HEMATOPOIETIC STEM CELL TRANSPLANTATION AND CORD BLOOD BANKING

Original article

Advantages of high cell concentration prior to cryopreservation of initial leukapheresis in CAR-T cell therapy

Diego Carbonell[1,2*], Silvia Monsalvo[1*], Eva Catalá[1,2], Ana Pérez-Corral[1,2], Carolina Villegas[1], Carmen Falero[1], Gloria Ruano[1], Monica Martinez[1], Mi Kwon[1,2], Cristina Muñoz-Martínez[1,2], José Luis Díez-Martín[1,2,3], Jorge Gayoso[1,2*], Javier Anguita[1,2*]

*Diego Carbonell, Silvia Monsalvo, Jorge Gayoso and Javier Anguita contributed equally to this manuscript.

[1]*Department of Hematology, Gregorio Marañón General University Hospital, Madrid, Spain;* [2]*Gregorio Marañón Health Research Institute (IiSGM), Madrid, Spain;* [3]*Department of Medicine, School of Medicine, Complutense University of Madrid, Madrid, Spain*

Background - Chimeric antigen receptor (CAR) T-cell therapy is increasingly used in patients affected by B-cell lymphoma and acute lymphoblastic leukemia. For logistical reasons, initial apheresis products may be cryopreserved for shipment to manufacturing centers. Due to the characteristics of these patients, cells are often collected in large volumes, meaning more bags must be cryopreserved. This requires increased storage, time and monetary costs. In this context, we aimed to evaluate a high cell concentration cryopreservation protocol by centrifugation to standardize the initial CAR-T manufacturing procedure.

Materials and methods - Sixty-eight processes of leukapheresis of 57 patients affected by refractory/relapsed B cell lymphoma and 9 patients affected by acute lymphoblastic leukemia who were eligible for anti-CD19 CAR-T cell treatment performed between June 2019 and October 2022 were analyzed. Whole blood count, percentage and number of T cells were assessed on the apheresis final product. The apheresis product, which was alternatively stored overnight at 4°C, was centrifuged, adjusting the volume to approximately 40 mL. The product was immediately cryopreserved to achieve a final cell concentration of 50-200×10^6 cells/ml for cryopreservation.

Results - Leukapheresis volume was reduced by almost fivefold (median: 185 to 40 mL), resulting in a higher product concentration in one bag. In addition, the number of non-target cells (monocytes, platelets and erythrocytes) was also reduced during the development of CAR-T cell therapy, thereby maintaining T lymphocyte levels and providing a purer starting material.

Discussion - The advantages of the protocol include reducing economic costs, saving storage space, simplifying the manufacturing process, and facilitating shipping logistics. In conclusion, we present a validated, simple, and cost-effective cell enrichment processing protocol that provides high-quality cryopreserved products as starting material for the CAR-T cell manufacturing process.

Keywords: *chimeric antigen receptor T cells, protocol, leukapheresis, high concentration, cryopreservation.*

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Correspondence: Diego Carbonell
e-mail: diegocarbonell3@gmail.com

INTRODUCTION

Chimeric antigen receptor (CAR)-T cells administration is a promising advance therapy for many cancer diseases[1]. Specifically, the Food and Drug Administration (FDA) and

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the European Medicines Agency (EMA) approved the administration of anti-CD19 CAR-T for the treatment of refractory/relapsed B-cell lymphomas and acute lymphoid leukemia (ALL)[2-4]. Anti-CD19 CAR-T manufacturing process consists of several phases. The first step is the collection of peripheral blood lymphocytes, in order to obtain the initial product. Once it has been verified that the collected product complies with the specifications, such as total nucleated cells (TNC) or percentage and number of T-cells, it is sent to the manufacturing laboratory. There, T cells are selected, activated, transduced and expanded and, after CAR-T cell generation is completed, the product is shipped to the center and administered to the patient[5,6]. Regarding the mononuclear cell (MNC) collection step, product could be shipped cryopreserved or not, with no great differences in product quality[7]. However, there is not a standardized protocol for this step that assures a homogeneous product handling. Cryopreservation and cell concentration techniques are usually reported in the setting of autologous peripheral blood stem cell transplantation (auto-HSCT) and, less frequently, in allogeneic hematopoietic stem cell transplantation (allo-HSCT) due to COVID-19 prophylaxis[8,9]. The currently used methodological approach to cryopreserve peripheral blood stem cells sets concentration limits for the nuclear cells, traditionally adjusted from 20-40×10^6 cells/mL to 150-200×10^6 cells/mL and 7.5-10 percent of dimethyl sulfoxide (DMSO) before cryopreserving[10,11]. In this sense, low cell concentrations imply higher volumes, increasing costs at the expense of a greater use of DMSO and number of bags, more storage space required, as well as complicating the logistics of shipping the product to the manufacturing center. Therefore, high concentration could be an alternative, without compromising the product, as it has been demonstrated in other therapies[12-14]. In addition, since the apheresis of these heavily treated patients involves processing large volumes of blood, they usually take a long time, making same-day cryopreservation difficult. Thus, we propose to evaluate a next-day cryopreservation protocol, including a centrifugation step, for assuring an optimal initial product for CAR-T cell manufacture that fits the available resources and requirements.

MATERIALS AND METHODS

Patients

Sixty-eight processes of leukapheresis of 57 patients affected by refractory/relapsed B cell lymphoma and 9 patients affected by ALL who were eligible for anti-CD19 CAR-T cell treatment performed between June 2019 and October 2022 were analyzed. The study was conducted in accordance with the Declaration of Helsinki and all patients signed the informed consent document.

Eligibility criteria

Eligibility criteria at the time of apheresis were as follows: an absolute lymphocyte count (ALC) of at least 100/µL in the peripheral blood (PB), no active severe infection or fever above 38.2°C, no clinical signs of infection within 48 hours of apheresis, platelets greater than 20,000/mL, hematocrit of 24% or greater.

MNC collection

Cell collection was performed on an apheresis COBE Spectra Optia platform (Terumo BCT, Tokyo, Japan) using the MNC collection program. The volume to be processed was estimated based on the whole blood count (WBC), the T-cell percentage and the minimum collection efficiency of the apheresis platform (40%). The anticoagulant (acid citrate dextrose solution A -ACDA-) ratio to blood was 12:1 in all cases. The collect pump rate was set at 0.8 mL/min. The collection line was kept at 1 to 2% based on a white blood cell colorgram provided by the manufacturer.

Patients' collection aimed for a range of 1-4×10^9 total T cells and for a minimum of 2×10^9 TNC. To monitor collection and patient status, a WBC was performed before and after each apheresis procedure with a DXH800 automated cell counter (Beckman Coulter, Brea, CA, USA). A WBC was also performed on the apheresis product after harvest to measure TNC and the total lymphocyte count. Percentage and number of T cells were assessed by flow cytometry of the PB samples prior to the MNC collection and in the apheresis products. T-cells were assessed by 8-color flow cytometry in a Navios (Beckman Coulter) and in a 10-color DxFlex cytometer (Beckman Coulter) using the following monoclonal antibodies: CD45 (FITC), CD4 (PE), CD8 (ECD) and CD3 (PC5.5) (Beckman Coulter). The number of viable nucleated cells was assessed by trypan blue dye (Sigma-Aldrich, St. Louis, MO, USA).

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Concentration cell processing

WBC count, percentage of T cells and viability were analyzed to assess the number of cells/mL and the total T cells in the initial apheresis product. If concentration was higher than 300×10^6 WBC/mL, concurrent autologous plasma was added to dilute the product. Subsequently, in the majority of cases, product was stored overnight at 4°C due to the institution's hours of operation. The apheresis product was centrifuged for 15 minutes in an Allegra X-15R centrifuge (Beckman Coulter) at the following parameters: acceleration=9, G=300, T=22°C, no brake. After centrifugation, product was assessed by direct observation for hemolysis, particulates or platelet clumps. Plasma was removed to adjust the cell pellet to an approximate volume of 40 mL and product was transferred to a 600 mL blood transfer bag (Grifols, Barcelona, Spain).

Cryopreservation

Leukapheresis product was cryopreserved immediately after centrifugation, within 24 h of collection, according to our cryopreservation procedure. Briefly, product was transferred to a 15-85 mL EVA cryopreservation bag (Macopharma, Tourcoing, France) and a cryopreservation solution composed of at least 20% of concurrent autologous plasma and 10% of DMSO (Research Industries Corp., Salt Lake City, UT, USA) of the total volume was gradually added within 5 minutes, obtaining a final cell concentration for cryopreservation of $50\text{-}200\times10^6$ cells/mL. Product was cooled at rate of 1°C/min to –80°C using a CM-2010 programmable controlled rate freezer (Carburos Metálicos, Barcelona, Spain) and immediately introduced in gas phase of liquid nitrogen with a temperature ≤159°C (**Figure 1**).

Statistical analysis

Quantitative variables were expressed as median and interquartile range. The Wilcoxon test was used for pairwise comparisons. Statistical significance was set at $p<0.05$ and all statistical test were performed with R (3.3.2 version [R Core Team, Vienna, Austria]) and Graphpad Prism (8.0.1 version [Graphpad Prism, Boston, MA, USA]).

Additionaly, a semiquantitative analysis was conducted on the difference in the number of bags and DMSO used between non-centrifuged and centrifuged leukapheresis in all cases. For this analysis, it was considered that the final volume of cells to be frozen consists of 70% of the total bag volume, with 20% representing plasma and 10% being DMSO. Estimation was based on bags of the same size as those used in this study (maximum volume 85 mL).

RESULTS

MNC collection and concentration cell processing

Apheresis product was collected in a median of 185 mL (147-222 mL), obtaining a median of 9.80×10^9 ($7.05\text{-}13.51\times10^9$) TNC with a median of 4.97×10^9 ($3.35\text{-}6.48\times10^9$) T-cells. Median hematocrit and platelets were 4.69% (3.59-5.69%) and 176.34×10^9 ($79.16\text{-}304.02\times10^9$) respectively (**Table I**). Concurrent autologous plasma was not added in any case, since the cell concentration was not higher than 300×10^6 WBC/mL in any of the collected apheresis.

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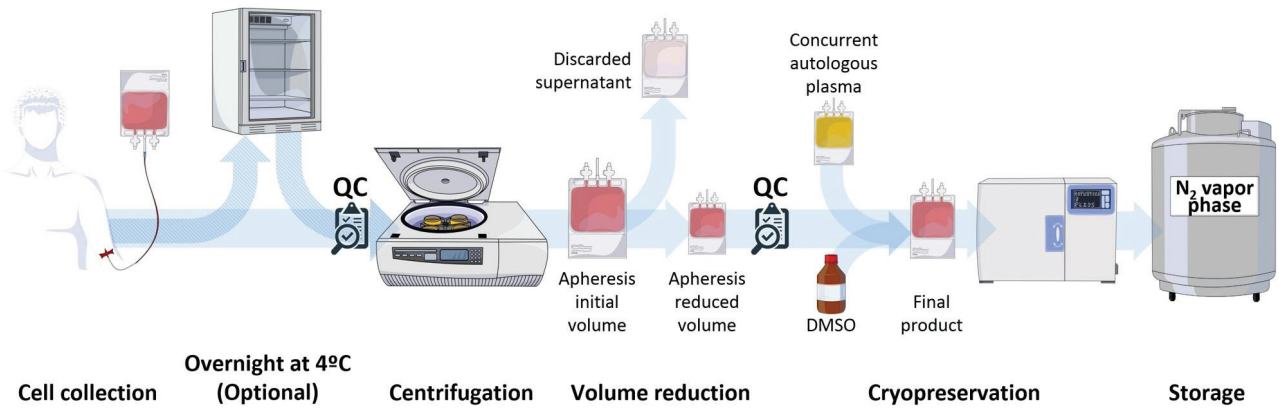


Figure 1 - General overview of the next-day cryopreservation protocol workflow

Leukapheresis volume was reduced 4.62 times after centrifugation, increasing the concentration of cells per milliliter 3.93 times. There were no statistically significant losses in absolute count of TNC, lymphocytes and T-cells post-centrifugation values were lower than pre-centrifugation, being the T-cell recovery percentage of 92.27%. Median hematocrit, total platelets and total monocytes were reduced after centrifugation, with these associations being statistically significant (**Table I**, **Figure 2**).

Table I - *Median and interquartile range of pre and post-centrifugation leukapheresis values*

Variable	Median (interquartile range)		p value
	Pre-centrifugation	Post-centrifugation	
Cell concentration (×10⁶ cells/mL)	58.30 (39.58-77.30)	229.50 (165.00-318.25)	-
Total nucleated cells (×10⁹ cells)	9.80 (7.05-13.51)	9.12 (5.94-12.70)	0.278
Total lymphocytes (×10⁹ cells)	6.10 (3.55-8.00)	4.94 (3.21-7.19)	0.219
Total T lymphocytes [CD3⁺] (×10⁹ cells)	4.97 (3.35-6.48)	4.42 (2.86-6.05)	0.316
Total monocytes (×10⁹ cells)	3.23 (2.11-4.26)	2.07 (0.82-3.75)	**0.013**
T lymphocytes [CD3⁺] recovery (%)	92.27 (84.91-97.46)		-
Total platelets (×10⁹ cells)	176.34 (79.16-304.02)	103.03 (51.99-161.58)	**<0.001**
Hematocrit (%)	4.69 (3.59-5.69)	4.18 (3.20-5.02)	**0.021**
Blood volume processed (L)	12 (10-15)		-
Apheresis volume (mL)	185 (147-222)	40 (36-41)	-
Cryopreserved product volume (mL)	60 (60-70)		-
Cryopreserved product cell concentration (×10⁶ cells/mL)	109 (86-141)		-
Time between end of apheresis and start of cryopreservation (hh:mm)	21:30 (20:50-22:16)*		-

*Including seven cases in which cryopreservation was performed approximately two hours after the end of the apheresis. Cryopreserved product includes apheresis product, DMSO and concurrent autologous plasma. Bold p values represent the statistically significant results.

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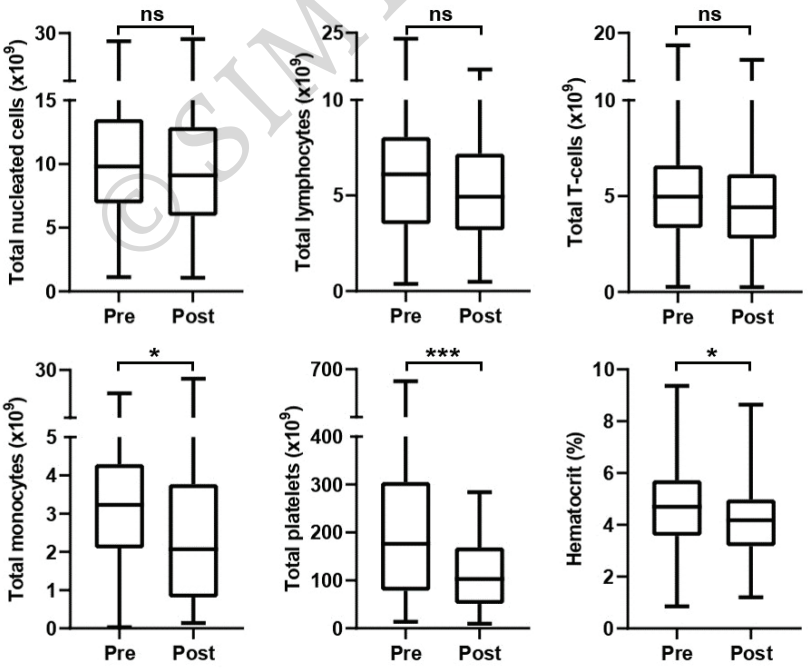


Figure 2 - Pre and post-centrifugation comparison of the variables analyzed
TNC: total nucleated cells. ns: p≥0.05, *p< 0.05, ***p<0.001

Cryopreservation

All leukapheresis products were cryopreserved within the 24 hours after end of cell collection, with a median of 21: 30 h (interquartile range 20: 50-22: 16 h [**Table I**]). In seven (10.3%) of the 68 cases, cryopreservation was performed the same day of apheresis, 2 hours after apheresis ended. As for the other 61 cases, collected cells were stored overnight at 4°C. No statistically significant differences in cell counts between same-day and next-day cryopreservation cases were found.

In regards to the semiquantitative analysis on the quantity of bags and DMSO, the results showed that if leukapheresis was not centrifuged, a median of 3.1 times more bags (interquartile range 2.47-3.73) and 4 times more DMSO (interquartile range 3.17-5) would have been required.

In all cases, only one bag from each batch was cryopreserved, fulfilling all the quality requirements.

Sterility

Sterility testing of the processed pooled leukapheresis products showed no bacterial or fungal contamination.

CAR-T cell manufacturing success

Out of the total 68 procedures, there were manufacturing failures in four cases (5.8%), two of them from the same patient. In these cases, products sent were at the lower limit of manufacturing requirements.

DISCUSSION

CAR-T cell manufacturing is mainly focused on hematological disorders, specifically CD19-positive B-cell lymphomas and LLA. In recent years, medical institutions have increasingly managed commercial CAR-T cells. The main role of these centers is to collect and prepare T cells for shipment. Once converted into CAR-T cells at the manufacturing center, they can receive and infuse them to the patient. As for the leukapheresis step, it is essential to collect the initial product under optimal conditions, complying with the requirements demanded by the CAR-T cell production center to ensure manufacturing success. In this way, collecting a sufficient number of T-cells can be challenging in patients who have undergone multiple lines of treatment or in those with a low white blood cell counts by the time of the apheresis[15]. In these cases, more volume of total blood volume processed is required, which increases the volume of the initial product, the procedure time and the material and human resources, leading to an increase in complexity and a loss of homogeneity among procedures. While cryopreservation of leukapheresis products may not always be necessary for shipment to the manufacturing center, in cases where it is required, there is currently no standardized protocol for the procedure[16]. In response to this need, we have developed and tested a protocol to standardize the processing of initial product for CAR-T therapy, which aims to increase cell concentration in order to simplify and homogenize the procedure, reducing time-consuming and costs. Regarding our results, all apheresis procedures were performed successfully and without complications, obtaining cell numbers similar to other studies[17-19]. Concerning manufacturing failures, they occurred in four of 68 cases (two of them from the same patient), most likely because the product characteristics were at the lower limit of the requirements. However, the overall manufacturing failure rate was 5.8%, which is below the 9% reported in the literature for similar products[20]. As for the procedures that were kept at 4°C overnight, no significant differences were found when compared to those procedures performed on the same day, coinciding with what has been described in the literature[21]. Thus, refrigeration of the product until it is cryopreserved increases the time flexibility of the procedure, allowing it to be carried out when it is not possible to do so on the same day.

Regarding cell concentration increasing before cryopreservation, in HSCT the volume is generally not reduced, as there is no specific range of cell concentration. However, in CAR-T cell therapy, there are usually specific ranges of cell number and cell concentration for shipment to manufacturing laboratories[22]. In auto-HSCT, several studies have shown that cryopreservation at a high concentration by prior centrifugation does not alter cell viability or functionality[12-14,23]. In the present study, we reduced the volume of the leukapheresis almost five times, allowing to obtain a more concentrated product in a single bag. In addition, the amount of non-target cells in the development of CAR-T cell therapy was also reduced, maintaining the levels of T lymphocytes, obtaining a purer starting material. In regards to the significant reduction in non-target cells, it may be attributed to multiple factors. Selective cell death could result from centrifugation, processing time, or the manual procedure

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itself. Additionally, automated cell quantification methods might also play a role. Moreover, in the case of monocytes, their inherent adhesion capacity to plastic surfaces could lead to a small but notable proportion remaining adhered to the transfer bag during the procedure. While further studies are needed to confirm these hypotheses, this reduction was consistently observed across all products in this study. Hence, we have acknowledged this finding in our study[24]. This would lead to an improvement of the procedure, since the presence of other cell populations has been reported to negatively affect CAR-T cell manufacturing. In the case of monocytes, it is known that they can reduce the efficiency of transduction, activation and proliferation of T lymphocytes[25-29]. Apheresis contamination by platelets and erythrocytes may compromise cell quantification by flow cytometry during quality control[30]. Thus, it was possible to reduce the leukapheresis volume without significantly affecting the initial product.

As for the advantages of this protocol over the standard protocol, semi quantitative analysis showed that if leukapheresis was not centrifuged, a median of 3.1 times more bags and 4 times more DMSO would have been needed, which implies several aspects. Firstly, in terms of the economic costs of cryopreservation, the number of needed bags, the volume of DMSO and the working time of the professionals are reduced. This also reduces the possibility of contamination as there is less handling of the product. Second, it saves storage space in the liquid nitrogen tanks, something that can be critical in the case of a high volume of work in therapies that require this type of conservation. Finally, reducing the number of bags simplifies the manufacturing process and it facilitates shipping logistics in case of outsourced manufacturing.

CONCLUSIONS

Our high cell concentration processing protocol achieves a high quality cryopreserved product as a starting material for the CAR-T cell manufacturing process, providing many advantages in terms of cost and time production.

Ethical consideration

The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki.

Written informed consent was obtained from each patient for study participation and data publication.

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AUTHORSHIP CONTRIBUTIONS

DC, SM: collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript. EC: administrative support, manuscript writing, final approval of manuscript; AP-C, CV, GR, MM, CF, MK, CM-M: collection and/or assembly of data, final approval of manuscript; JLD-M, JG: financial support, final approval of manuscript; JA: conception and design, financial support, final approval of manuscript.

The Authors declare no conflicts of interest.

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