI developed transient cutaneous infection. No severe complications were seen in the other seven dogs. Necropsy revealed a reduction in cell numbers of bone marrow in two dogs treated with 12 Gy TLI. In the allogeneic HSCT experiment, donor-derived PBSCs were successfully engrafted in the recipient dog, and this chimerism was maintained until the end of the follow-up period. No major complication was observed in the recipient dog during the experimental period. These results showed that TLI with VMAT may be safe and feasible for canine allogeneic HSCT conditioning.

To improve treatment outcomes of hematologic malignancies, adopting transplantation therapy is an important element in veterinary medicine. However, ensuring the safety of the procedure is absolutely essential for growing the availability of canine HSCT treatment. In the present study, no severe complications were observed throughout the procedures. These results prove that this trial procedures were safe for healthy dogs. In clinical practice, it is supposed that cancer patients will be treated with multidrug chemotherapy as induction therapy before HSCT. Because cumulative chemotherapy can affect a patient's hematopoietic capacity, adverse effects of bone marrow caused by pretransplant

conditioning therapy may be more severe in healthy dogs. Further studies including clinical cases are warranted to fully demonstrate the safety of this allogeneic HSCT procedure and its ability to contribute to the improvement of treatment outcome in canine cancer patients.

Acknowledgments

I would like to express my deepest gratitude to professors, Dr. Masahiro Okumura and Dr. Takafumi Sunaga, in Laboratory of Veterinary Surgery for their continuous support and thoughtful guidance. I am also particularly grateful to Dr. Kazuhiko Ohashi (Laboratory of Infectious Diseases), Dr, Mitsuyoshi Takiguchi (Laboratory of Veterinary Internal Medicine), and Dr. Kenji Hosoya (Laboratory of Veterinary Advanced Medicine) for their constructive suggestions.

I would like to express my appreciation to research partners, Syouki Murakami, Ryou Owaki, Ayumi Kobayashi, Masaru Kato, and Natsuki Fukayama, for their devoted supports. I am deeply grateful to Dr. Yumiko Kagawa (North Lob) for her precise histopathological diagnoses.

I would like to extend my sincere condolences on the death of experimental dogs that were used for my research.

Special thanks are extended to all members in Laboratory of Veterinary Surgery and Hokkaido University Veterinary Teaching Hospital.

Finally, I would like to express my sincere gratitude to my family and all peoples who helped me to complete this study.

References

1. **Bair, S. M., Brandstadter, J. D., Ayers, E. C., and Stadtmauer, E. A.** Hematopoietic stem cell transplantation for blood cancers in the era of precision medicine and immunotherapy. *Cancer* 2020;126:1837-1855.

2. **Baron, F., and Sandmaier, B. M.** Chimerism and outcomes after allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning. *Leukemia* 2006;20:1690-1700.

3. **Baron, F., and Storb, R.** Allogeneic hematopoietic cell transplantation following nonmyeloablative conditioning as treatment for hematologic malignancies and inherited blood disorders. *Mol Ther* 2006;13:26-41.

4. **Belka, C., Ottinger, H., Kreuzfelder, E., Weinmann, M., Lindemann, M., Lepple-Wienhues, A., Budach, W., Grosse-Wilde, H., and Bamberg, M.** Impact of localized radiotherapy on blood immune cells counts and function in humans. *Radiother Oncol* 1999;50:199-204.

5. **Bolan, C. D., Greer, S. E., Cecco, S. A., Oblitas, J. M., Rehak, N. N., and Leitman, S. F.** Comprehensive analysis of citrate effects during plateletpheresis in normal donors. *Transfusion* 2001;41:1165-1174.

6. **Burroughs, L., Mielcarek, M., Little, M. T., Bridger, G., Macfarland, R., Fricker, S., Labrecque, J., Sandmaier, B. M., and Storb, R.** Durable engraftment of AMD3100-mobilized autologous and allogeneic peripheral-blood mononuclear cells in a canine transplantation model. *Blood* 2005;106:4002-4008.

7. **Cancelas, J. A., Scott, E. P., and Bill, J. R.** Continuous $CD34^{+}$ cell collection by a new device is safe and more efficient than by a standard collection procedure: results of a two-center, crossover, randomized trial. *Transfusion* 2016;56:2824-2832.

8. **Cashen, A., Lopez, S., Gao, F., Calandra, G., MacFarland, R., Badel, K., and DiPersio, J.** A phase II study of plerixafor (AMD3100) plus G-CSF for autologous hematopoietic progenitor cell mobilization in patients with Hodgkin lymphoma. *Biol Blood Marrow Transplant* 2008;14:1253-1261.

9. **Chalfon, C., Martini, V., Comazzi, S., Aresu, L., Stefanello, D., Riondato, F., Ferrari, R., and Marconato, L.** Minimal residual disease in lymph nodes after achievement of complete remission predicts time to relapse in dogs with large B-cell lymphoma. *Vet Comp Oncol* 2019;17:139-146.

10. **Clift, R. A., Buckner, C. D., Appelbaum, F. R., Bryant, E., Bearman, S. I., Petersen, F. B., Fisher, L. D., Anasetti, C., Beatty, P., and Bensinger, W. I.** Allogeneic marrow transplantation in patients with chronic myeloid leukemia in the chronic phase: a randomized trial of two irradiation regimens. *Blood* 1991;77:1660-1665.

11. **Clift, R. A., Buckner, C. D., Appelbaum, F. R., Sullivan, K. M., Storb, R., and Thomas, E. D.** Long-term follow-up of a randomized trial of two irradiation regimens for patients receiving allogeneic marrow transplants during first remission of acute myeloid leukemia. *Blood* 1998;92:1455-1456.

12. **Copelan, E. A., Chojecki, A., Lazarus, H. M., and Avalos, B. R.** Allogeneic hematopoietic cell transplantation; the current renaissance. *Blood Rev* 2019;34:34-44.

13. **Diaconescu, R., Little, M. T., Leisenring, W., Yunusov, M., Hogan, W. J., Sorror, M. L., Baron, F., and Storb, R.** What role is there for antithymocyte globulin in allogeneic nonmyeloablative canine hematopoietic cell transplantation? *Biol Blood Marrow Transplant* 2005;11:335-344.

14. **DiPersio, J. F., Ho, A. D., Hanrahan, J., Hsu, F. J., and Fruehauf, S.** Relevance and clinical implications of tumor cell mobilization in the autologous transplant setting. *Biol Blood Marrow Transplant* 2011;17:943-955.

15. **DiPersio, J. F., Micallef, I. N., Stiff, P. J., Bolwell, B. J., Maziarz, R. T., Jacobsen, E., Nademanee, A., McCarty, J., Bridger, G., and Calandra, G.** Phase III prospective randomized double-blind placebo-controlled trial of plerixafor plus granulocyte colony-stimulating factor compared with placebo

plus granulocyte colony-stimulating factor for autologous stem-cell mobilization and transplantation for patients with non-Hodgkin's lymphoma. *J Clin Oncol* 2009;27:4767-4773.

16. **Duarte, R. F., Shaw, B. E., Marín, P., Kottaridis, P., Ortiz, M., Morante, C., Delgado, J., Gayoso, J., Goterriz, R., Martínez-Chamorro, C., Mateos-Mazón, J. J., Ramírez, C., de la Rubia, J., Achtereekte, H., Gandhi, P. J., Douglas, K. W., and Russell, N. H.** Plerixafor plus granulocyte CSF can mobilize hematopoietic stem cells from multiple myeloma and lymphoma patients failing previous mobilization attempts: EU compassionate use data. *Bone Marrow Transplant* 2011;46:52-58.

17. **Even-Or, E., Eden-Walker, A., Di Mola, M., McDougall, E., Schechter, T., Ali, M., Svajger, G., Gassas, A., Licht, C., and Krueger, J.** Comparison of two apheresis systems for autologous stem cell collections in pediatric oncology patients. *Transfusion* 2017;57:122-130.

18. Fatobene, G., Rocha, V., St Martin, A., Hamadani, M., Robinson, S., Bashey, A., Boumendil, A., Brunstein, C., Castagna, L., Dominietto, A., Finel, H., Chalandon, Y., Kenzey, C., Kharfan-Dabaja, M., Labussière-Wallet, H., Moraleda, J. M., Pastano, R., Perales, M. A., El Ayoubi, H. R., Ruggeri, A., Sureda, A., Volt, F., Yakoub-Agha, I., Zhang, M. J., Gluckman, E., Montoto, S., and Eapen, M. Nonmyeloablative Alternative Donor Transplantation for Hodgkin and Non-Hodgkin Lymphoma: From the LWP-EBMT, Eurocord, and CIBMTR. *J Clin Oncol* 2020; 38:1518-1526.

19. **Francisco, L. V., Langston, A. A., Mellersh, C. S., Neal, C. L., and Ostrander, E. A.** A class of highly polymorphic tetranucleotide repeats for canine genetic mapping. *Mamm Genome* 1996;7:359-362.

20. **Giralt, S., Costa, L., Schriber, J., Dipersio, J., Maziarz, R., McCarty, J., Shaughnessy, P., Snyder, E., Bensinger, W., Copelan, E., Hosing, C., Negrin, R., Petersen, F. B., Rondelli, D., Soiffer, R., Leather, H., Pazzalia, A., and Devine, S.** Optimizing autologous stem cell mobilization strategies to improve patient outcomes: consensus guidelines and recommendations. *Biol Blood Marrow Transpl* 2014;20:295–308.

21. **Gunn, N., Damon, L., Varosy, P., Navarro, W., Martin, T., Ries, C., and**

Linker, C. High $CD34^+$ cell dose promotes faster platelet recovery after autologous stem cell transplantation for acute myeloid leukemia. *Biol Blood Marrow Transpl* 2003;9:643–648.

22. **Haynes, L. D., Coonen, J., Post, J., Brunner, K., Bloom, D., Hematti, P., and Kaufman, D. B.** Collection of hematopoietic CD34 stem cells in rhesus macaques using Spectra Optia. *J Clin Apher* 2017;32:288-294.

23. **Jantunen, E., and Kvalheim, G.** Mobilization strategies in hard-to-mobilize patients with lymphoid malignancies. *Eur L Hematol* 2010;85:463-471.

24. **Kessans, M. R., Gatesman, M. L., and Kockler, D. R.** Plerixafor: a peripheral blood stem cell mobilizer. *Pharmacotherapy* 2010;30:485-492.

25. **Kohrt, H. E., Turnbull, B. B., Heydari, K., Shizuru, J. A., Laport, G. G., Miklos, D. B., Johnston, L. J., Arai, S., Weng, W. K., Hoppe, R. T., Lavori, P. W., Blume, K. G., Negrin, R. S., Strober, S., and Lowsky, R.**TLI and ATG conditioning with low risk of graft-versus-host disease retains antitumor reactions after allogeneic hematopoietic cell transplantation from related and unrelated donors. *Blood* 2009;114:1099-1109.

26. **Körbling, M., and Freireich, E. J.** Twenty-five years of peripheral blood stem cell transplantation. *Blood* 2011;117:6411-6416.

27. **Lange, S., Altmann, S., Brandt, B., Adam, C., Riebau, F., Vogel, H., Weirich, V., Hilgendorf, I., Storb, R., Freund, M., and Junghanss, C.** Investigation of immunological approaches to enhance engraftment in a 1 Gy TBI canine hematopoietic stem cell transplantation model. *Exp Hematol* 2009;37:143-150.

28. **Lee, S. N., Sohn, J. Y., Kong, J. H., Eom, H. S., Lee, H., and Kong, S. Y.** Comparison of Two Apheresis Systems of COBE and Optia for Autologous Peripheral Blood Stem Cell Collection. *Ann Lab Med* 2017;37:327-330.

29. **Leung, A. Y., and Kwong, Y. L.** Haematopoietic stem cell transplantation: current concepts and novel therapeutic strategies. *Br Med Bull* 2010;93:85-103.

30. **Lupu, M., Gooley, T., Zellmer, E., Graves, S. S., and Storb, R.** Principles of peripheral blood mononuclear cell apheresis in a preclinical canine model of

hematopoietic cell transplantation. *J Vet Intern Med* 2008;22:74-82.

31. **Lupu, M., Sullivan, E. W., Westfall, T. E., Little, M. T., Weigler, B. J., Moore, P. F., Stroup, P. A., Zellmer, E., Kuhr, C., and Storb, R.** Use of multigeneration-family molecular dog leukocyte antigen typing to select a hematopoietic cell transplant donor for a dog with T-cell lymphoma. *J Am Vet Med Assoc* 2006;228:728-732.

32. **McKay, C., Knight, K. A., and Wright, C.** Beyond cancer treatment - a review of total lymphoid irradiation for heart and lung transplant recipients. *J Med Radiat Sci* 2014;61:202-209.

33. **Mohty, M., Azar, N., Chabannon, C., Le Gouill, S., Karlin, L., Farina, L., Milkovich, G., Ostermann, H., Glaß, B., Noppeney, R., Kron, F., Kron, A., and Hübel, K.** Plerixafor in poor mobilizers with non-Hodgkin's lymphoma: a multi-center time-motion analysis. *Bone Marrow Transplant* 2018;53:246-254.

34. **Ocanto, A., Escribano, A., Glaría, L., Rodríguez, I., Ferrer, C., Huertas, C., Pérez, A., and Morera, R.** TLI in pediatric patients. *Clin Transl Oncol* 2019; 22;884-891.

35. **Paix, A., Antoni, D., Waissi, W., Ledoux, M. P., Bilger, K., Fornecker, L., and Noel, G.** Total body irradiation in allogeneic bone marrow transplantation conditioning regimens: A review. *Crit Rev Oncol Hematol* 2018;123:138-148.

36. **Pandey, S., and Cottler-Fox, M.** Optia® continuous mononuclear collection (CMNC) system is a safe and efficient system for hematopoietic progenitor cells-apheresis (HPC-a) collection and yields a lower product hematocrit (HCT%) than the COBE® spectra system: A retrospective study. *J Clin Apher* 2018;33:505-513.

37. **Patel, P., Aydogan, B., Koshy, M., Mahmud, D., Oh, A., Saraf, S. L., Quigley, J. G., Khan, I., Sweiss, K., Mahmud, N., Peace, D. J., DeMasi, V., Awan, A. M., Weichselbaum, R. R., and Rondelli, D.**Combination of linear accelerator-based intensity-modulated total marrow irradiation and myeloablative fludarabine/busulfan: a phase I study. *Biol Blood Marrow Transplant* 2014;20:2034-2041.

38. **Posner, L. P., Willcox, J. L., and Suter, S. E.** Apheresis in three dogs weighing <14 kg. *Vet Anaesth Analg* 2013;40:403-409.

39. **Punzel, M., Kozlova, A., Quade, A., Schmidt, A. H., and Smith, R.** Evolution of MNC and lymphocyte collection settings employing different Spectra Optia® Leukapheresis systems. *Vox Sang* 2017;112:586-594.

40. **Putensen, D., Smith, R., Pilcher, L., and Trandafir, G.** Comparison of the CMNC and MNC apheresis protocol for the collection of T-cells showed comparable outcome: An observational study in a single centre. *J Clin Apher* 2018;33:349-356.

41. **Rezvani, A. R., Kanate, A. S., Efron, B., Chhabra, S., Kohrt, H. E., Shizuru, J. A., Laport, G. G., Miklos, D. B., Benjamin, J. E., Johnston, L. J., Arai, S., Weng, W. K., Negrin, R. S., Strober, S., and Lowsky, R.** Allogeneic hematopoietic cell transplantation after failed autologous transplant for lymphoma using TLI and anti-thymocyte globulin conditioning. *Bone Marrow Transplant* 2015;50:1286-1292.

42. **Rosenthal, J., Wong, J., Stein, A., Qian, D., Hitt, D., Naeem, H., Dagis, A., Thomas, S. H., and Forman, S.** Phase 1/2 trial of total marrow and lymph node irradiation to augment reduced-intensity transplantation for advanced hematologic malignancies. *Blood* 2011;117:309-315.

43. **Sancho, J. M., Morgades, M., Grifols, J. R., Juncà, J., Guardia, R., Vives, S., Ferrà, C., Batlle, M., Ester, A., Gallardo, D., Millà, F., Feliu, E., and Ribera, J. M.** Predictive factors for poor peripheral blood stem cell mobilization and peak $CD34^+$ cell count to guide pre-emptive or immediate rescue mobilization. *Cytotherapy* 2012;14:823-829.

44. **Schmitz, N., Pfistner, B., Sextro, M., Sieber, M., Carella, A. M., Haenel, M., Boissevain, F., Zschaber, R., Müller, P., Kirchner, H., Lohri, A., Decker, S., Koch, B., Hasenclever, D., Goldstone, A. H., and Diehl, V.** Aggressive conventional chemotherapy compared with high-dose chemotherapy with autologous haemopoietic stem-cell transplantation for relapsed chemosensitive Hodgkin's disease: a randomised trial. *Lancet* 2002;359:2065-2071.

45. **Schultheiss, T. E., Wong, J., Liu, A., Olivera, G., and Somlo, G.** Image-

guided total marrow and total lymphatic irradiation using helical tomotherapy. *Int J Radiat Oncol Biol Phys* 2007;67:1259-1267.

46. **Somlo, G., Spielberger, R., Frankel, P., Karanes, C., Krishnan, A., Parker, P., Popplewell, L., Sahebi, F., Kogut, N., Snyder, D., Liu, A., Schultheiss, T., Forman, S., and Wong, J. Y.** Total marrow irradiation: a new ablative regimen as part of tandem autologous stem cell transplantation for patients with multiple myeloma. *Clin Cancer Res* 2011;17:174-182.

47. **Sorror, M. L., Leisenring, W., Mielcarek, M., Baron, F., Diaconescu, R., Hogan, W. J., Graves, S. S., and Storb, R.** Intensified postgrafting immunosuppression failed to assure long-term engraftment of dog leukocyte antigen-identical canine marrow grafts after 1 gray total body irradiation. *Transplantation* 2008;85:1023-1029.

48. **Spinner, M. A., Kennedy, V. E., Tamaresis, J. S., Lavori, P. W., Arai, S., Johnston, L. J., Meyer, E. H., Miklos, D. B., Muffly, L. S., Negrin, R. S., Rezvani, A. R., Shizuru, J. A., Weng, W. K., Hoppe, R. T., Strober, S., and Lowsky, R.** Nonmyeloablative TLI-ATG conditioning for allogeneic transplantation: mature follow-up from a large single-center cohort. *Blood Adv* 2019;3:2454-2464.

49. **Springer, A., Hammer, J., Winkler, E., Track, C., Huppert, R., Böhm, A., Kasparu, H., Weltermann, A., Aschauer, G., Petzer, A. L., Putz, E., Altenburger, A., Gruber, R., Moser, K., Wiesauer, K., and Geinitz, H.** Total body irradiation with volumetric modulated arc therapy: Dosimetric data and first clinical experience. *Radiat Oncol* 2016;11:46-55.

50. **Stewart, D. A., Smith, C., MacFarland, R., and Calandra, G.** Pharmacokinetics and pharmacodynamics of plerixafor in patients with non-Hodgkin lymphoma and multiple myeloma. *Biol Blood Marrow Transplant* 2009;15:39-46.

51. **Stiff, P. J., Micallef, I., Nademanee, A. P., Stadtmauer, E. A., Maziarz, R. T., Bolwell, B. J., Bridger, G., Marulkar, S., Hsu, F. J., and DiPersio, J. F.** Transplanted $CD34^{+}$ cell dose is associated with long-term platelet count recovery following autologous peripheral blood stem cell transplant in patients with non-Hodgkin lymphoma or multiple myeloma. *Biol Blood Marrow*

Transpl 2011;17(8):1146-1153.

52. **Storb, R. F., Champlin, R., Riddell, S. R., Murata, M., Bryant, S., and Warren, E. H.** Non-myeloablative transplants for malignant disease. *Hematology Am Soc Hematol Educ Program* 2001;375-391.

53. **Storb, R., Yu, C., Barnett, T., Wagner, J. L., Deeg, H. J., Nash, R. A., Kiem, H. P., McSweeney, P., Seidel, K., Georges, G., and Zaucha, J. M.** Stable mixed hematopoietic chimerism in DLA-identical littermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. *Blood* 1997;89:3048-3054.

54. **Storb, R., Yu, C., Barnett, T., Wagner, J. L., Deeg, H. J., Nash, R. A., Kiem, H. P., McSweeney, P., Seidel, K., Georges, G., and Zaucha, J. M.** Stable mixed hematopoietic chimerism in dog leukocyte antigen-identical littermate dogs given lymph node irradiation before and pharmacologic immunosuppression after marrow transplantation. *Blood* 1999;94:1131-1136.

55. **Suter, S. E.** Collection of peripheral blood $CD34^+$ progenitor cells from healthy dogs and dogs diagnosed with lymphoproliferative diseases using a Baxter-Fenwal CS-3000 Plus blood cell separator. *J Vet Intern Med* 2011;25:1406-1413.

56. **Suter, S. E., Hamilton, M. J., Sullivan, E. W., and Venkataraman, G. M.** Allogeneic hematopoietic cell transplantation in a dog with acute large granular lymphocytic leukemia. *J Am Vet Med Assoc* 2015;246:994-997.

57. **Symons, K., Morrison, C., Parry, J., Woodings, S., and Zissiadis, Y.** Volumetric modulated arc therapy for total body irradiation: A feasibility study using Pinnacle^3 treatment planning system and Elekta Agility™ linac. *J Appl Clin Med Phys* 2018;19:103-110.

58. **Teusink, A., Pinkard, S., Davies, S., Mueller, M., and Jodele, S.** Plerixafor is safe and efficacious for mobilization of peripheral blood stem cells in pediatric patients. *Transfusion* 2016;56:1402-1405.

59. Veterinary cooperative oncology group - common terminology criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1. *Vet Comp Oncol* 2016;14:417-446.

60. **Warry, E. E., Willcox, J. L., and Suter, S. E.** Autologous peripheral blood hematopoietic cell transplantation in dogs with T-cell lymphoma. *J Vet Intern Med* 2014;28:529-537.

61. **Willcox, J. L., Pruitt, A., and Suter, S. E.** Autologous peripheral blood hematopoietic cell transplantation in dogs with B-cell lymphoma. *J Vet Intern Med* 2012;26:1155-1163.

62. **Wong, J. Y., Forman, S., Somlo, G., Rosenthal, J., Liu, A., Schultheiss, T., Radany, E., Palmer, J., and Stein, A.** Dose escalation of total marrow irradiation with concurrent chemotherapy in patients with advanced acute leukemia undergoing allogeneic hematopoietic cell transplantation. *Int J Radiat Oncol Biol Phys* 2013;85:148-156.

63. **Zaucha, J. M., Yu, C., Zellmer, E., Takatu, A., Junghanss, C., Little, M. T., and Storb, R.** Effects of extending the duration of postgrafting immunosuppression and substituting granulocyte-colony-stimulating factor-mobilized peripheral blood mononuclear cells for marrow in allogeneic engraftment in a nonmyeloablative canine transplantation model. *Biol Blood Marrow Transplant* 2001;7:513-516.

64. **Zhao, Q., Li, T., Chen, G., Zeng, Z., and He, J.** Prognosis and risk factors of radiation-induced lymphopenia in early-stage lung cancer treated with stereotactic body radiation therapy. *Front Oncol* 2020;9:1488-1498.

Summary in Japanese

和文要旨

造血幹細胞移植（HSCT）は、医学領域ではリンパ腫および白血病などの造血器系腫瘍に対する重要な治療技術である。これらの疾患における HSCT は、治療強度の増強が目的のひとつである。抗がん剤および放射線療法は骨髄などの造血臓器に対して強い毒性を示すため、薬剤の容量、照射方法、あるいは線量が制限される。従来の移植治療では、導入治療が終了した症例に対して、造血能が失われるほどの高い強度の抗がん剤投与あるいは放射線照射が移植前処置として行われる。これらの処置により造血能を失った患者に対して造血幹細胞を移植し、喪失した造血能を生着した移植片から補填させる。さらに、非自己の造血幹細胞を移植（同種 HSCT）に用いることで、ドナー由来の移植片がレシピエント体内の腫瘍細胞を異物として認識して免疫反応を示す、移植片対白血病効果も治療成績の改善に大きく寄与することが知られている。

犬リンパ腫は発生頻度が高く、犬に発生する造血器系腫瘍のうち 8 割以上を占める疾患である。犬のリンパ腫は人と同様に抗がん剤への反応性が良く、ほぼ全ての症例で初期治療に反応し、完全寛解に至る。しかし、治療開始から一年未満で半数の症例が再発し、死亡に至る。この治療成績は数十年間にわたり改善が得られておらず、これは抗がん剤以外の治療法に関する研究が医療と比べて大幅に遅れていることが大きな要因と考えられる。

これまで犬の HSCT に関する報告は、人の前臨床モデルとしてのものが多く、実際にそれらの条件が獣医臨床へ適応可能かを検討した報告は限られている。そこで本研究では HSCT の犬リンパ腫治療への普及を最終目的とし、ドナーおよびレシピエントのそれぞれにおいてより安全な HSCT を確立すべく、以下の研究を行った。第 I 章では、末梢血に動員した造血幹細胞を採取する Apheresis 技術を確立し、レシピエントから移植必要量の造血幹細胞を採取するための効果的な動員法について検討を行った。第 II 章では、より副作用の少ない安全な移植前処置として全リンパ照射（TLI）の条件検討を行い、2 頭の同腹犬を用いて同種 HSCT を実施した。

第 I 章では、体重 10 k 前後の犬における Apheresis 技術を確立し、末梢血造血幹細胞（PBSC）採取において、これまで犬ではそれぞれ単剤で用いられてきた granulocyte-colony stimulating factor（G-CSF）および Plerixafor の併用による PBSC 動員に対する増強効果について検討を行った。実験には正常ビーグル犬 9 頭を用い、動員薬として G-CSF および Plerixafor をそれぞれ単剤あるいは併用し、実験を実施した（各プロトコル 3 頭ずつ）。動員薬の投与から Apheresis 開始までの総白血球、単核球数、好中球数、および末梢血および Apheresis 産物中の PBSC 数を比較解析した。Apheresis 条件は全頭で一定とし、回路プライミングには Apheresis 実施の 2-3 週間前に採取した自己全血を用いた。また、Apheresis に関連した合併症を検討するために、Apheresis 中から処置後にかけて血液学的および血液化学検査を実施した。Apheresis 産物中の PBSC 数は、Plerixafor 単剤、G-CSF 単剤、および併用群において、それぞれ 1.3 ± 0.24、 4.2 ± 0.47、 および 6.4 ± 0.9 × 10^6 cells/kg

であった。Apheresis 中および終了時にみられた合併症は、貧血、血小板減少および低カルシウム血症などであった。これらの合併症は全身麻酔、体外循環、血液分離装置の構造および輸液製剤などが影響したと考えられたが、そのすべてが重篤な臨床徴候を示さず、無処置で 5 日以内に正常値まで回復した。以上より、体重 10 kg 前後の犬において本研究で定めた Apheresis 条件は安全に実施可能であり、さらに G-CSF および Plerixafor の併用により採取可能な PBSC が増加する傾向が認められた。

第 II 章では、強度を減弱させた治療前処置とされる骨髄非破壊的前処置としての TLI の条件、およびその有効性を検討することを目的とした。正常ビーグル犬 6 頭を TLI の計画処方線量ごとに 8Gy 群、および 12Gy 群の 2 群に分け、照射後の血液学的変化の観察、および末梢血単核球を用いたリンパ球サブセット解析、およびリンパ球増殖能試験を実施した。観察期間は照射後 2 ヶ月間とし、実験終了時に剖検を実施した。次いで、12Gy の TLI を用い、白血球抗原型の一致した同腹犬において同種 HSCT を行った。移植後よりレシピエントには免疫抑制剤 2 剤の投薬を行い、移植後 1 から 16 週目の間に生着判定を目的としてマイクロサテライトマーカーを用いたキメリズム解析を行った。6 頭全頭で一時的な好中球減少症、および血小板減少症が確認され、末梢血中のリンパ球数は観察期間中低値が持続した。8Gy 群の 1 頭では観察期間中に感染徴候が認められたが、抗生剤により治癒した。その他の 5 頭では臨床徴候を伴う、重篤な合併症は認められなかった。剖検後の病理組織学的検査では、12Gy 群の 3 頭で骨髄内の細胞成分の減少が認められた。同種 HSCT では、ドナーから 6.67×10^{6}cell/kg の PBSC が採

取され、全量を TLI 実施後のレシピエントに輸注した。移植後に実施したキメリズム解析においては、移植後 2 週目から単核球層、および顆粒球層の両細胞分画でキメリズムが認められた。また、観察期間中に移植の副作用と考えられる重篤な臨床徴候は認められなかった。以上より、検討した TLI の条件は健常犬において重篤な副作用を示すことなく安全に実施可能であることが示された。また、観察期間が短期的ではあるが、犬においても TLI を用いた同種 HSCT による移植片の生着が可能であることが示された。

本研究から、犬においても Apheresis および TLI が実施可能であることが示された。また、本法が同種 HSCT を用いた治療を行う上で、安全な移植技術になる可能性が示唆された。