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Dear transplant colleagues

In 2019 we celebrated the 40th anniversary of the first bone marrow transplant (BMT) in our country, with the pioneering spirit of Professor Ricardo Pasquini, Eurípides Ferreira and his team, a fact that was undoubtedly a milestone and the driving force for us to arrive where we are. Today, we are 84 BMT-enabled centers in Brazil and we have seen the great success of these teams, demonstrating a process of maturation of our transplant recipients.

Our company was founded in 1996 by a group of specialists and within this same premise. Today we are prominent in the worldwide transplanting community, having entered into several partnerships with international entities, such as ASCT, LABMT, CIBMTR, FACT, among others.

We have a research group at GEDECO (Grupo de Estudo Doença Enxerto Contra o hospedeiro e complicações tardias) ,coordinated by our dear Dr. Mary Flowers and Dr Afonso Celso Vigorito. This started small as a group of studies on graft disease and because of its quality and empathy, it has now become the gateway to cooperative studies on various topics in our society. SBTMO also maintains a Pediatrics Group, a flow cytometry group, a multidisciplinary group and one of data managers. Every two years, a consensus of indications and complications of transplants is performed, which serves as a guide for the guidance of specialists and public policies.

Faced with this scenario, in a natural way, arose the need to have a journal that could disseminate the work of this scientific community, doctors and multidisciplinary professionals, thus strengthening our interaction with transplantation professionals from various countries.

It is with this spirit of joy and hope that we launched this volume of JBMCT, Journal of Bone Marrow Transplantation and Cellular Therapy, which will certainly be a periodical to publicize the work of all those who believe that science , research and caring for patients, is the best way to improve our walking.

Fernando Barroso Duarte *Nelson Hamerschlak*

Summary

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PRECLINICAL STUDIES USING CAR-T CELLS

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ABSTRACT

The therapy with genetically modified T cells to express chimeric antigen receptors (CAR) is a promising strategy for immunotherapy against cancer. CAR-T cells can specifically recognize antigens on the surface of tumor cells and then effectively kill those cells. Several researchers have presented the development of CAR-T cells for various hematological targets and the treatment of solid tumors. Quality control and preclinical evaluation of these products are essential to demonstrate their safety and efficacy and allow development to the clinical trial phase. This chapter will present relevant guidelines regarding pre-clinical research of CAR-T cell products. Preclinical research on cell therapy products should include in vitro and *in vivo* pharmacodynamics studies (antitumor activity), pharmacokinetics (proliferation, distribution, and persistence of CAR-T cells *in vivo*), and animal safety studies.

Keywords: Immunotherapy, Adoptive.

OBJECTIVE

Describe relevant requirements for preclinical studies (*in vitro* and *in vivo*) in order to evaluate the functionality and safety of CAR-T cells.

INTRODUCTION

The therapy with genetically modified T cells to express chimeric antigen receptors (CAR) is a promising strategy for immunotherapy against cancer. CAR-T cells can specifically recognize antigens on the surface of tumor cells and then effectively kill those cells[1, 2]. Several researchers have presented the development of CAR-T cells for various hematological targets and the treatment of solid tumors[2-4]. Quality control and preclinical evaluation of these products are essential to demonstrate their safety and efficacy and allow development to the clinical trial phase. This chapter will present relevant guidelines regarding pre-clinical research of CAR-T cell products.

Preclinical research on cell therapy products should include *in vitro* and *in vivo* pharmacodynamics studies (antitumor activity), pharmacokinetics (proliferation, distribution, and persistence of CAR-T cells *in vivo*), and animal safety studies.

- Good Manufacturing Practices

It is recommended that pharmacodynamics and pharmacokinetics studies as well as *in vivo* safety analysis of CAR-T cell products be carried out under

good manufacturing practices (GMP) conditions. If GMP conditions cannot be met at this stage of CAR-T cell development, pre-clinical experiments must follow the guidelines of good laboratory practices (GLP). This experimental rigor is necessary to guarantee the reliability, integrity, and traceability of the trial results to obtain a final preclinical study report. The results should be robust and adequate to support the analysis of the advanced cell therapy product development by the research team, regulatory agencies, and other stakeholders.

- Starting material and reagents

Starting material to be used in CAR-T cells manufacturing for animal studies does not necessarily need to be derived from a patient; healthy donor samples can also be used in this case. In addition, animal-derived reagents should be avoided or replaced with clinical, pharmaceutical, or GMP-certified products whenever possible. Thus, the homogeneity of the manufacturing process and a better comparability of the final advanced cell therapy product results can be later evaluated regarding safety and efficacy in clinical protocols[5].

- Analysis of the cells before infusion

The manufacturing process of CAR-T cells for animal studies must be accompanied by a complete analysis of the quality and stability of the product. An immunophenotypic evaluation is recommended for detecting CAR expression and T lymphocyte cell populations. It is also necessary to perform a functional evaluation of the antitumor activity of CAR-T cells through potency tests (detailed below) and tests to detect possible contamination of the cell preparation by microorganisms.

The stability study must include all the dosages that will be administered, the expected storage temperatures, and the transport process simulation before animal administration. Complementary analyses must be performed at the time of administration of CAR-T cells product to verify cell viability, total number of cells, and particles presence in suspension.

- In vitro potency assays

The potency assay is used to determine the effectiveness of the manufactured CAR-T cells. Among the factors that can affect CAR-T cells activity are the type of vector and its design, the transduction rate of T cells, the manufacturing process, the source of the starting material, the percentage of cell populations at the beginning of the culture process, expansion profile *ex vivo* and T cell phenotype at the end of the expansion, including effector, memory or exhausted populations, among others[6-9].

The percentage of CAR-T cells in the final product is an essential factor in determining the effectiveness of antitumor activity; this analysis is usually performed by flow cytometry. The classical method for determining the efficacy of CAR-T cells is the chromium release assay. It is based on the use of Chromium[51] isotope loaded into target cells and measuring its release to assess T cell-mediated cytotoxicity. Alternative methods based on fluorescent agents, such as carboxyfluorescein diacetate (CFSE), have been used to avoid radioisotopes, labeling the target cells and evaluating the target the effector/target ratios before and after the period of co-cultivation[10]. Another example is the use of calcein, also practical for the analysis of cytotoxicity mediated by CAR-T cells. Calcein can tag target cells, and its release into the culture medium represents cell death under attack by CAR-T cells. Its detection/quantification can be performed in fluorimeters[11].

The functionality and antitumor activity of CAR-T cells can also be evaluated by detecting the production of specific cytokines such as INF-γ, TNF-α, IL2, in addition to other proteins associated with the cytotoxic activity of T cells, such as perforin and granzyme. Flow cytometry can detect these molecules after co-culture of CAR-T cells with tumor cells[12-14]. Alternatively, cytokines can also be identified by enzyme-linked immunosorbent assay (ELISA), bead-based detection panels (with cytometer readout), and enzyme immunospot assay (ELISpot)[14,15].

- Animal model selection

When administered to animals with a normally functioning immune system, the host can eliminate the human cells. Previous studies have shown that using immunodeficient mice, such as NOD/SCID or NOD/SCID knockout gamma chain (NSG), is appropriate. The grafting and growth of hematologic neoplasia and human immune system cells such as T lymphocytes are possible and pertinent in this animal model[16], preserving the characteristics of the primary tumors and the human cells used for the treatment[17,18]. Human T cells can cause a xenogeneic response in animals, causing graft-versus-host disease. An animal variation that can avoid this effect is NSG mice that are knockouts for the invariant beta chain MHC class I, which prevents the expression of MHC class I in the cell membrane[19].

Another animal model widely used for this purpose is based on humanized mice, with partial human immune function or producing cytokines that help the engraftment of human hematopoietic cells. This animal model was previously established by administering human hematopoietic cells, lymphocytes, or tissues in immunodeficient mice[20].

The use of immunosuppressive agents to eliminate the immune response to cells is less recommended since these agents can interfere in evaluating efficacy or toxicity, requiring careful analysis. The use of animal-derived CAR-T rather than human cells may also be considered for animal studies. However, potential differences between the functionality of murine and human CAR-T cells may compromise the analysis and interpretation of results[21]. The use of murine cells expressing CARs in these immunocompetent mouse models is recommended when the tumor microenvironment must be reproduced, as is the case especially for solid tumors. Models that determine, as far as possible, the natural history of the disease, such as transgenic animals such as MMTV-PyMT (mouse mammary tumor virus - Polyoma Virus middle T antigen) for breast cancer[22], are recommended especially for the evaluation of 4th generation CARs that are also intended to modulate the microenvironment[23].

Clinical signs of animals should be recorded throughout the *in vivo* study, which includes, but are not limited to: changes in skin and fur; changes in attitude, posture, and reaction to handling, eating, and stool patterns, as well as stereotypies (e.g., excessive licking, repetitive movement) or abnormal behaviors (e.g., self-mutilation).

Other animals such as primates, pigs, and rats can be used to evaluate the pharmacodynamics and pharmacokinetics of CAR-T cells. For all models, it is necessary to consider an adequate weight and age pattern, healthy animals, and preferably SPF (Specific-pathogen-free). The food and environment must remain constant during the preclinical trial. These more complex models, in general, are not widely used and have limited exploration from the regulatory point of view. In addition, in the design of the *in vivo* study, other factors that may affect the study's outcome must be evaluated, as described in Table 1.

TABLE 1 - Factors to be considered for the in vivo study using CAR-T cells.

Vector	Animal Model	Experimental Design	Advanced Therapy Product
integration	immune response	number of animals	dose
transduction	pre-existing immunity	control groups	fresh/thawed product
replication		sex	cell viability
infectivity		route of administration	potency
		treatment regimen	

- Pharmacodynamics/Efficacy Study in vivo

As described, experimental models with immunodeficient mice for xenotransplantation of human cells are the most commonly used to evaluate the antitumor effect of CAR-T cells. In the case of CAR-T cells used to treat lymphocytic leukemia or lymphoma, cell lines derived from human tumors (e.g., Raji cells – Burkitt's lymphoma) or genetically modified cell lines that express the molecular target under analysis can be used for the establishment of an animal model of the target disease. In this approach, it is essential to establish appropriate control groups: a group with unmodified T cells, since they may affect tumor cells and a group that only receives the infusion of tumor cells. Increasingly, tumor cell lines expressing reporter genes such as green fluorescent protein (GFP) or luciferase are being used. With these reporters, tumor cells can be easily detected in the mouse by flow cytometry or immunohistochemistry (GFP) or even by imaging the animal with ultra-sensitive cameras to detect bioluminescence in case of grafted animals with luciferase-expressing tumors that have received luciferin substrate injections. This last technique, in particular, allows the longitudinal follow-up of the same animals, quantifying the tumor burden, and has been the method of choice in preclinical studies to validate CAR-T products.

Other complementary assessments are equally important, such as analyzing the number of tumor cells in the animal by flow cytometry, macroscopic and microscopic analysis of the mice's organs, and de-

tecting tumor-associated cytokines in the animal's serum[24]. In the case of subcutaneously grafted tumors, calculating the tumor volume using a caliper is also a standard practice. Table 2 presents some of the general parameters for evaluating the pharmacodynamics/efficacy of CAR-T cells. If the mice show any signs of suffering, severe pain, or are moribund, they should be euthanized, and the effectiveness of the treatment will also be evaluated through the survival curve.

TABLE 2 - Parameters commonly evaluated in the analysis of the pharmacodynamics/efficacy of CAR-T cells

Parameters
Tumor volume
Tumor weight
Location of tumor cells
Tumor cell analysis - bioluminescence
Serum cytokine analysis
Global animal survival
Macroscopic and microscopic analysis of organs
Effective dose with less toxicity

- Pharmacokinetic study in vivo

The *in vivo* study is essential for the preliminary analysis of the safety and efficacy of the produced CAR-T cells. The pharmacokinetic study includes analysis of proliferation, distribution, and persistence of CAR-T cells *in vivo* (Table 3). The CAR-T cells can also be detected in the animal body by preclinical imaging tests, using, for example, CAR-T cells labeled with fluorochromes, nanoparticles, or radioisotopes, in addition to their modification with different versions of luciferase. Flow cytometry can also be applied to detect CAR-T cells in these experimental animal tissues and organs[25]. The PCR technique can detect trace amounts of DNA or RNA from CAR-T cells in tissue or organ samples, as well as hybridization *in situ* can also be used to analyze the presence of CAR-T cells in tissues.

Pharmacokinetics also analyze the excretion of the advanced cell therapy product and the risk of transmission to the germline.

TABLE 3 - Evaluation of the pharmacokinetic profile in preclinical in vivo studies.

Biodistribution	Persistence	Clearance
Dose	Cells duration	Excretion cells
Route of administration	Level of expression	
Treatment regimen		

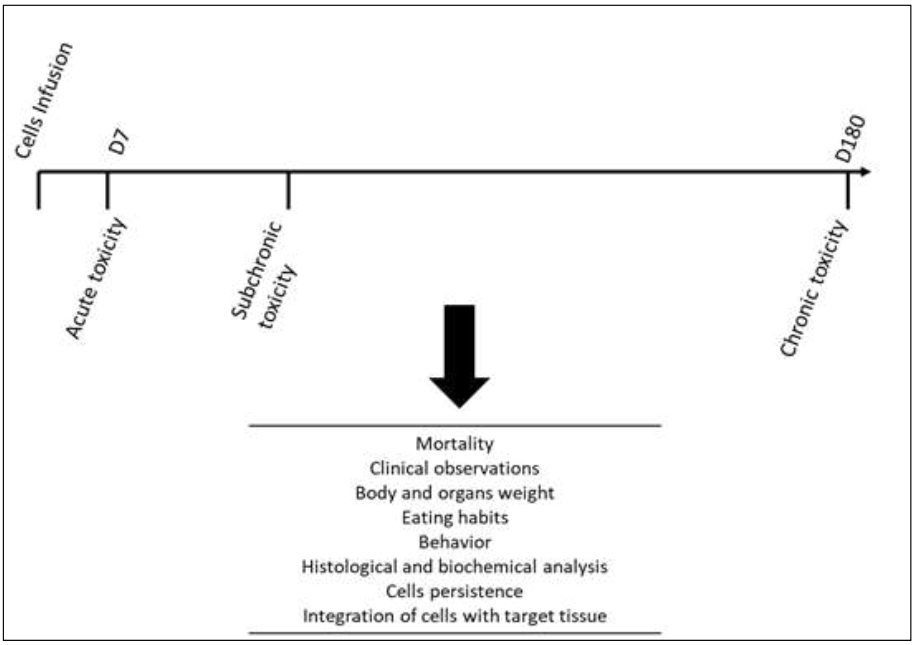
- Safety in vivo study

Safety of the treatment with CAR-T cells *in vivo* must be analyzed against the following items:

- Immunogenicity
- Risk of transmission to the germline
- Immunotoxicity
- Genotoxicity
- Tumorigenicity
- Tropism and biodistribution

In addition, routine toxicological indices such as clinical symptoms, body weight, food intake rate, serum biochemistry, hematology, and histopathology should be analyzed. Figure 1 shows a basic outline of a preclinical study for safety assessment.

FIGURE 1 - Schematic representation of the timeline and parameters to be considered in preclinical studies intended for safety/toxicity assessment.



It is essential to highlight that, although widely used to assess the potency of the antitumor effect of CAR-T cells, models of immunodeficient mice grafted with human tumors are not models that allow the prediction of CAR-T off-target effects, since cross-reactivity between antigens of mice and humans is not guaranteed. The pattern of antigen distribution can also vary between the two species, not guaranteeing that results in the preclinical model can be transposed to the result in clinical trials. Likewise, these models are not suitable for assessing cytokine release syndrome (CRS) and neurotoxicity. Adaptations such as pre-engraftment of NSG animals that produce human cytokines with human hematopoietic cells can be made to create a framework that reproduces the interrelationship between CAR-T cells and the human myeloid compartment. When these models receive the tumor and CAR-T cells, it becomes possible to reproduce some aspects of CRS and neurotoxicity[26], serving as an investigational model, although still little used due to its complexity.

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QUALITY AND ACCREDITATION IN CELLULAR THERAPY

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ABSTRACT

Quality in a Health System as we know it today can trace its origins back to the early twentieth century, when a number of measures were taken to address great variations in medical education and care. The complexity of hematopoietic cell transplantation (HCT) as a medical technology and the frequent need for close interaction and interdependence between different services, teams and external providers (donor registries, typing laboratories, etc.) distinguish it from many other medical fields. The implementation of a Quality Management program with its components including quality control, quality assurance, quality assessment and quality improvement advances the quality of service provided for patients and helps programs to address external threats and internal weaknesses, which could negatively impact services and products. In HCT, different stakeholders have been identified as holding an interest in ensuring that patients receive quality care: patients and their families, referring physicians, payers, other community healthcare providers, commercial suppliers, regulatory authorities, insurance payers and professional and patient organizations. Evidence does exist for HCT, where studies using European HCT registry data have correlated the different phases of preparation for and achievement of accreditation at centre level with incremental improvements in patient survival and reduction in procedural mortality. It also proves the level of commitment to high-quality measures and monitoring cellular therapy practice and patient care. This Manual chapter will deepen the theme of quality in cell therapy, as discussed in the Consensus 2021 of SBTMO, in order to stimulate the development of quality in the centers, as well as to give a north in the proper deployments. We will briefly go through all the steps of the implementation of quality in a Stem Cell and Cell Therapy Center.

Keywords: Stem Cells. Cell Therapy. Hematopoietic Cell Transplantation.

INTRODUCTION

Quality in healthcare as we know it today can trace its origins back to the early twentieth century, when a number of measures were taken to address great variations in medical education and care. The WHO defines quality of health care as "the extent to which health care services provided to individuals and patient populations improve desired health outcomes"[1].

The complexity of haematopoietic cell transplantation (HCT) as a medical technology and the frequent need for close interaction and interdependence between different services, teams and external providers (donor registries, typing laboratories, etc.) distinguish it from many other medical fields. At around the turn of the millennium, recognition of these challenges led to efforts by the HCT community to standardize processes based on consensus to better manage quality, including the inherent risks of HCT[1].

The implementation of a Quality Management pro-

gram with its components including quality control, quality assurance, quality assessment and quality improvement advances the quality of service provided for patients and helps programs to address external threats and internal weaknesses, which could negatively impact services and products. In HCT, different stakeholders have been identifed as holding an interest in ensuring that patients receive quality care: patients and their families, referring physicians, payers, other community healthcare providers, commercial suppliers, regulatory authorities, insurance payers and professional and patient organizations[1,2].

Evidence does exist for HCT, where studies using European HCT registry data have correlated the different phases of preparation for and achievement of accreditation at centre level with incremental improvements in patient survival and reduction in procedural mortality. It also proves the level of commitment to high-quality measures and monitoring cellular therapy practice and patient care[1,2].

STANDARDS AND QUALITY

A standard has been defined as "a desired and achievable level of performance against which actual performance is measured". Standard-setting organisations also consider themselves as facilitators of the evolution from compliance towards improvement[1,3].

Conformance to generally accepted quality standards and external accreditation and regulation rules is fundamentally important for patient safety, efficient use of resources and best possible process outcome. Compliance with such requirements includes developing a quality plan for the organization. It sets the conditions for a review of objective evidence to demonstrate that processes and products consistently meet predetermined specifications[2].

A quality management system (QMS) is a mechanism to ensure that procedures are being carried out in line with agreed standards with full participation by all staff members. In a cell transplant programme, this ensures that the clinical, collection and laboratory units are all working together to achieve excellent communication, effective common work practices and increased guarantees for patients. It is a means of rapidly identifying errors or accidents and resolving them so that the possibility of repetition is minimised. It assists in training and clearly identifies the roles and responsibilities of all staff. The quality system should monitor processes and operations through the performance of self-assessment audits, error management, and customer feedback[1,3,4].

ACCREDITATION

AABB, FACT and JACIE set voluntary cellular therapy standards,with accreditation cycles of two, three and four years, respectively. The College of American Pathologists (CAP) transfusion medicine checklist includes cellular therapy requirements. The National Donor Marrow Program (NMDP) standards set forth basic guidelines and requirements for programs working with the NMDP. The standards encompass network participation criteria with requirements for transplant centers, recruitment centers and product collection centers. Lastly, The Circular of Information for the Use of Cellular Therapy Products is jointly written by the multiple organizations[2].

FACT (Foundation for the Accreditation of Cellular Therapy) was founded in 1996 by the American Society for Transplantation and Cellular Therapy (ASTCT) and the International Society for Cell and Gene Therapy (ISCT), published the first edition of Hematopoietic Cell Standards that year, and initiated the North American inspection and accreditation program based on these Standards in 1997. JACIE (Joint Accreditation Committee of ISCT and EBMT) was established in 1999, adopted the first edition of FACT Standards, and jointly reviewed the second edition in 2002. Subsequent editions of Standards have been jointly developed, and published by FACT and JACIE[5].

The accreditation process is divided into three phases:

The first phase is a preinspection phase where the applicant submits the relevant documentation, Application Form, Self-Assessment Standards Checklist and the inspectors review it in advance of the inspection.

The second phase is an inspection phase, where the inspectors assess on-site if the documentation from the preinspection phase meets the reality of the day-to-day work in the centre through interviews with key personnel, tour of the facilities and review of additional documentation. Inspectors document fndings and observations in the inspection report, which is reviewed by the accreditation committee which decides on the next steps for the centre to achieve the accreditation.

The third phase is a post-inspection phase, where the applicant submits evidence of corrections for the deficiencies identifed in the report. The programme achieves compliance once the inspectors assess the evidence of corrections, the standards are compliant and the accreditation committee gives the approval[1].

The JACIE and FACT accreditation systems are based on the regular update of standards covering the entire transplantation process, from the selection of the donor/ patient to the follow-up, including collection, characterization, processing and storage of the graft. And have included new items specifically developed for other cellular therapy products, with special reference to immune effector cells (IEC). Considering the different competences included in the process, the standards are articulated in 4 parts: Clinical Programme, Bone Marrow Collection, Apheresis Collection and Processing Facility. A Quality Management section is embedded in each part, aimed to provide a tool for both the applicants to develop a comprehensive quality system and the inspectors to check the compliance of the transplant programme to the standards[1].

GENERAL STANDARDS

Here, there is a summary of the main FACT/JACIE standards:

- The Program shall consist of an integrated medical team, with common staff training, protocols, Standard Operating Procedures, quality management systems, clinical outcome analyses, and regular interaction among all clinical sites[5];

- It shall use cell collection and processing facilities that meet FACT/JACIE Standards. Each facility (Clinical, Collection and Processing Lab) shall have a designated team that includes a Program Director, a Quality Manager, and a minimum of one (1) additional staff member[5].

- Apheresis and Processing Facilities shall have also a Medical Director. This team shall have been in place and performing cellular therapy for at least twelve (12) months preceding initial accreditation[5].

FACILITY

Adequate environmental conditions must be maintained at all times, with adequate equipment and materials for the procedures performed. Most supplies and equipment have required storage temperatures for optimal performance. A process should be in place to notify the appropriate departments when conditions do not meet established criteria. Facility cleaning and sanitation should also be documented. The Program shall have a written safety manual that includes instructions for action in case of exposure to different hazards, instructions for waste disposal and for use of personal protective equipment[3-5].

The Collection and Processing Facilities shall be divided into defined areas of adequate size to prevent improper labeling, mix-ups, contamination, or cross-contamination of cellular therapy products and shall have a written assessment of critical parameters (as temperature, humidity, air quality, and surface contaminants). There shall be secured and controlled access to designated areas for the collection and processing procedures and for storage of equipment, supplies, and reagents. Oxygen sensors shall be appropriately placed and utilized in areas where liquid nitrogen is present[5].

There shall be designated outpatient and inpatient care areas that protects the patient from transmission of infectious agents and allows for appropriate patient isolation; confidential examination and evaluation; and administration of intravenous fluids, medications, or blood products[5].

Another important point, specially in case of complications, is the prompt access of patients to an intensive care unit or emergency services, renal support, use of appropriate blood products and a pharmacy providing 24-hour availability of medications needed for the care of cellular therapy patients[5].

PERSONNEL

- Facility Director and Medical Director: the Director shall be a physician or a person with equivalent degree in a relevant science, appropriately licensed and specialized, with a minimum of two (2) years of experience; and shall participate in a minimum of ten (10) hours of educational activities related to cellular therapy annually. The Facility Director shall be responsible for all elements (administrative and clinical operations) of the design of the facility, including quality management. The Facility Medical Director is a licensed physician with a minimum of two (2) years postgraduate certification, with training and practical and relevant experience for the scope of activities carried out[5].

- Attending Physicians: shall be appropriately licensed and specialized and shall have had a minimum of one (1) year of supervised training in the management of transplant and cellular therapy patients[5].

- Programs performing pediatric transplantation shall have a transplant team trained in the management and collection of pediatric patients[5].

- Nurses: The Program shall have adequate number of nurses formally trained and experienced in the

management of patients receiving cellular therapy, with specific training and maintainance of competence in the transplant and cellular therapy-related skills that they practice[5].

- Pharmacists: shall be licensed and knowlegdeable in the care of patients receiving cellular therapy, including adverse events, therapeutic drug interactions and adjustments[5].

- Consulting Specialists: from key disciplines who are capable of assisting in the management of recipients and donors requiring medical care[5].

- Quality Manager: to establish and maintain systems to review, modify, and approve all policies and Standard Operating Procedures intended to monitor compliance with the Standards[5].

- Data Management Staff: sufficient to comply with the Standards.

Support Services: Dietary, Social Services, Psychology and Physical therapy staff with appropriate training and education to assist the patients[5].

- Training must be completed prior to an employee performing a task independently and must be repeated at defined intervals.Personnel must be assessed following training to ensure they are competent to perform the tasks for which they are responsible. Elements of competency assessment include direct observation of routine procedures, evaluation of problem-solving skills, written or oral tests[3,4].

- The following policies and processes are required by regulatory and accreditation agencies: Job descriptions and employee qualifications; Orientation; Training; Assessments of competence; Continuing education. Continued competency for each critical function performed has to be assessed annually at a minimum[3-5].

QUALITY MANAGEMENT

- There shall be an overall Quality Management Program that incorporates key performance data from clinical, collection, and processing facility quality management; and the Program shall establish and maintain a written Quality Management Plan[5].

- The Program Director shall review the quality management activities with representatives in key positions in all elements of the cellular therapy program, at a minimum, quarterly and annually review its effectiveness. Performance data and review findings shall be reported to key positions and staff[5].

Planned deviations shall be pre-approved by the Program Director and reviewed by the Quality Manager[5].

- Data and records: The Program shall collect and maintain complete and accurate data necessary to complete the Transplant Essential Data Forms of the CIBMTR or the Minimum Essential Data-A forms of the EBMT[5].

- The Program shall maintain a current listing of all critical electronic record systems and system elements to maintain the accuracy, integrity, identity, and confidentiality of all records[5].

- The Quality Management Plan shall include or reference:

  - An organizational chart of key positions

  - Personnel requirements for each key position in the Program

  - A comprehensive system for document control, with identification of the types of documents that are considered critical (SOPs, worksheets,forms, labels, etc). Critical documents should have a standardized format, an unique identifier, review every two years and a system for document approval, change control, archival, retraction and protection from unauthorized modification.

  - Maintenance of written agreements with external parties providing critical services

  - Review of outcome analysis and cellular therapy product efficacy, including time to neutrophil and platelet engraftment, incidence of GVHD, catheter infection and overall and treatment-related morbidity and mortality at thirty (30) days, one hundred (100) days, and one (1) year after cellular therapy product administration.

  - A schedule of audits of the Program's activities to verify compliance with elements of the Quality Management Program.

  - Management of cellular therapy products with positive microbial culture results.

  - Management of occurrences (errors, accidents, deviations, adverse events, adverse reactions, and complaints)

  - Cellular therapy product chain of identity and chain of custody that allow tracking from the donor to the recipient or final disposition and tracing from the recipient or final disposition to the donor.

- Actions to take in the event the Program's operations are interrupted.

- Qualification of critical manufacturers, vendors, equipment, software, supplies, reagents, facilities, and services.

- Validation or verification of critical procedures: processing techniques, cryopreservation procedures, marrow or other cellular collection procedures, testing, labeling, storage, distribution, preparation for administration, and infusion at minimum.

- Evaluation of risk in changes to a process.

- Methods for obtaining feedback[5]

AUDITS

- An audit can be defined as a documented, systematic evaluation to determine whether approved policies or standard operating procedures have been properly implemented and are being followed[1].

- Audits shall be conducted by an individual with sufficient knowledge in the process and competence in auditing to identify problems and shall be used to recognize problems, detect trends, identify improvement opportunities, implement corrective and preventive actions when necessary, and follow-up on the effectiveness of these actions. They shall be performed annually at a minimum[5].

OCCURRENCES

- The management of occurrences shall include:

  - Detection

  - Investigation: shall identify the root cause and a plan for short- and long-term corrective and preventive actions.

  - Documentation

  - Reporting: to the donor's and recipient's physician(s), other facilities participating in the manufacturing of the cellular therapy product and governmental agencies as required by Applicable Law.

  - Corrective and preventive action[5].

VALIDATIONS

- Validation is a documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes[3,4].

- Each validation shall include:

  - An approved plan.
  - Acceptance criteria.
  - Data collection.
  - Evaluation of data.
  - Summary of results.
  - References.
  - Review and approval5.

STANDARD OPERATING PROCEDURES (SOPS)

- The Program shall establish and maintain policies or Standard Operating Procedures addressing critical aspects of operations and management[5].

- SOPs shall be sufficiently detailed, unambiguous and describe clear objectives, equipment and supplies used, acceptable end-points, a stepwise description of the procedure, references and documented approval. SOPs that are relevant to processes being performed shall be readily available to the facility staff. Staff review, training and competency shall be documented[5].

- When genetically modified cellular therapy products are utilized in the Clinical Program, the program shall incorporate or reference institutional or regulatory requirements relating to biosafety practices, including disposal. There shall be policies and Standard Operating Procedures addressing the administration of immune effector cells and management of complications[5].

- The organization must establish a process to adress emergency preparedness. It's important to have a written disaster plan, that should be reviewed and tested on a regular basis[3,4].

DONOR AND RECIPIENT CARE

- There shall be written criteria for allogeneic and autologous donor selection, evaluation, and management by trained medical personnel[5].

- The donor and the recipient have to sign an informed consent. Informed consent is a process wherein the physician engages the donor/patient in a discussion and discloses information in a manner permitting the patient to make a knowledgeable decision about the proposed procedure/treatment. The following should be discussed: the nature and purpose of the procedure, its risks and benefits, the alternatives, the likelihood of achieving the treatment goals. The procedures shall be explained in

terms the donor can understand and he shall have the opportunity to ask questions, to refuse to donate or withdraw consent[3-5].

- Laboratory testing of all donors shall be performed by a laboratory that is accredited or licensed, using donor screening tests, HLA typing and pregnancy test in accordance with Applicable Law[5].

- Collection from a donor who does not meet collection safety criteria or an ineligible allogeneic donor shall require documentation of the rationale for his/her selection by the donor's physician and documentation of the informed consent of the donor and the recipient. If central venous access is required, the rationale shall be documented in the donor's records[5].

LABELS

- Cellular therapy products shall be identified by name according to ISBT 128 standard terminology or Eurocode[5].

- ISBT 128 provides a uniform coding and labeling system that is used worldwide. It provides traceability of all medical products of human origin including blood, bone marrow and tissues. ISBT128 provides a standard format and appearance for labeling that includes a unique Donation Identification Number (DIN) and standardized product descriptions, as well as information such as ABO and RhD blood groups, the appropriate biohazard and warning labels, collection date and time and expiration date and time[3-5].

- Label systems shall be validated to confirm accuracy regarding identity, content, and conformity of labels, with a version control and checks in labeling procedures to prevent errors in the transferrence of information. Labeling elements required by Applicable Law shall be present[5].

PROCESS CONTROLS

- An organization's process control measures must include checks and balances that assist in identifying when all is well and when the process is in danger of failing or has failed. Written policies for all operational tasks must exist and should be reviewed regularly to ensure what is written is actually what's being practiced[3,4].

EQUIPMENT

- All equipment must be uniquely identified and its use documented as far as which tests, processes, or patient procedures were performed on it. This allows for traceability and troubleshooting. The following documentation should always be available: Selection (evaluation of the equipment before the purchase with a Supplier Qualification Process); Installation Qualification; Operational qualification; Performance qualification; Calibration; Preventive maintenance; Cleaning[3,4].

- When the equipment arrives, it first needs to be evaluated to ensure the device performs per the manufacturer established specifications, the process of **qualification**. Once qualified, the equipment is then inserted into it intended role in a process. The process is then **validated**. Data is collected to ensure that the process with the new equipment is functioning according to the acceptance criteria[2].

- The department's computers and software are also considered equipment. The quality plan should state who is responsible for installing, managing and maintaining the computer systems. Important processes like validation, staff access and downtime must be clarified, along with identifying the process for upgrades, backups and access removal of unauthorized personnel[3,4].

SUPPLIER AND CUSTOMER ISSUES

- Characteristics or functional requirements for critical materials have to be defined along with the ability of vendors/suppliers to meet these requirements. There should be processes in place for: Contract or agreement review; Service review; Receipt, inspection, and testing of incoming supplies. Criteria must be established for accepting critical materials[3,4].

CELLULAR THERAPY PRODUCT STORAGE

- Collection and Processing Facilities shall control and secure storage areas to prevent mix-ups, deterioration, contamination, cross-contamination, and improper release or distribution of cellular therapy products. Conditions and duration of storage of all cellular therapy products shall be validated with a written stability program that annually evaluates the viability and potency of cryopreserved cellular therapy products. Processes for storing cellular therapy products in quarantine shall be defined in Standard Operating Procedures[5].

TRANSPORTATION AND SHIPPING

- Shall be designed to protect the integrity of the product and the health and safety of individuals in

the immediate area, using a validated container at a temperature defined in a SOP[5].

RECEIPT AND DISTRIBUTION

- Standard Operating Procedures shall be established and maintained for acceptance, rejection, inspection, verification of appropriate transport and quarantine of cellular therapy products.[4]

- Each cellular therapy product shall meet pre-determined release criteria prior to distribution. The cellular therapy product distribution records shall permit tracking and tracing of the cellular therapy product.[4]

RECORDS

- A records management system shall be established and maintained to facilitate the review of records, and preserve their integrity, preservation, retrieval and confidentiality.The facility shall follow good documentation practices. For all critical electronic record systems, there shall be an alternative system for all electronic records to allow for continuous operation in the event that critical electronic record systems are not available[5].

- Retention of records is dictated by regulatory and accreditation standards although an organization can choose to be more stringent[3,4].

DISPOSAL

- Disposal of cellular therapy products shall include a pre-collection written agreement between the storage facility and the designated recipient or the donor defining the length of storage and the circumstances for disposal[5];

- Documentation of no further need for the cellular therapy product before any product is discarded and a method of disposal and decontamination that meets Applicable Law for disposal of biohazardous materials and/or medical waste are necessary[5].

Note: Some parts of the text, either highlighted by the bibliographic indication or in quotation marks, were often written as the source text of the manual FACT-JACIE. This was necessary, because there are definitions, statements and standards , that cannot be interpreted. They have to reported *Ipsis litteris* as at source.

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REGULATORY CONSIDERATIONS FOR CELLULAR THERAPY

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ABSTRACT

Advanced therapy products can be an alternative treatment for several disease. Manufacturing steps and product release are critical points to avoid unsafe use of products. Quality controls tests, manufacturing practice, safety testing and efficacy trials need to be properly accessed before releasing to patients. Regulatory system for cell therapy products determines guidelines for production, clinical trials and registration, considering risk-benefit ratios. This article aims to discuss main aspects of National Regulatory for advanced therapy products.

Keywords: Regulatory; advanced therapy products; registration; good manufacturing practice

INTRODUCTION

Therapeutic potential of cellular therapy products has been shown in growing number of clinical trials and can be an alternative treatment to improve outcomes of several disease. However, development of this new approach needs to be faced at the risk of unsafe use of products. Quality controls tests, manufacturing practice, safety testing and efficacy trials need to be properly accessed before releasing to patients. These considerations have led to implementation of specific regulatory system for cell therapy products which incorporates consideration of risk-benefit ratios and includes quality controls tests to release products.

REGULATORY FRAMEWORK

In 1999, Brazilian National Agency of Health Surveillance (Anvisa) was established in order to regulate, control and inspect products and services that involve risk to human health[1,2].

After approval of studies with embryonic cells, in 2005, Anvisa was designated to create rules for collection and processing procedures, storage, transportation, quality control tests and use of human embryonic stem cells. This initial attribution triggered other regulations to improve development of cell therapies products and in 2011, Anvisa published Resolution of the Collegiate Board of Directors (RDC) nº 9, which provides guidelines for operation of Cellular Technology Centers (CTC) for clinical research and use in conventional therapy[1]. The RDC nº 09/2011 established minimum requirements for provision of cells by CTC, in addition to making sanitary licensing mandatory of these establishments. This regulation determined criteria for quality control and cell safety and also determined that human cells could only be made available for research clinic after approval of research project by Research Ethics Committee/ National Ethics Commission in Research (CEP/ Conep)[3].

Similarly, in 2007, the European Medicines Agency (EMA) and the US Food Agency and medicines (Food and Drug Administration – FDA) published their main regulations and guides for production and control of Advanced Therapies Products[4,5].

In 2016, Federal Attorney's Office with Anvisa approved registration and commercialization of cell therapy products conditioned to establishment of a normative framework to guarantee ethical use of human cells[6].

Considering risks involved in production and use of cell therapy products, Anvisa proposed national

regulatory framework based on: 1) Good Practices in Human Cells for Therapeutic and Clinical Research – Resolution of the Collegiate Board of Directors nº 508; 2) Clinical trials with advanced cell therapy products - Resolution of the Collegiate Board of Directors nº 506; 3) Registration of Advanced Therapies Products - Resolution of the Collegiate Board of Directors nº 505; and 4) Certification of Good Practices for producing establishments[7-9].

The regulatory framework aims to ensure quality, safety and effectiveness of these new products and, in this way, guarantee safe access for future users. Several guidelines are outlined in these resolutions to provide good manufacturing practice, such as infrastructure, biosafety strategies, waste disposal, validation process, qualification of equipment and reagents and a quality management system.

It is worth highlighting the difference between concept of minimal manipulation and extensive manipulation of cells. First consists of a cell processing technique or tissues that do not significantly alter their biological characteristics, including differentiation and activation state, proliferation potential and metabolic activity. Stem cells processing for bone marrow transplantation purposes is considered minimal cell manipulation. Extensive manipulation, on the other hand, consists of processing of cells and tissues that alters any of their biological characteristics, including state of differentiation and activation, proliferation potential and activity metabolic and will be focus of our discussion[10].

GOOD MANUFACTURING PRACTICES

Good manufacturing practices (GMP) involve all aspects of manufacturing to guarantee quality and safety of products supplied for therapeutic and research use. To achieve these practices, Cell Therapy Center should have qualified and trained personnel; adequate physical infrastructure; equipment, instruments, suppliers and support services qualified and approved; approved computerized systems that guarantees data traceability; materials, reagents and products for in vitro diagnostics validated and all activities described in operational procedure approved by director of center.

Quality management system is crucial to success of cell therapy centers, monitoring all activities, reviewing release criteria of all products, identifying non-conforming products and determine root cause of non-conformance or error and determine appropriate corrective action. Quality management will be addressed in another chapter.

INFRASTRUCTURE

Cell therapy center must consist of environments for carrying out administrative activities, receiving biological products area, processing lab, including production of gene therapy vectors or the manipulation of genetic modified products, storage area and a quality control lab[8].

The Cell Processing Center that process Genetic Modified Organisms (GMO) must be certified by National Technical Biosafety Commission (CTNBio). GMO requires dedicated rooms or isolated environments for manipulation, ensuring that structure is adequate to avoid environmental and professional risks in handling products. GMO and vectors risks are classified considering their characteristics, proposed use and adverse effects on human health and on environment[11]. Cell Therapy Centers must be planned according to which GMO and vectors they will handle, as physical infrastructure is different for each risk group. As an example, Centers that handle class 2 risk products must have an autoclaving system for their waste before disposing of materials handled in this area.

A Clean Room and controlled environments with air particle count is required to minimize risk of contamination during product manipulation. ISO classification of air is given in Table 1. For advanced cell therapy products manipulation, an ISO 5 condition must be maintained surrounded by environment with ISO 8 classification[8,12].

TABLE 1 – ISO class number based on particle concentration[12]

ISO class number	Maximum Allowed Concentration Particles Equal Or Greater Than The Considered Size					
	0,1µm	0,2 µm	0,3 µm	0,5 µm	1 µm	5 µm
1	10					
2	100	24	10			
3	1.000	237	102	35		
4	10.000	2.370	1.020	352	83	
5	100.000	23.370	10.200	3.520	832	
6	1.000.000	237.000	102.000	35.200	8.320	
7				352.000	83.200	293
8				3.520.000	832.000	2.930
9				35.200.000	8.320.000	29.300

Clean environments must perform microbiological monitoring regularly. The limits expressed in colony forming units (CFU) in operation are described in Table 2.

TABLE 2 – Limits of microbiological monitoring in clean environments[8]

	Sedimentation Plate (90 MM; CFU/4 HOURS)	Contact Plates (55 MM; CFU/ PLATE)	Air Sampling (CFU/M³)
ISO 5	<1	<1	<1
ISO 8	50	25	100

BIOSAFETY

Cell Therapy Center must keep procedures to assure occupational and environmental safety, including waste disposal and transportation of biological material.

Cell Processing Center must have an advice regarding classification of the biosafety level of Environments, as well as the hygiene rules and necessary personnel protective equipment at entrance of each sector.

Transportation of biological products and samples must be packaged in a way that preserves their integrity and stability during transport, as well as guarantees safety of personnel involved in this process. GMO and its samples have and additional rule for transportation and center that will receive these products have to be certified to handle GMO class risk[13,14].

Reagents and products for in vitro diagnostics

All materials used in advanced cell therapy must be regularized with Anvisa and suppliers must be qualified by center. Materials used in collection and processing cells must be sterile, non-pyrogenic and, when applicable, non-cytotoxic, of pharmaceutical grade and for single use. Centers must respect the manufacturer's recommendations for use, conditions of preservation and storage and the expiration dates. Products of animal origin must be avoided[8,15].

Growth factors, measures of identity, purity and potency must be established, to ensure reproducibility of the characteristics of the cell culture that will be addressed in other chapter[8].

Qualification and Validation

Qualification and validation are necessary to prove that all processes defined as critical are under control, providing security to products and users.

Qualification program should include: 1) Project qualification; 2) Installation qualification; 3) Operation qualification; 4) Performance qualification.

Project qualification provides evidence for acquiring, installing, and operating a new equipment or system have met initial requirements. Installation Qualification requires: 1) identification of installed items; 2) maintenance and calibration requirements; 3) list of

operating and work instructions given by supplier; 4) cleaning requirements. Operation Qualification corresponds to the evaluations or studies of critical parameters of operation of an equipment or system, with objective of evidencing, through documents, that all the functions of the equipment/system are in accordance with the manufacturer's manual. Performance Qualification must provide documented evidence that the equipment or systems and all your components can work consistently with your specifications and routine of work[16].

Validation aims to demonstrate that critical processes, using specified materials and equipment, systematically achieve their goals, ensuring that consistent results are obtained, and final products are safe and of good quality. Validation can be based on evidence obtained through testing (prospective and concurrent validation) or on analysis of data accumulated over a given period (retrospective validation). Whenever possible, prospective validation should be opted for, since retrospective validations are no longer recommended[16].

Quality control tests

In order to release final advanced therapy products for patient use there are tests that must be performed to guarantee quality and safety. This issue will be addressed in specific chapter.

CLINICAL TRIALS

All clinical trials with advanced therapy products must have Anvisa and respective ethics committees the CEP/CONEP approval.

Advanced therapy products are classified in two types: 1)Class I product: advanced cellular therapy product that undergoes minimal manipulation and performs a different function in recipient from that performed in donor; 2) Class II product: advanced cell therapy product subjected to extensive manipulation, tissue engineering product and gene therapy product. [7] In class I products trial, Anvisa approval will be based in documents about clinical investigation plan, containing description of the product and a summary description of indications, outcomes and population to be studied; and product's critical parameters of processes and critical attributes of quality that will be analyzed.

In class II products trial, documents with extensive description of product, including composition; biological and toxicological effects on animals and humans; information on safety and efficacy in humans; and possible risks and adverse events related to use of investigational product should be added to documents described in class I product trial for Anvisa approval[7].

Clinical trials should be registered on clinical trial database of the "International Clinical Trials Registration Platform/World Health Organization" (ICTRP/WHO), the Brazilian Registry of Clinical Trials (ReBEC) or another entity[7].

Cell Therapy Products Registry

Commercialization of advanced therapy products after efficacy and harmlessness are proven is possible by registration in Brazil. Requirements for registration for a class I product are: 1) studies to prove therapeutic effect and effective dose; 2) studies on the interaction of product with other tissues, with evaluation of potential side effects; 3) studies aimed to determine parameters of viability, shelf life, distribution, metabolism and excretion of the advanced therapy product; 4) product toxicity studies, including cellular component, excipients and any impurities related to process; 5) studies to determine potential immunogenic effects; 6) studies on tumorigenic potential of the product; 7) safety studies, which address aspects related to biodistribution and grafting, shelf life, oncogenetic transformation and cell line stability; and 8) clinical efficacy studies[9].

Registration of class II product will be necessary to include documents with product manufacturing, containing: 1) information on starting material, material and excipients; 2) information about active component and final advanced therapy product; 3) Information about manufacturing steps; 4) protocol and report of stability studies performed; 5) additional comparability studies, considering possible changes in the advanced therapy product manufacturing process; and 6) description of storage of final advanced therapy product[9].

CONCLUSION

National regulatory framework for advanced cell therapy products aim to ensure quality, safety and effectiveness of these new products and promote health of the population. Good manufacturing practices, clinical trials approval by Anvisa and registration of advanced therapy products guarantee safe access for future users and create a stable and transparent regulatory environment, to promote technological development.

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COLLECTION OF LYMPHOCYTES BY APHERESIS

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ABSTRACT

The first step in the manufacturing of cell therapy products is the collection of cells from donors or patients. This point should be given special attention by all professionals involved in the development of these therapies because it is critical to guarantee the quality of the final product. Lymphocytes, especially T-cell lineage, have been used for treatment in a large number of pathologies, including oncological and infectious diseases, especially after approval by regulatory agencies in several countries for the use of Chimeric Antigen Receptor Therapies (CAR T). This article describes the steps for the procedure of collecting lymphocytes by apheresis, defining indications and contraindications as well as the planning and preparation measures for the procedure.

Keywords: Lymphocytes. Blood Component Removal. Immunotherapy, Adoptive.

OBJECTIVE

Describe the steps for the procedure of collecting lymphocytes by apheresis. Define indications and contraindications as well as planning and preparation measures for the procedure.

INTRODUCTION

The first step in the manufacturing of cell therapy products is the collection of cells from donors or patients. This point should be given special attention by all professionals involved in the development of therapies because it is critical to guarantee the quality of the final product[1].

Unlike the collection of cells for hematopoietic progenitor cell transplantation, in which there is a possibility of collection by apheresis of peripheral blood or directly from the bone marrow with its well-known risks, benefits and preparation of the donor, cell collection for other types of cell therapy, especially to produce CAR cells (chimeric antigen receptor), must be done by apheresis, however, the ideal procedure, the quantitative and qualitative characteristics to be achieved for the collected product, as well as the risks specifically related to this type of procedure are less known[1].

The apheresis procedure involves the application of a centrifugal force to a continuous or semicontinuous blood flow so that the different cellular components and plasma can be separated by density difference[2].

The apheresis equipment used to collect mononuclear cells differs slightly in the programming and interaction mode between the operator and the equipment, and in their cell separation technologies with Spectra Optia (Terumo BCT) equipment being the most frequently used in the United States, and COM.TEC equipment (Fresenius HemoCare) the mostly used in Europe[3,4].

Lymphocytes, especially T-cell lineage, have been used for treatment in a large number of pathologies, including oncological and infectious diseases, especially after approval by regulatory agencies in several countries for the use of Chimeric Antigen Receptor Therapies (CAR T) [5].

During the centrifugation procedure the mononuclear cells, including lymphocytes, are located between the erythrocyte and polymorphonuclear layers, which are denser, and the platelet layer, which is less dense. Thus, it is possible to return to the donor the cells that are not of interest to the collection and

store the cells of interest containing a small number of other cell types[4,6].

Most studies and commercial products that use lymphocytes as a starting cell for the manufacturing of cell therapy products use autologous cells, therefore interest in the use of allogeneic cells has grown[7], for this reason the article will focus on this type of procedure, however the interest in the use of allogeneic cells has grown and some relevant information regarding this type of donor will be presented separately.

For the collection of lymphocytes intended for the manufacture of CAR T, the donor does not need to be stimulated with growth factors, but there is still little knowledge about the clinical and technical factors that influence the results of the collection, as well as on the methods for optimizing the procedure, therefore there is much to be studied in this area[8].

We know that not all experience gained in the collection of hematopoietic progenitors can be transferred directly to the collection of mononuclear cells, mainly due to the fact that immobilized donors, for the most part, have low leukocyte counts often making the selection of target cells challenging.

The procedure for collecting lymphocytes from allogeneic donors for the infusion of lymphocytes from the donor after bone marrow transplantation is the procedure that most resembles that of the collection of lymphocytes for the production of cell therapy products. However, as most patients involved in studies and treatments with CAR T use autologous lymphocytes, these donors often have intense secondary lymphopenia related to the treatment, and this may also be associated with the presence of circulating blasts, which makes the cell separation procedure more complex, and may result in less uniform collection products[8,9].

PROCEDURE

Indications and contraindications

Patients or allogeneic donors, who have an indication of lymphocyte collection by apheresis for the manufacture of cell therapy products, and who have signed the form of the terms of free consent are indicated for the procedure.

Autologous donors, because they are patients, should have their clinical conditions evaluated on a case-by-case basis to measure the risk-benefit ratio. The fitness for the apheresis procedure should be discussed at the time of indication of CAR T cell treatment. Comorbidities such as severe heart and liver failure, in addition to ischemic heart disease with recent manifestation, may contraindicate the apheresis procedure, but this decision should be made by the physician responsible for the apheresis procedure together with the team responsible for treating the patient.

3.2 Necessary human resources

The apheresis procedure should be performed by a trained and qualified health professional for the procedure and, due to the frequent need for central venous catheter manipulation, it is advisable that the professional be a nurse, but this is not a requirement.

Moreover, a doctor, preferably a hematologist or hemotherapist, experienced in apheresis procedures, and who is technically responsible for the procedure is required.

Pediatric care should preferably be performed in an environment with a pediatrician available to care for any complications.

3.3 Minimum requirements for the procedure

Team trained to perform the procedure

Apheresis equipment available

Physical space suitable for performing an apheresis procedure

Donor approved for the procedure: attention to signs of recent infection, especially in the last 24 hours, and blood count results from the day of collection mainly for autologous donors. Be alert to the need for red blood and platelet transfusion before the start of the procedure.

3.4 Material

- Disposable kit for mononuclear cell collection (suitable for the equipment that will be used)
- Anticoagulant solution
- Saline
- Skin or catheter asepsis material (sterile gloves, gauze, alcohol, or chlorhexidine solution).

3.5 Pre-and post-procedure guidance

Pre-procedure:

Fasting is not required of the donor whose peripheral venous access is suitable for collecting by apheresis. On the contrary, they should be instructed to eat before the beginning of the procedure. It is recommended that the donor attend the collection accom-

panied by someone, or that at least someone can collect them after the end of the procedure in cases of outpatient collections. For those who require central venous access, it is important to inform the appropriate fasting time, if necessary, and the flow to catheter implantation.

Post-procedure:

Avoid performing physical exertion in the first 24 hours after donation. In the case of donors who received a central venous catheter only for the procedure, the catheter should be removed as soon as it is confirmed that the collected cellular dose has reached the target. After catheter removal, a compressive dressing should be applied appropriately to the site where the catheter was implanted, and the donor should remain at rest for at least 60 minutes to avoid bleeding at the site. For autologous donors, the medical team that is responsible for treating the patient should decide on the removal or maintenance of the catheter.

3.6 Description of the procedure

The institution should create a notification flow of donors who are candidates for the collection of lymphocytes by apheresis to manufacture cell therapy products for the hemotherapy team that will perform the procedure. Preferably, this request should be formalized through a signed document that, at minimum contains the patient identifiers and the target cells to be collected. Based on this information, the team responsible for the collection should start the procedure planning process.

Venous access:

Adequate venous access is essential for performing the procedure and the possibility of peripheral venous access should be accessed by a trained team.

In case there are no conditions for collection by peripheral venous access, the implantation of a central venous catheter must be planned.

For adult patients, a catheter with a caliber between 10 and 13.5Fr usually provides adequate flow, and the choice of using tunneled or non-tunneled catheters should take into account the planned length of catheter permanence, with the tunneled catheters being more suitable for patients who will use it to receive medications after the collection, and non-tunneled catheters more suitable for patients who will use it only for the collection procedure[2].

The experience of the team that will perform the implant should be taken into consideration for the catheter implantation site. But as a general rule, short-term catheters, should be implanted in the femoral region due to the lower risk of complications related to local hemorrhage[2].

Proper planning for venous access is important because failure in this process can lead to significant delays in collection, which can have a major impact on cell manipulation and transport logistics, as many manufacturing laboratories require a maximum time between collection and the start of cryopreservation, and others require cells to arrive fresh for manufacture.

Procedure planning:

Another important point of attention during the procedure planning is the clinical assessment of the donor and the sample collection to perform the screening for transfusion-transmissible infectious diseases.

In the case of autologous donors, the assessment can be summarized as an aptitude assessment of the apheresis procedure[10].

Mandatory laboratory tests to be performed on cell donors for advanced cell therapy products by current legislation in Brazil are:

- Hepatitis C: anti-HCV antibody and NAT for HCV

- Hepatitis B: HBsAg, anti-HBc total with differentiation of IgG and IgM or just IgG and NAT for HBC

- HIV: anti-HIV (subtypes 1 and 2) and NAT for HIV

- HTLV: serology for HTLV 1 and 2

- Chagas disease: serology for Chagas disease

- Syphilis: one treponemic or non-treponemic test

Carrying out NAT tests is not mandatory for autologous donors if handling and infusion takes place with fresh cells and there is no risk of cross-contamination during storage.

Testing for plasmid or plasmid antigens may also be required if the donor lives or travel to a malaria-endemic region in the last 12 months.

RDC 508/21 authorizes the use of an autologous donor product regardless of the test that is altered, as long as the patient's physician is aware of this alteration, however manufacturing laboratories may have their own criteria for the inability of the donor that may be stricter than national legislation, therefore, in these cases, it is recommended that screening tests for infectious diseases be carried out before the day

of cell collection, as the product may be considered unfit for use after it has already been collected.

It is worth mentioning that the current legislation in relation to cell therapy products (RDC 508/2021) cites that the screening for infectious diseases for the collection of cells other than hematopoietic progenitor cells obtained for the purpose of perform conventional transplantation must be carried out until 7 days before or 7 days after the day of collection. Ordinance 158 /2016, which defines the technical regulations for hemotherapy procedures, mentions that these tests for the collection of lymphocytes must be performed up to 72 hours before the procedure.

Other tests to determine the donor`s fitness are not a consensus among the services, being that *the European Society for Blood and Marrow Transplantation* (EBMT) indicates that hemoglobin levels should be higher than 8g/dL, total leukocytes above 1,000cells/mm3, lymphocyte count above 200 cells/mm3, platelet count greater than 30,000/mm^3, and the ejection fraction of the left ventricle should be greater than 40%[3].

Selection and determination of donor fitness:

Each service must have a POP describing the acceptable selection criteria and relevant assessments to define the donor's fitness, and these criteria may vary depending on the laboratory that will manufacture the cell therapy product.

It is important to highlight that cells that need to be exported for manipulating, or products manipulated abroad that are imported for use in Brazil must meet the legal requirements of both countries.

It is also important that after determining the fitness of the donor, he or she must be informed about the procedure, answer any questions that they may have, and that the terms of free consent form be applied, and TCLE clarification given regarding the cell collection procedure.

The TCLE should contain, at a minimum, information on the procedure for collecting cells by apheresis, risks related to the procedure, laboratory tests to which the donor will be submitted, the purpose of cell collection, and authorization of the donor for access to the team that will use the cells for manufacture to clinical and laboratory data, authorization for the storage of cell aliquots, blood and/or plasma for future tests that may be necessary, as well as the possibility of withdrawal at any stage of the process, and when it fits, what are the consequences of such withdrawal.

Special donors:

Pediatric donors

Children, especially those under 25 Kg, often require central catheter implantation. A diameter of at least 7Fr is suitable for the procedure, and unless the catheter is needed for use at other stages of treatment, a femoral implantation is recommended.

If the donor or patient is under 18 years of age, the TCLE must be signed by the parents or legal guardian.

Allogenic donor

According to RDC 508/21, allogeneic donors must be clinically evaluated and released according to criteria similar to those used for releasing blood donors.

If the donor does not have peripheral venous access compatible with the procedure, central venous catheter implantation is allowed, although not recommended, and the preference is for implantation in the femoral region performed by a vascular surgeon with experience in central catheter implants.

Allogeneic donors must perform all the tests for detecting infectious diseases mentioned for autologous donors, in addition to serology for cytomegalovirus (CMV) with titration of total antibodies + IgM or IgG + IgM and the pregnancy test is mandatory and must be performed within, maximum, 7 days before the day of the procedure.

Although the acceptance criteria for donors with altered/positive serology are different between laboratories that manufacture cell therapy products, RDC 508/21[10] says that donors are unfit for allogeneic donation: with HIV or HTLV positive test, HBsAg non-reactive with anti-HBc reactive (unless the donor also has anti-HBs reactive), HBsAg reactive and/or NAT positive for virus B, anti-HCV reactive and/or NAT positive for Virus C, or with a positive test for Trypanosoma cruzi.

Execution procedure:

On the day of collection, the correct identification of the patient and the bag should be a point of special attention.

According to general rules of safe identification, at least 2 identifiers should be used, and preferably conferred positively with the donor, i.e., the donor himself must say the two identifiers (e.g., full name and date of birth).

The bag should also be identified with a specific numbering per procedure, preferably using ISBT

encoding or, in case the service does not use ISBT encoding, a single, sequential numerical, or alpha-numeric code of the service. Some cell therapy product manufacturing laboratories also provide a specific identifier for the patient, and the bag. A secure identification procedure must be defined by the institution for safe identification, and the procedure should be defined by the institution, as later on no other type of identification check will be possible during the transportation, manufacturing, and infusion processes.

Ideally, the identification of the bag that will store the product should be done after the assembly of the disposable system of the apheresis equipment, immediately before the beginning of the procedure, and after conferring the donor`s identification to avoid any change of identification of the bag in case there is more than one patient being submitted to cell collection on the same day, or in the same service.

The procedure must be performed in apheresis equipment with a validated program for the collection of mononuclear cells, and there is diverse equipment available in the national market. All available equipment can be used with advantages and disadvantages being that the experience of the service in using the equipment is a factor that should be taken into consideration when choosing the equipment to be used. The table in Annex 2 shows some of the leading manufacturers of apheresis equipment as well as a comparison of their main characteristics.

Technically, the procedure is very similar to the collection of donor lymphocytes for infusion after allogeneic transplantation, and, to date, neither the companies that produce the apheresis equipment nor the manufacturing laboratories have defined a specific protocol for the collection of lymphocytes for the collection of T cells for cell therapy, therefore each service must validate its process based on the information provided by the manufacturer of the apheresis equipment for the collection of lymphocytes.

The best way to validate the collection process is the systematic calculation of the collection efficiency of the procedures performed in the service followed by the comparison with benchmarks. In addition, the achievement of collection objectives is also a very important indicator, since, in practice, this is the objective of the entire procedure.

A collection efficiency greater than 40% is generally considered adequate but may vary depending on the type of equipment used as showed in Annex 2[3].

The efficiency calculation is obtained using the following formula:

Collection efficiency: total CD3+ cell count in the bag /(CD3+ peripheral cell count per litre x volume of processed blood in litres) x 100

When the average collection efficiency of the service is known, or, ideally, the efficiency of the equipment being used, it is also possible to calculate the volume to be processed to reach the volume of target cells for the patient. However, not all laboratories require a minimum target of CD3+ cells or lymphocytes, some request that a certain blood volume be processed according to the weight and peripheral lymphocytes count of the donor or patient, and others request a minimum volume of mononuclear cells.

Thus, each service must determine its protocol for calculating the volume to be processed to reach the target requested by the laboratory that will manufacture the product that will be collected.

Although the procedure can be performed in patients with an absolute count of lymphocytes in peripheral blood below 100 cells /mm^3 the probability of obtaining a number of lymphocytes that is sufficient for the manufacture of the cell therapy product greatly increases when this value is above 500. However, most adult patients with peripheral lymphocyte count equal to or greater than 200/mL can obtain the minimum required amount of cells T for manufacturing in only one collection procedure with the processing of 3 to 4 blood cells and in cases where a collection is not sufficient, it is possible to perform a second collection on the following day.

In addition, to avoid the adverse effects related to the accumulation of exposure to chemotherapeutics, either in the reduction of T-cell production and in the quality of these, the collection of cells should be scheduled, whenever possible, before the start of rescue chemotherapy. Where such a procedure is not possible, a minimum interval between the administration of rescue medications and collection should be carefully considered.

Before the initiation of an apheresis procedure, the

donor must be clinically assessed for vital signs and medical solicitation documents for the procedure, determination of the fitness of that donor, and a consent form must be checked for the necessary content and signatures. Any lack of documentation or incorrect information should result in a temporary suspension of the procedure until the matter is resolved.

The donor must also be reminded about the donation procedure, possible symptoms related to the procedure, and any inquiries should be clarified before starting the procedure.

After initial checks, the procedure should take place according to the guidelines provided by the manufacturer of the equipment used for the collection of lymphocytes and the final liquid balance must be as close to zero as possible, and the positive balance must not exceed the limit of 20% of the total blood volume of the patient[4].

Anticoagulation:

For the apheresis procedure, anticoagulation is a primordial process and ensures the quality of the collected product. In general, a 1:10 to 1:12 rate of citrate is sufficient to promote adequate anticoagulation. Typically, with this anticoagulant ratio, electrolyte replacement is not required if processed up to 2 times the total blood volume. If a lower ratio is required, which results in greater use of citrate, the processing of higher blood volume, or in children (especially those below 20 kgs) prophylactic calcium replacement may be recommended and should be part of the protocol developed by the service.

Many laboratories manufacturing cell therapy products discourage the use of heparin as it can interfere with cell culture, therefore, anticoagulation should be discussed with the laboratory that will manufacture in cases of patients with hypersensitivity to citrate, or patients with severe renal or liver failure.

Collection objectives:

The coloration of the collected product should be light salmon, with a hematocrit expected between 2-3%[7].

The minimum and ideal cellularity for collection vary according to the laboratory that will do the manufacturing, however, minimum cellularity of $0.5x10^9$ CD3+ cells and target cellularity of $2x10e^9$ meet most of the available manufacturing protocols.

And at the end of the apheresis procedure, it must be ensured that the bag containing the collected product is correctly identified. Moreover, some laboratories request that milking pliers not be used in the bag segment after sealing and separating the apheresis kit.

Records:

Always pay attention to ensure that all data related to the procedure were carefully and adequately recorded on its form, or on an electronic system containing, at a minimum, the donor`s identifiers, bag number, date, and time of start and end of the procedure, and the volume of the collected product. An example worksheet where the procedure data is recorded can be viewed in Annex 1.

CRITICAL POINTS AND PROCESS RISKS

Adequate evaluation of venous access so that the central access is not indicated beyond what is necessary, but also not running the risk of starting the procedure with peripheral access, which can prove to be inadequate.

- Correct identification of the collection bag.

- Risk of secondary hydro-electrolytic disorders in the use of citrate-based anticoagulants.

- Risk of anemia and thrombocytopenia, during and after the procedure.

- Risk of hypothermia, due to extracorporeal circulation in young children.

STANDARD OF PRACTICE

The instituition must determine its efficiency goal according to the equipment used, as well must define goals in relation to the number of procedures necessary to achieve the collection objective.

The rates of serious adverse events (grade 3-4, classified according to CTC-NCI) should be lower than 1% of the procedures.

PERIODICITY OF TRAINING OR ASSESSMENT OF COMPETENCIES

The instituition should define the basic competencies for hiring the professional who will work in the apheresis sector. After hiring, it is necessary to define a procedure that includes the initial training and the methodology in the preparation of a professional to perform the function. Furthermore, the service quality program must define a regular training and upgrading program.

The professional qualified to perform the apheresis procedure must be a trained healthcare professional that is qualified to do this task, due to the frequent need for manipulation of central venous catheters. Preference should be given to nursing professionals, but such training is not mandatory.

We suggest the following steps in the initial training:

Observation of at least 2 collections by apheresis after reading the service's POP, followed by a written assessment to record the effectiveness of the training.

Performance of 3 to 5 procedures under supervision, and if the professional and supervisor are sure that the professional is fit, free to work on their own.

Training routine should be defined, at least annually with a recorded or registered participation of professionals.

SUGGESTED QUALITY INDICATORS

Collection efficiency

Average number of procedures per patient to reach the collection target

Rate of procedure-related adverse events

ANNEXES

Annex 1. Example of worksheet for recording the cell collection procedure by apheresis.

GRUPO GESTOR EM SERVIÇOS DE HEMOTERAPIA

FORMULÁRIO DE COLETA DE CÉLULAS POR AFÉRESE

Local de Coleta: _____ Data:___/___/_____ Nº Sessões:_____
Início:____:____ Temperatura Ambiente:_____°C Umidade Ambiente: _____
Término:____:____ Temperatura Ambiente:_____°C Umidade Ambiente: _____

Tipo de Coleta: ( ) Autólogo ( ) Alogênico

Se Alogênico:
( ) Aparentado ( ) Não Aparentado
Nome do Receptor:_____
Data de Nascimento:___/___/___ Peso:_____ Altura:_____Sexo:_____ABO/RH:_____
Diagnóstico/Indicação:_____

1. Dados do Doador

Nome: _____
Atendimento:_____ Leito:_____ Data de nascimento:___/___/_____
Convênio:_____ Nº: _____
Sexo:_____ Peso:_____ Altura:_____ VST: _____ ABO/RH:_____
Diagnóstico/Indicação:_____
Dupla Checagem na Identificação do Doador? ( ) Sim ( ) Não Responsáveis: _____
Acesso Venoso: ( ) Periférico ( ) Central Tipo:_____
Se Cateter: ( ) Apto ( ) Não Apto Checado por: _____

2. Exames Laboratoriais

Data dos Exames: ___/___/_____ CD34+/CD3+ Pré:_____ %:_____ Ht:_____
Hb:_____ Leucócitos: _____ Plaquetas:_____ Cai:_____ K:_____Mg:_____
Uréia:_____ Creatinina:_____ Outros:_____

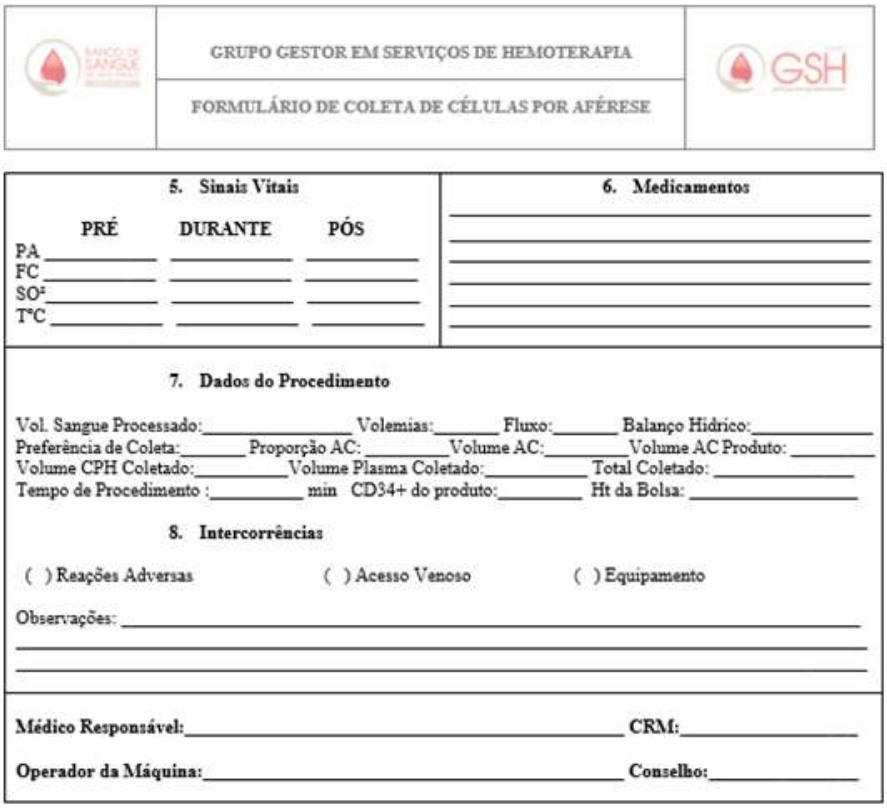
3. Mobilização

G-CSF Data de início:___/___/_____ QT Prévia: ( ) Não ( ) Sim - Data de início: ___/___/_____
Plerixafor: ( ) Não ( ) Sim – Data de início: ___/___/_____
Observação:_____

4. Materiais e Equipamentos

Equipamento: ( ) MCS+ ( ) Cobe Spectra ( ) Optia
Nº do Equipamento:_____Higienizado em:___/___/_____Responsável:_____

Kit:_____ Fabricante: _____ Lote: _____Validade:_____
Anticoagulante Fabricante:_____Lote:_____Validade:_____
Soro Fisiológico Fabricante:_____ Lote: _____Validade:_____
Bolsa Térmica – Identificação:_____Higienizada em: ___/___/_____Responsável: _____



GRUPO GESTOR EM SERVIÇOS DE HEMOTERAPIA

FORMULÁRIO DE COLETA DE CÉLULAS POR AFÉRESE

5. Sinais Vitais

	PRÉ	DURANTE	PÓS
PA			
FC			
SO²			
T°C			

6. Medicamentos

7. Dados do Procedimento

Vol. Sangue Processado:_____ Volemias:_____ Fluxo:_____ Balanço Hídrico:_____
Preferência de Coleta:_____ Proporção AC: _____Volume AC:_____Volume AC Produto: _____
Volume CPH Coletado:_____Volume Plasma Coletado:_____ Total Coletado: _____
Tempo de Procedimento :_____ min CD34+ do produto:_____ Ht da Bolsa: _____

8. Intercorrências

( ) Reações Adversas ( ) Acesso Venoso ( ) Equipamento

Observações: _____

Médico Responsável:_____ **CRM:**_____

Operador da Máquina:_____ **Conselho:**_____

Annex 2. Main models of apheresis equipment and their performance characteristics (adapted from Cellular Therapy: Principles, Methods, and Regulations)[4]

Equipament	Collection process	Extraction flow rate (mL/min)	Extracorporeal Volume	Platelet reduction after procedure (%)	Average collection efficiency for MNC (%)
Spectra Optia (Terumo BCT)(11)	Continuous blood flow, continuous collection	40-80	191mL*	13-42%	60%
COM.TEC (Fresenius)	Continuous blood flow, cyclic collection	NI	130mL	22-50%	21,4%
Haemonetics (MCS)	Intermittent blood flow, cyclic collection	20-30	480mL	39-46%	NI

* Typical extracorporeal volume147mL, can reach 191mL
NI: not informed by the manufacturer

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CAR-T CELL PRODUCTION

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ABSTRACT

The treatment of patients affected by hematologic neoplasms with autologous T cells expressing a chimeric antigen receptor (CAR) is one of the most promising adoptive cellular therapy approaches. Reproducible manufacturing of high-quality, clinical-grade CAR-T cell products is a prerequisite for the wide application of this technology. Product quality needs to be built-in within every step of the manufacturing process, including the choice of the vectors to modify T cells, such as viral vectors: lentiviral or gamma-retrovirus, and non-viral vectors, especially those based on transposons. Additionally, the CAR-T cell quality control must be in accordance to local Regulatory prior to infusion. Herein we summarize the state of art manufacturing platforms available. CAR-T cell therapy may be on the verge of becoming standard of care for a few clinical indications. Challenges in the manufacturing standardization and product characterization remain to be overcome in order to achieve broad usage and eventual commercialization of this therapeutic modality.

Keywords: Immunotherapy, Adoptive.

AIMS

Describe different techniques for the production of CAR-T cells, using viral vectors or non-viral techniques.

INTRODUCTION

Cell therapy with T cells expressing chimeric antigen receptor (CAR-T) is a type of immunotherapy that involves the manipulation and reprogramming of immune cells (T lymphocytes) in order to recognize and kill tumor cells. In its classic configuration, CAR represents a monoclonal antibody fragment, called a single-chain variable fragment (scFv), which resides in the extracellular portion of the T cell membrane and guides the cell to its target antigen. The scFv is linked to a loop, followed by a transmembrane portion and this portion, which to intracellular signaling domains[1,2] . Once the CAR-T cell connects with the antigen present in the target cell, the stimulatory molecules provide the necessary signals for the cell to become fully activated. T cells can effectively proliferate and attack cancer cells[3-5].

In most clinical studies and commercial CAR-T cell products, the cells are genetically modified using viral vectors. The viral vectors commonly used for the production of CAR-T cells are lentiviral or gamma-retrovirus derivatives. The viral vector is used to deliver a gene specifically, with high efficiency and, in this way, permanently integrate the transferred DNA into the genome allowing for long-term gene expression[6]. When a viral vector is used to modify the cellular genome for gene therapy, this vector must not induce allergic reactions or severe inflam-

matory processes, still fulfilling its therapeutic role, whether it exacerbates normal functions, corrects deficiencies, or inhibits deleterious activities (Table 1). Despite the profile of random integration and risk of mutagenesis, the use of viral vectors for the production of CAR-T lymphocytes has been shown to be safe[7,8]. These therapeutic vectors must be produced under Good Manufacturing Practices (GMP) conditions, purified in large quantities and high concentrations to be available on a large scale, which results in high production costs[9-11]. The vector must still be safe not only for the patient but also for the handler in the production process.

Despite the wide use of gamma-retroviral and lentiviral vectors for the manufacturing of CAR-T cells, there is a recent trend towards developing of non-viral vectors, especially those based on transposons[6]. Non-viral vectors generally consist of non-infectious DNA fragments that are carried into the target cell through physical methods, such as electroporations or microinjections, or chemically, through transfection reagents. Due to the low efficiency of lipofection protocols in T lymphocytes, the method of choice has been electroporation. The most used non-viral methods for the generation of CAR-T cells include Sleeping Beauty[12-14] and PiggyBac[15] transposons. Both have already had clinical use, in addition to the pre-clinical development carried out by different groups[16-18]. In general, gene delivery consists of transposons that carry the transgene of interest (in this case, CAR) in the form of DNA (either as plasmid[16] or minicircles[19]). The minicircles-based engineering CAR represents an advantage, since smaller masses of DNA are necessary for the delivery of the transgene, reducing the toxicity resulted from electroporation at high concentrations of DNA. The transposase can be delivered in the form of plasmid DNA, mini-circle, mRNA, or protein[6]. Following the same logic, decreased toxicities are found when using mini-circles and mRNA. The recent development of a transposase with greater solubility and potential for penetration into cells suggests that the efficiency of the system can be improved[20].

Although transposon-based systems present a genomic integration pattern considered to be less prone to insertional mutagenesis than that found in viral vectors, and Sleeping Beauty-based protocols have recently presented relevant clinical results with cells expressing anti-CD19 CARs, a clinical protocol based on PiggyBac carrying anti-CD19 CARs led to the transformation of CAR+ T cells causing the development of lymphomas in 2 of 10 treated patients[21]. This recent result was accompanied by antitumor response in 5 patients, demonstrating the potential for CAR-T cell response accompanied by a risk of T cell transformation not previously observed in any CAR-T cell generation protocol. The transformation mechanism and its potential association with the PiggyBac system are still under investigation[22]. No similar adverse events have been reported yet in protocols, including transposons Sleeping Beauty or PiggyBacs.

TABLE 1 - Viral vector used gene therapy and CAR-T cell manufacturing

	Retrovirus	Lentivirus	Herpes Vírus	Adenovirus	Adeno associated	Plasmid
Provírus	RNA	RNA	RNA	DNA	DNA	DNA
Capacity	~ 9 kB	~ 10 kB	> 30 kB	~ 30 kB	~ 4,6 kB	No limit
Integration into host genome	Yes	Yes	Yes	No	Rare	No
Time of transgene expression.	Long	Long	Transient	Transient	Long in post mitotic cells	Transient
Pre existing immunity	No	No	Yes	Yes	Yes	No
Adverse effects	Insertional Mutagenesis	Insertional Mutagenesis	Inflammatory response	Inflammatory response	Slighty Inflammatory response	No
Germline transmission	Possible	Yes	No	No	Possible	No
Applications	CAR-T, gene therapy as Gaucher, Fanconi anemia, B hemophilia	CAR-T	Neuronal diseases	Cystic fibrosis	Sickle cell disease	Cloning and protein isolation

Adapted from Misra S. Human gene therapy: a brief overview of the genetic revolution. J Assoc Physicians India. 2013;61(2):127-33 (9)

PROCEDURE DESCRIPTION

Indication: patients eligible for treatment who consented to participate in a clinical study case. In the production of CAR-T cells for clinical use, the service responsible for manufacturing must present official approval regarding technical standards. For clinical research situations, all steps involving ethical evaluation should be approved by CEP/CONEP and safety and quality should be approved by ANVISA and CTNBio agencies.

Contraindication: patients not eligible for treatment with CAR-T cells and lack of approval and regularization with the official norms and current techniques for the production of CAR-T cells.

HUMAN RESOURCES

It is recommended that the procedures should be carried out in a GMP environment or equivalent approved according to the legislation and with the leadership of multidisciplinary teams with proven ability in the manufacture of CAR-T cells.

MINIMAL REQUIREMENTS TO THE PROCEDURE

Lymphocytes collected by leukapheresis or blood collection are used as starting material. The collection from patients in the good clinical condition is recommended, with negative test for infectious agents such as C, C/II or B hepatitis, HIV, NAT, anti-HTLVI II, HBsAG, HBV NAT, anti-HCV, NAT for HCV, serological test for syphilis, antibody anti-*T. cruzi* for non-reactive Chagas disease and no signs of infection in the last 24 hours before collection.

Additionally, a checklist of all materials and reagents is suggested to perform the procedure.

CRITICAL POINTS AND RISKS

The manufacturing process of CAR-T cells must comply with good practices using human cells established by Resolution RDC number 508, of May 27, 2021, as described in the Regulation chapter of this Manual (ANVISA, RDC N° 508, 2021).

Reagents for the manufacturing process must be classified as GMP or for clinical use. Exceptions may be accepted by regulatory agencies for selected reagents under justification and risk minimization in reagent selection.

For manufacturing in closed and automated cellular processing systems, such as the CliniMACS Prodigy equipment, the critical points and risks are: inspection and proper handling of the equipment, identification and fixation of bubble and pressure sensors, fixation of the temperature control unit and the cell culture chamber.

STANDARD OPERATING PROCEDURE

Transduction efficiency, represented by the percentage of CAR positive T lymphocytes, generally identified by flow cytometry, can be used as a standard operating procedure. The median transduction of the product consisting of anti-CD19 CAR-T cells, such as tisagenlecleucel, when manufactured from cells from patients diagnosed with acute lymphocytic leukemia, is around 23% (5%-56%)[23]. When manufacturing in the automated Prodigy system is used, mean transduction of 30.6% ± 13.44[24] and a median of 46.88% (29.02–61.09%)[25] is observed.

The manufacturing failure rate can also be considered a parameter to be evaluated in the standard operating procedure. Studies have shown manufacturing failure of CAR-T cells around 1 to 7%[26-28].

MATERIAL

Biological safety cabinet, human recombinant cytokines, culture medium, reagent for activating T lymphocytes, syringes, human AB serum, or defined chemical alternative. For manufacture on CliniMACS Prodigy: MACSQuant Flow Cytometer, CliniMACS Prodigy/TCT Software, CliniMACS Prodigy Tubing Set.

PROCEDURE

Autologous cells collected from peripheral blood or leukapheresis are the most used starting materials for manufacturing of CAR-T cells. Leukapheresis is the preferred technique in cases of lymphopenia or high tumor burden with low lymphocyte numbers[29,30]. Lymphocytes can be cryopreserved for shipment to the specialized manufacturing center or can be used as fresh starting material.

Some protocols establish the separation of lymphomononuclear cells by density gradient or elutriation, as well as enrichment of T lymphocytes or selection of subpopulations of T lymphocytes, such as CD4 and CD8, with the use of conjugated antibodies associated with magnetic beads for positive or negative selection[31,32]. These processes improve product purity and yield. T lymphocytes are then activated using reagents such as: anti-CD3 or anti-CD3/an-

ti-CD28 antibodies, antibodies linked to paramagnetic beads, reagents such as Transact, which uses anti-CD3 and anti-CD28 antibodies conjugated to a colloid of polymeric nanomatrix or via hydrogel "stimulation matrix" incorporating antibodies[33-35]. After the activation process, the T cells are genetically modified in a process known as transduction. In manufacturing protocols using viral vectors, the quality, the titer of the vectors, and their rapid use after thawing are determining factors for the efficiency of transduction, including high expression of CAR in T lymphocytes. Reagents such as protamine, retronectin, poly-L-lysine, and vectofusin can be used to facilitate this process[33,34,36].

CAR-T cells can be cultivated in culture medium containing recombinant human cytokines alone or in combinations (such as IL-2, IL-7 and/or IL-15) in an open system such as flasks known as G-REX bioreactors or by closed system cell expansion system such as Xuri, Wave or Quantum[33,37,38]. Currently, the production of CAR-T cells has become more automated with the development and implementation of new equipment that can perform an increasingly diverse set of functions, such as the CliniMACS Prodigy equipment, marketed by the company Miltenyi Biotec or the Cocoon device, provided by the company Lonza. Both systems operate in a closed and automated system, indicated for cell separation, T cell activation, transduction, expansion and cell collection. The equipment also records parameters at all stages of production, ensuring process traceability[24,39-41].

Although the generation of CAR-T cells with non-viral vectors generally follows the same standards of cell isolation, genetic modification, *in vitro* activation, and cell expansion in the laboratory, the use of non-viral systems allows T cells to be modified genetically without requiring prior activation. This feature allows ultra-fast CAR-T cell generation protocols to be generated through the infusion of freshly electroporated cells. Some initiatives in this direction describe CAR-T cells generated in less than 24h with antitumor potential demonstrated in preclinical models[42,43]. The automated systems mentioned in this chapter have the possibility of coupling electroporators, which allows the generation of T cells with non-viral platforms in these systems, as well as gene editing protocols with systems such as CRISPR/Cas9.

After the manufacture of CAR-T cells, the product is submitted to the quality control process, prior to infusion or cryopreservation, following the the National Health Surveillance Agency (ANVISA - RDC 506/2021 and 508/2021). The expected results are as follow and must be in the corresponding release document (Supplementary file):

1) High viability (above 70% is suggested - from flow cytometry analysis or trypan blue count of the transduced product)

2) Endotoxin: ≤ 5 EU/kg

3) Detection of microorganisms in the final product: no growth of bacteria or fungi after 14 days of incubation

4) Gram stain (for fresh infusion): absence of visualization of microorganisms

5) qPCR for mycoplasma: negative

6) VSVG and GAG qPCR for viral vector replication assessment: negative

7) Dose of CAR-T cells/kg: per cohort (indicated in processing and infusion orders)

8) Potency assay confirming tumor cell death induced by CAR-T cells

9) Absence of karyotypic changes

CAR-T cells can be infused fresh or after cryopreservation. The most used cryopreservation protocols include 5-10% dimethyl sulfoxide, human albumin, and plasmalyte followed by storage in vapor-phase liquid nitrogen (44), as described in the freezing of lymphocytes after leukapheresis, at the time of pre-manufacturing. Several studies demonstrate no significant differences in T cell percentage, transduction efficiency, and CD4:CD8 lymphocyte ratios pre- and post-cryopreservation and thawing[45,46].

FREQUENCY OF TRAINING OR COMPETENCE ASSESSMENT

Frequent training and competency assessment is suggested for the manufacturing process and product quality control.

QUALITY INDICATORS

The efficiency of T cell transduction by the vector of choice, potency test confirming CAR-T-induced cytotoxicity of target cells, and absence of contaminants for clinical infusion can be used as indicators.

SUPPLEMENTARY FILE
CAR-T CELLS MANUFACTURING

1.0 General Information	
Study:	Patient ID:
Patient Weight:	Dose: kg
Initial day of culture: / /	Time of culture: days
Performed by:	Checked by:

2.0 Product reception and verification		
2.1 Time and temperature details upon receiving the product		
Time:	Transportation temperature: °C	Overnight: ( ) Yes ( ) No
Temperature overnight: °C	Location overnight:	
Confirm if the product concentration is ≤ $2x10^8$/mL for overnight storage		
Show calculation to adjust the concentration (if necessary)		
Date:	Initials:	
Performed by:	Checked by:	

3.0 Preparation of reagents and samples	
3.1 Culture medium preparation (48h in advance)	
3.1.1	
Calculation:	
Date:	Initials:
:	
24 hours:	48 hours:
3.2 Buffer Preparation (24h to 48h in advance)	
3.2.1	
3.2.2	Name this buffer as: Processing buffer
Calculation:	

Date:	Initials:	
3.3	Getting the starting material samples	
3.3.1	Remove 0.5 mL for cell count	
3.3.2	Remove 2x10^6 cells for flow cytometry analysis	
Calculation (if necessary):		
Performed by:	Checked by:	

4.0 Pre-processing			
4.1	Leukapheresis volume: mL		
4.2	Calculation of starting material		
4.2.1.	Leucocyte count (WBC): x10^6/mL		
4.2.2	_____ x10^6/mL x _____ mL= WBC x10^6/mL (Item 4.2.1) Volume (Item 4.1)	_____x10^9 Total # WBC	
	Note: Starting material should not be more than 20x10^9 leucocytes (WBCs)		
Calculation (if necessary):			
4.3	Flow Cytometry analysis		
4.3.1	Markers: CD4+, CD8+, and CD4+/CD8+	%	
4.3.2	_____ _____ _x_____ _____ ____= Total WBC (Item 4.2.2) Stained cells (Item 4.3.1)	_____x10^9 Total stained cells	
	Note: Material should not exceed 3x10^9 CD4+ and CD8+ cells		
Checked by:		Initials:	
Comments (if necessary):			
Performed by:	Checked by:		

5.0 Fresh infusion			
5.1	**Only for fresh infusion:**		
5.1.1	Remove adequate volume for release tests (endotoxin, Gram, sterility)		
	_____mL x _____1_____ = _____mL mL from final product (Item 4.1) number of doses **Total volume**		
5.1.2	An additional amount of $20x10^6$ WBC must be added prior to final formulation for release testing.		
	$20x10^6$ WBC / _____10^6/mL = _____mL Correct WBC /mL (Item 4.2.2) Additional volume to be pipetted		
5.1.3	CAR-T cell total volume for final formulation		
	_____mL + _____/mL = _____mL (Item 5.1.1) (Item 5.1.2)		
5.1.4	Adjust WBC concentration to 4-$8x10^6$ WBC/mL		
	(_____x10^6/mL x _____/mL) / _____x10^6 = _____mL Corrected WBC /mL (Item 5.1.2) Final product total volume (Item 5.1.3) Concentration (4-$8x10^6$) Formulation total volume		
5.1.5	Calculate the final volume for distribution and infusion. Remove the volume needed for one dose, send the remainder release tests.		
	_____mL - _____mL = _____mL Formulation total volume (Item 5.1.4) Volume for release tests (Item 4.2.3) Final volume for infusion		
5.2	Take a note from date, time and final volume from formulated final product		
	Date: _____/_____/_____	Time:_____	Volume:_____mL
	Expiration date:	Expiration time:	
Date:		Initials:	
Performed by:		Checked by:	

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PERIPHERAL BLOOD MONONUCLEAR CELLS CRYOPRESERVATION AND TRANSPORTATION

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ABSTRACT

Chimeric antigen receptor-T (CAR-T) cells have emerged as a treatment option for patients with hematologic malignancies. Usually, their manufacture has been carried out by specialized and centralized laboratories. A useful strategy, which allows a better logistical organization, is the peripheral blood mononuclear cells (PBMC) cryopreservation and transportation. This article aims to discuss the most relevant points related to the PBMC cryopreservation and transportation process. In addition, it proposes a kind of standard operational protocol (SOP) that can be very helpful for the cell processing labs.

Keywords: Immunotherapy, Adoptive. Leukocytes, Mononuclear. Cryopreservation. Transportation.

OBJECTIVES:

Describe the steps for the peripheral blood mononuclear cells cryopreservation procedure to obtain the maximum recovery of viable cells.

Describe the steps of the cryopreserved product transportation and shipping.

INTRODUCTION

Chimeric antigen receptor-T (CAR-T) cells are an option for patients with advanced hematological neoplasms.[1] Born from academic research, the manufacture of CAR-T cells is currently carried out chiefly by pharmaceutical industries. Because the production of CAR-T cells is complex and expensive, companies centralize patients' cell manufacture in a few specialized laboratories, allowing cell harvesting in different countries. One strategy to reduce viability loss in patients' leukapheresis products is cryopreservation prior to transportation.[1]

Logistics is the main advantage of cryopreservation. It allows the collection of peripheral blood mononuclear cells (PBMC) in patients' best clinical condition, better planning of transportation, and manufacture into CAR-T cells.[1, 2] Therefore, some companies chose to cryopreserve cell products over fresh products to manufacture CAR-T cells.[2] In this sense, several cell processing labs have been organized to be part of this chain of custody, maintaining product quality and traceability at all stages of the process.[3-5]

Few studies address PBMC cryopreservation.[6-8] Most services use protocols similar to peripheral blood hematopoietic stem cells (PBSC) cryopreservation. This recommendation paper will address the most relevant aspects of the PBMC cryopreservation and their preparation for transportation to centralized CAR-T cell manufacturing facilities. For a comprehensive review on cryopreservation, we suggest De Santis *et al.* and Meneghel *et al.* [9, 10]

FACTORS THAT INFLUENCE THE CELL PRESERVATION

Product volume:

The product volume collected is usually estimated by the apheresis equipment, but it must always be measured in the laboratory by weighing the product's bag. Some services convert from grams to milliliters considering a 1:1 ratio. Some prefer to use the monocytes (1.062 g/mL) or lymphocytes (1.07 g/mL) specific density to perform this calculation.[11, 12]

Volume reduction:

Some products require plasma excess removal to adjust the nucleated cells (NC) concentration. The product is centrifuged and the plasma excess extracted into a transfer bag. Each service must validate the centrifugation parameters: gravitational force (g), time, and temperature. These parameters usually vary between 400 and 1000 g, for 10 to 15 minutes, at refrigerated temperature (2 to 8º C), respectively.[13, 14] Some services use the 1:1 ratio for converting grams to milliliters of the plasma amount to be removed. Others prefer to use the specific plasma density (1.026 g/mL) in this calculation.[12]

To minimize the rupture risk during centrifugation, it is crucial to transfer the product from the original collection bag to a transfer bag that can be centrifuged. Each service must define and validate its maximum volume (usually less than 2/3 of the bag's nominal volume).

Nucleated cell concentration

There is no consensus on the ideal nucleated cells concentration (density) for PBMC cryopreservation. Many centers cryopreserve PBMC in vials, with the final concentration varying between 2 and 100 x 10^6 cells/mL.[6, 7, 13] Stroncek *et al.* described PBMC cryopreservation in bags, with concentrations varying from 20 to 300 x 10^6 cells/mL.[7] The main advantages of concentrated products are reducing dimethyl sulfoxide (DMSO) dose (less DMSO toxicity), cost, and storage space utilization[15]. However, too high cell concentration can increase the risk of clumping during the cryopreservation and/or during the thawing and infusion period.[13]

Cryopreservation solution

Cryoprotective agents reduce cell dehydration and mechanical injury induced by the cryopreservation process, maintaining cell viability.[9] The most widespread PBMC cryopreservation solution consists of a protein solution (autologous plasma or human albumin) with 20% DMSO. When combined with an equal volume of cell suspension, it results in a final concentration of 10% DMSO.[6] Reported alternative solutions are 10% DMSO in Plasmalyte®,[16] and 5% DMSO, 6% hydroxyethyl starch (HES) derivatives, 4% human albumin final concentrations.[7] For PBSC cryopreservation, the EBMT (European Society for Blood and Marrow Transplantation) recommends the ACD-A association with the cryopreservation solution at a dose of 0.05 to 0.25 mL per product mL in order to reduce the risk of clumps.[17]

Cryopreservation solutions are hypertonic, and the osmotic stress caused by its introduction into the recipient might induce cell death. Moreover, DMSO is toxic (biochemical toxicity), and DMSO exposure time at room temperature further contributes to cell loss.[18] To reduce the osmotic stress, the cryopreservation solution must be added into the cells' recipient, never the other way around. This process must be refrigerated, systematic, and standardized. Furthermore, it is mandatory to have strict control over the cryopreservation solution addition time and the interval between the completion of it and the initial freezing time.[3] Each service must validate these parameters. Specific data concerning these intervals are lacking, but the ones used in the PBSC cryopreservation can be used as a reference in the validation.[14]

Cryopreservation velocity

The gold standard for PBMC freezing is using equipment programmed to freeze bags at 1º C per minute decay,[3, 6, 18] similar to PBSC.[17] However, passive freezing in mechanical freezers (minus 80º C) is an option.[8] In both cases, the service must validate the process. Physical factors, such as bag configuration, product volume, cell concentration, storage cassette, bag's direct contact with the freezer or on polystyrene layer, may interfere with the cryopreservation product speed and must be considered in the process validation.[9, 19]

To carry out the programmed freezing, one must configure the equipment beforehand, following literature data,[20] service validation, or protocol provided by the CAR-T cell manufacturing service. **Table 1** provides data that can be used as an initial reference for process validation.

When placing the bags in the programmed freezing equipment, it is crucial to observe the placement of the temperature probe. The probe must be in contact with the central region of one among the product bags to be cryopreserved or inside a bag containing cryopreservation solution (periodically changed). The probe must not be placed in the label pocket or in contact with the air.[20]

TABLE 1: PBSC freezing curve example[20]

Step	Rate	Target temperature	Location
1	Wait	0º C	Chamber
2	1º C/minute	- 4º C	Sample
3	25º C/minute	- 55º C	Chamber
4	15º C/minute	- 24º C	Chamber
5	1º C/minute	- 45º C	Chamber
6	2º C/minute	- 80º C	Chamber
7	End		

STORAGE FOR CLINICAL USE

Ideally, PBMC should be stored at temperatures below minus 150º C, in tanks containing liquid or, preferably, steam nitrogen,[3, 7, 18, 21, 22] similar to the EBMT recommendations for PBSC storage.[17] Tanks containing liquid nitrogen (LN_2) seem to be safer in terms of temperature stability.[19] However, additional care is required to avoid cross-contamination between products.[23, 24] For this reason, some industries have demanded the storage of PBMC intended for CAR-T cells in nitrogen steam.

The mandatory criteria to be observed in the product cryopreservation process must be defined following the CAR-T cells manufacture's protocol, together with additional criteria established by each cell processing lab. For example, a) the maximum cell concentration for product overnight storage between the completion of the procurement and the initial of the cryopreservation process; b) the maximum interval between the completion of the procurement and the initial of the cryopreservation process; c) the minimum dose of CD3+ cells to be cryopreserved; d) the maximum concentration of nucleated cells/mL in the final cryopreserved product; e) the cryopreservation solution to be used; f) the freezing method; f) the storage freezer type until transport; g) pre and post-cryopreservation quality control tests and their respective reference values.

TRANSPORTATION AND SHIPPING

The transport of cryopreserved products must be carried out in dry shippers. These containers must comply with local, sender, and receiver regulations.[25]

Dry shippers must be supplied following the manufacturer's guidelines. This process may take longer than a day, as more than one filling cycle may be required according to the type of dry shipper. When properly loaded, they are capable of maintaining the appropriate temperature for transport (below minus 150º C) for 5 to 15 days.[5, 25]

The dry shipper temperature must be monitored continuously throughout the transport. For this purpose, previously validated and calibrated temperature monitors (data loggers) that allow data monitoring printing should be used.[5, 25]

Each service must validate the transport procedure. Dry shippers must be requalified and inspected at regular intervals, at least a visual check, at each procedure.[25]

INDICATION AND CONTRAINDICATIONS

- Patients presented at a multidisciplinary meeting and approved for CAR-T cells treatment, with a medical prescription for PBMC cryopreservation.
- Suitability and eligibility documented duly signed by the responsible medical team.
- No contraindication for this protocol
- Signed informed consent.

MINIMAL REQUIREMENTS

- Medical prescription for the PBMC cryopreservation.
- Initial training of all employees involved in the PBMC cryopreservation and transportation. The annual competence of all staff is required.
- Product cryopreservation forms and freezing

curves approved by the Cell Processing Lab Director and Quality Manager.

- Product release report approved by the Cell Processing Lab Director and Quality Manager.
- In case of exceptional release – report and release approved by Cell Processing Lab Medical Director.
- Cryopreservation and transportation process validated.
- Qualified, calibrated and cleaned equipment's.
- Critical material inspected and approved for use.

PROCEDURE

- Equipment's
- Balance
- Biological safety cabin
- Cryogloves
- Data logger for temperature monitoring
- Dry shipper
- LN_2 source
- Nitrogen tank for storage
- Plasma extractor
- Programmable controlled-rate freezer with a probe
- Refrigerated centrifuge
- Refrigerator
- Sterile connection device
- Tubing sealer

SUPPLIES AND REAGENTS

- Assay sample tubes
- Blood culture medium
- Cryogenic tubes
- Disposable needles
- Cryobags
- Cryopreservation solution with DMSO
- Labels
- Reusable refrigerated ice brick
- Sampling-site couplers
- Sterile syringes
- Storage cassettes
- Transfer bags
- Tube stripper

CRYOPRESERVATION PROCEDURE:

- Programmable controlled-rate freezer (CRF) equipment preparation:

  - Confirm that the CRF equipment is clean.
  - Check the CRF LN_2 source for sufficient LN_2 to perform the entire freezing procedure.
  - Turn on the equipment and verify if it is working correctly.
  - Ensure the dry shipper is loaded without nitrogen residue inside.

- Start filling in the forms to maintain the traceability of the entire process, which includes equipment, supplies, and reagents used, critical calculations, and verification of product identification by two people or one person and one computerized system. Where available, reference values of critical data and formulas for calculations should be included in the forms to minimize the risk of errors.

- Ensure that the ice brick that will be used to maintain the product temperature during the cryopreservation solution addition is in the refrigerator as previously validated.

- Collect samples for pre-processing quality control testing:
  - Properly homogenize the product, as previously systematized.
  - Proceed with collecting the minimum volume necessary to perform tests (relevant cell counts, cell viability, and other tests according to the service routine or manufacturer's protocol), inside the biological safety cabinet (BSC).

- Determine the initial product volume:

  - Properly tare the balance and then weigh the product.
  - Calculate the initial product volume by dividing its weight by its density or use the 1:1 ratio, as previously defined in the institutional or manufacturer's protocol.

- Determine the cell concentration:

  - Check the NC count on the complete cell blood count (CBC). Remember $10^3/\mu L = 10^6/mL$.
  - Calculate the total nucleated cells (TNC) number of the product using the following formula:

TNC = initial product volume (mL) x NC x 10^6/mL.

  - Check the desired NC/mL concentration, as pre-

viously defined in the institutional or manufacturer's protocol.

- Calculate the cell concentrate final volume using the following formula:

$$\text{Cell concentrate volume (mL)} = \frac{\text{total nucleated cells}}{\text{NC concentration/mL}}$$

- Check the maximum product volume of each cryobag.

- Calculate the number of cryobags to be used.

- Prepare the cryopreservation solution:

- The solution volume to be prepared will be the same as the cell concentrate to be cryopreserved.

- Check the previously validated cryopreservation solution.

- Calculate the amount of each reagent, maintaining the ratio of the standard solution to the target volume to be prepared.

- Add the reagents to a previously identified transfer bag using a sampling-site coupler, syringe, and needle. This process must be carried out inside the BSC.

- Place the bag containing the cryopreservation solution in the refrigerator for temperature stabilization as previously defined in the institutional or manufacturer's protocol.

- When relevant, proceed with the excess plasma removal from the product for cell concentration.

- Calculate the plasma volume to be removed using the following formula:

Plasma volume to be removed = initial product volume – cell concentrate volume

- Calculate the plasma bag weight to be removed by multiplying its volume by the plasma density or using the 1:1 ratio, as previously defined in the institutional or manufacturer's protocol.

- Make a sterile connection between the collection bag and a previously identified transfer bag.

- Drain the product to the bag where it will be centrifuged, respecting its maximum volume, as previously defined in the institutional or manufacturer's protocol. If necessary, use more than one transfer bag.

- Centrifuge the product as previously defined in the institutional or manufacturer's protocol.

- Carefully place the product in the plasma extractor. Place the empty bag on the balance (previously positioned next to the extractor) and tare it.

- Remove the predetermined plasma volume with care. Do not lose cells.

- Heat-seal and separate the bags.

- Weigh the product and the plasma bags and register these data.

- Calculate the product volume by dividing its weight by its density or use the 1:1 ratio, as previously defined in the institutional or manufacturer's protocol.

- Carefully homogenize the product.

- Inside the BSC, collect the minimum necessary product to perform the quality control tests, as previously defined in the institutional or manufacturer's protocol.

- Place the product bag into the refrigerator to stabilize the temperature as previously defined in the institutional or manufacturer's protocol.

According to the service routine, calculate the cell recovery after the plasma extraction.

- Freezing

- Identify and double check all labels used to identify bags, segments, cryovials, and storage cassettes.

- Confirm that the CRF equipment is operating, and its internal temperature is stable at 0º C.

- If necessary, resolve all intercurrences with the equipment before proceeding with the addition of the cryopreservation solution.

- Remove the ice brick from the refrigerator and place it on the local where the cryopreservation solution will be added to the cells.

- Remove the product containing the bag and the cryopreservation solution from the refrigerator.

- Make a sterile connection between the cell concentrate and the cryopreservation solution bags.

- Place the bag containing the product over the ice brick and add the cryopreservation solution to the cell concentrate as previously validated by the service. Register the addition of cryopreservation solution start and endpoint times.

- Inside a CBS, proceed with collecting the minimum product necessary to perform the quality control tests (e.g., sterility) and manufacture cryovials as previously defined in the institutional or manufacturer's protocol.

- Proceed with the division of the cell concentrate in the freezing bags.

- Remove air bubbles according to the institutional protocol.
- Seal the product segments and the cryobags.
- Place the cryobags in an overwrap bag to prevent cross-contamination.
- Proceed with positive identification of bags and cassettes.
- Place the cryobags into a labeled storage cassette.
- Place the cryobags and the cryovials into their specific place in CRF.
- Ensure the cryobags are covered by the top plate and that the nitrogen port is free.
- Correctly position the probe. The flat end needs to be against the bag and centered over the central portion of cryobags (not label pocket).
- Run freezing at programmed temperature.
- Register freezing start and endpoint times.
- Calculate the cryopreservation solution addition duration and register it.
- Calculate the time between the completion of the cryopreservation solution addition and the initial freezing time. Register it.
- These data are critical and must comply with the maximum period previously validated.
- Monitor the freezing, following the curve generated by the CRF equipment.

- Storage

  - Identify the location where the bags and cryovials will be stored.
  - Open the CRF door at the completion of freezing.
  - Remove the bags and cryovials using cryogenic gloves. Transport them in a dry shipper to the tank where they will be stored.
  - Carefully store the cells in predetermined locations.
  - Evaluate the cryopreservation curve, verify if it conforms to previously approved models.

- Final check

  - Complete the organization of the forms and forward the folder containing all the procedure data for verification by the responsible professionals

- Post-cryopreservation quality control tests

Perform post-cryopreservation product quality control tests according to the service protocol.

The minimum parameters to be evaluated are the CD3+ viability, the product identification, and the visual check of the bag.

PRODUCT TRANSPORT AND SHIPPING PROCEDURE:

- Proceed with a new verification of all forms generated in the process, including the request to send the product.

- Preparation of the dry transport container:

  - Select a dry shipper that is suitable for use and fill it with antecedence according manufacturer's guidelines.
  - Prepare the labels, including those affixed to the dry shipper cover.
  - Prepare the necessary documentation.
  - Confirm the dry shipper has been correctly loaded and LN2 excess has been removed.
  - Check the bags' and cryovials' location in the tanks.
  - Remove the bags and cryovials from the tanks and carefully place them in the dry shipper using cryogenic gloves.
  - Place polystyrene boards inside the dry shipper to stabilize the products to not move during transport.
  - Place the data logger probe inside the container, wait for the temperature stabilization, and, after that, activate the data logger.
  - Weigh the dry shipper (inside the cover) and register this data.
  - Ask the receiving service to return the transport data (the arrival temperature and the dry shipper's weight at minimum) as soon as they receive the cells.

- Dry shipper return

  - Perform visual check for external damage.
  - Keep monitoring the temperature until it reaches positive parameters.
  - Download the data logger information and print the graph.
  - Verify if the data complies with validation, including time to maintain the proper temperature for transportation.

CRITICAL POINTS AND RISKS:

- Checking of product identification by two people or one person and a computerized system upon receipt of the product, identification of documentation, at each bag exchange, making segments or cryovials, and cassette insert time. Risk: product identification and exchange error.

- Start of cryopreservation processing after 24 hours of collection. Risk: loss of cell viability with compromised quality of CAR-T cells.

- Bag breakage during centrifugation. Risk: product loss with the need for a new collection.

- Cell loss in plasma extraction. Risk: product loss with the need for a new collection.

- Quick addition of cryopreservation solution. Risk: cells heating with consequent loss of viability due to toxicity to DMSO.

- Delay in starting the freezing after cryopreservation solution addition. Risk: loss of cell viability due to DMSO toxicity.

- Liquid nitrogen supply interruption. Risk: interruption of the cryopreservation process with cell viability loss and CAR-T cells compromised quality.

- Inadequate dry shipper filling. Risks: inadvertent product heating, with loss of cell viability and CAR-T cells' quality; skin injury of people involved in transportation due to contact with LN_2; fracture of the product bag, which becomes unsuitable for CAR-T cell manufacture.

STANDARD OF PRACTICE

- Reference values for each critical process step must be established according to the validation of each service and agreement with the CAR-T cells manufacturer's protocol.

- Literature data that can guide these criteria:
  - TNC recovery after plasma extraction greater than 90%[11]
  - Cryopreserved TNC recovery: 92 ± 17%[7]
  - CD3+ cells recovery: 79 ± 19%[7]
  - Total cell viability (flow cytometry, 7AAD): 84 ± 6%[7]

QUALITY INDICATORS

- Cell loss in plasma extraction

- Cryopreserved CD3+ cell viability – test performed on segment or cryovial sample.

- Sterility test negative for aerobic, anaerobic bacteria, and fungi

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CONFLICT OF INTEREST

The authors have disclosed no conflict of interest.

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ANNEX

Receipt form of incoming cells

Patient data	Name: Date of birth: Age: Weight:
Collection data	Procurement facility name: Donation identification number: Product description code and division code: Product name and attributes Blood cell separator model & software version: Date and time of procurement: Anticoagulant used: Product anticoagulant volume
Transportation data	Date: Date and time of departure and receipt: Duration: Departure and receipt temperature: Acceptable temperature during transportation? ☐ yes ☐ no Observations: Signature and stamp:
Receipt data	Receipt data and time: Product inspection upon receipt: Usual product appearance: ☐ yes ☐ no Visible evidence of contamination: ☐ yes ☐ no Container integrity? ☐ yes ☐ no Appropriate labeling? ☐ yes ☐ no Product classification ☐ acceptance ☐ rejection ☐ quarantine Disposition ☐ cryopreservation ☐ discard Responsible person (signature and stamp):
Temporary storage	Equipment: Date and time (start/endpoint): Duration: Observations

Cryopreservation registry

	Type	Identification code	Calibration[&#]	Preventive maintenance[#]
Equipment	Balance			
	Biological safety cabin			
	Centrifuge			
	Dry shipper			
	Plasma extractor			
	Sterile connection device			
	Programmable controlled-rate freezer			
	Refrigerator			
	Tubing sealer			
	Nitrogen tank			
	LN_2 source			

& if pertinent; # validity

	Type	Supplier[$]	Lot[$]	Validity date[$]	Numbered used[$]
Supplies and Reagents	Assay sample tubes				
	Blood culture medium				
	Cryogenic tubes				
	Disposable needles				
	Cryobags				
	Cryopreservation solution				
	Labels				
	Ice brick refrigerated				
	Sampling-site couplers				
	Sterile syringes				
	Storage cassettes				
	Transfer bags				

$ Mandatory registration if considered critical

Patient data	Name: Date of birth: Age: Weight:
Product data	Donation identification number: Product description code and division code: Product name and attributes: Procurement data and time Product anticoagulant volume
Temporary storage	Procedure start at: Duration between the completion of the procurement and cryopreservation process initial: _____ h Responsible person (signature and stamp):
Pre-procedure product inspection	Usual product appearance: ☐ yes ☐ no Visible evidence of contamination: ☐ yes ☐ no Clumps? ☐ yes ☐ no Container integrity? ☐ yes ☐ no Appropriate labeling? ☐ yes ☐ no Responsible person (signature and stamp):
Sample collection	Volume: _____ mL
Volume determination	Initial weight _____ g Calculated volume: _____ mL Responsible person (signature and stamp):
Cell concentration determination	TNC = _____ Cell concentrate final volume: _____ mL Number of bags to be cryopreserved: _____ Responsible person (signature and stamp):
Cryopreservation solution preparation	Volume to be prepared: _____ mL Reagents proportion: - DMSO 100%: _____ mL - Plasma or albumin: _____ mL - _____ mL Preparation time: _____: _____ Lot: _____ Bag insertion time into refrigerator: _____: _____ Bag removal time from refrigerator: _____: _____ Responsible person (signature and stamp): Responsible person (signature and stamp) for double check:
Plasma removal	Volume to be removed: _____ mL Calculated weight to be removed: _____ g Centrifugation time: Initial: _____: _____ end: _____: _____ Plasma extraction: Initial: _____: _____ end: _____: _____ Bag weight: _____ g Calculated plasma volume: _____ mL Cell concentrate bag weigh: _____ g Calculated cell concentrate volume: _____ mL Sample tests volume: _____ mL Cell recuperation: _____ % Responsible person (signature and stamp): Responsible person (signature and stamp) for double check:

Freezing	Cell concentrate and cryopreservation solution bags removal time from refrigerator: ____: ____ Cryopreservation solution addition to the cell concentrate times: Initial: _____: _____ end: _____: _____ Freezing times: Initial: _____: _____ end: _____: _____ Time between the completion of the cryopreservation solution addition and the initial freezing time: _____ min. Responsible person (signature and stamp): Responsible person (signature and stamp) for double check:
Storage	Time: _____: _____ Local: _____ Responsible person (signature and stamp):

Product transportation and shipping

Patient data	Name: Date of birth: Age: Signed request for sending? ☐ yes ☐ no Responsible person for checking (signature and stamp):
Cryopreservation data	Date: Procedure approved? ☐ yes ☐ no Cryopreservation curve approved? ☐ yes ☐ no Responsible person for checking (signature and stamp):
Product visual check	Cracks or bubbles? ☐ yes ☐ no Usual appearance? ☐ yes ☐ no Appropriate labeling? ☐ yes ☐ no Responsible person (signature and stamp):
Post-cryopreservation quality control	Cell viability: _____ %
Sending preparation	Select dry shipper identification: _____ Filling date and time: ___/___/___ ___: ___ Responsible person (signature and stamp): Labels preparation: ___/___/___ Responsible person (signature and stamp): Forms preparation: ___/___/___ Responsible person (signature and stamp):
Dry shipper preparation for sending	LN_2 excess removed (date and time): ___/___/___ ___: ___: Double identification check (bags and cryovials): Dry shipper weight: _____ g Temperature: _____ ºC Responsible person 1 (signature and stamp): Responsible person 2 (signature and stamp):
Dry shipper return	Visual check usual? ☐ yes ☐ no Temperature: _____ ºC Responsible person (signature and stamp): Transportation temperature graph: Responsible person (signature and stamp) for printing: Responsible person (signature and stamp) for its evaluation: _____

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ADVANCED CELL THERAPY PRODUCT RELEASE CONTAINING CAR-T CELLS

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ABSTRACT

Immunotherapy consisting of genetic modification of T cells such as CAR-T cells need to address several tests and exams attesting the quality of the advanced therapy product. These tests are based on the Resolution of the Collegiate Board (RDC – Resolução da Diretoria Colegiada - Anvisa) number 508 of May 27, 2021 that guide the necessary release tests of advanced cells/gene therapy for use in humans. These tests include the total count number of the cells, identity of cells populations, cell viability, purity test, aseptic tests, cytogenetics, potency test and nucleid acid detection for some virus in case of allogenic use and for gene therapy product, tests carrying out identity, integrity, purity and potency of the vector used for the genetic modification. The final release of the advanced cell/gene therapy product should attest the safety and characteristics of the cells.

Keywords: Immunotherapy, Adoptive.

AIMS

Describe the release tests required after the manufacturing of CAR-T cells in accordance with current regulations.

INTRODUCTION

Manufacturing of customized gene or cell therapy products such as CAR-T cells is complex and depends on release tests andexams that can attest to a consistent quality standard for each product. The quality of CAR-T cell products is subject to donor variation, but also includes the manufacturing environment, as well as the quality and availability of materials and reagents. Quality must be carefully monitored and integrated into the manufacturing process.

Risk-based approach should guide the advanced cell therapy product manufacturing process so that quality assurance is achieved[1] (EMA/CAT/CPWP/686637/2011). Unintentional variability in cell cultures such as differences in starting material, vector conditions and concentration, transduction efficiency, multiplicity of infection (MOI) can result in quantitative and/or qualitative differences in product quality.

According to Resolution of the Collegiate Board number 508 of May 27, 2021[2], the release of advanced cells/gene therapy for use in humans must follow the Good Practices in Human Cells for therapeutic, as summarized in the table below:

TABLE 1 - Release Tests on a Sample of the Advanced Cell/Gene Therapy Final Product

Release Tests on a Sample of the Advanced Cell/Gene Therapy Final Product
a. total count of relevant cells
b. identity testing or appropriate phenotyping for the product and quantification of cell populations present
c. cell viability
d. purity test
e. microbiological tests
f. cytogenetics
g. potency test
h. nucleic acid detection of CMV, HIV-1 and HIV-2, HTLV-I and HTLV-II, EBV, HBV, HCV and B19 viruses in case of allogeneic use
i. Gene Therapy: carrying out tests of identity, integrity, purity and potency, related to the stem cell line and vector.

ADVANCED THERAPY PRODUCT RELEASE TESTS

According to the Good Manufacturing Practices (GMP) regulations, quality is incorporated into the design of the manufacturing process[3]. A carefully list of release tests is required to provide adequate evidence of identity, safety, purity, potency and cytogenetics. The identity of CAR-T cell products is commonly characterized by surface expression of CAR but it also could be assessed by qPCR[4,5]. Safety is related specially to the absence of any possible contamination such as endotoxin, mycoplasma, microorganisms and, also to the lack of lentiviruses replicating[4,5]. Purity is specified by number of viable T cells and, also' CD3+ and CAR-T+ cells; regarding purity, a panel could be done in flow cytometry to identify the populations within the final cell therapy product. Impurities may be present in CAR-T cell product and it could be evaluated by observing under the microscopy for residual possible magnetic beads; CD19+ B cells could be determined by flow cytometry and expressed by % of unwanted cells. Potency of CAR-T cells could be determined by in vitro cytotoxicity assay or cytokines secretion, such as interferon-γ, when CAR-T cells are cultured with cells expressing the target such as CD194,[5]. Table 2 summarizes examples of release assays for CAR-T cells using different genetic modifications.

TABLE 2 - Quality Controlo f CAR-T cells product[4,5].

Advanced Therapy Product Release Tests			
Safety	Genetic modification by retroviral or lentiviral vector	Genetic modification by transposon	Genetic modification by electroporation (mRNA)
	Gram stain/microbiologicals	Gram stain/microbiologicals	Gram stain/microbiologicals
	Mycoplasma by qPCR	Mycoplasma by qPCR	Mycoplasma by qPCR
	Endotoxin quantification	Endotoxin quantification	Endotoxin quantification
	Determination of VSV-G DNA by qPCR		
Purity	% viable T cells	% viable T cells	% viable T cells
	% CD3+ T cells	% CD3+ T cells	% CD3+ T cells
	% CAR-T cells	% CAR-T cells	% CAR-T cells
Identity	% CAR-T cells – by PCR or flow cytometry		
cytogenetics	T lymphocyte karyotype		
Potency	in vitro cytotoxicity assays or interferon-γ release in response to cells expressing the target molecule		
Quantity	Number of viable cells and calculation of the dose		

SAFETY

- Gram staining/microbiological assays

Microbiological tests are performed at determined key points in the manufacturing process; the method and timing of testing will provide assurance of sterility of the advanced therapy product. If the final product is cryopreserved, the tests must be performed before freezing process so that all results will be released at the time of infusion in the patient; however, if there is any type of manipulation of this product after thawing before infusion such as washing, there will be a need to repeat the microbiological tests or to perform a rapid test before infusion and follow-up of the standard microbiological culture.

If the product must be administered right after manufacturing (fresh infusion) before the results of microbiological tests are ready, there is a need for validation of an additional test that guarantees the sterility of the product. Some regulatory agencies, such as the Food and Drug Administration (FDA), recommend certain tests:

- Microbiological test on a sample 48 to 72 hours before the end of the manufacturing process;
- A rapid microbial detection test such as the Gram stain;
- 21 CFR 610.12 compliant sterility test (sterility) in the final product formulation6.

In this way, the advanced therapy product would be released based on the results described above; the culture of the final product must be continued until 14 days of incubation even after the product has been administered to the patient, and a final result, without growth of microorganisms, will confirm that the aseptic technique was maintained. If there is a positive result, an investigation should be carried out to determine the cause of the sterility failure.

The principal investigator should evaluate the patient for any signs of infection that may be related to the product. If the patient has any serious adverse reaction, which may be due to the failure of sterility of the advanced therapy product, a report must be sent to the health surveillance.

According to Brazilian Health Surveillance (Anvisa), The Cell Processing Center must have mechanisms to identify, investigate and execute corrective and preventive actions related to Technical Complaints and Adverse Events.

MYCOPLASMA

Mycoplasma contamination can happen from 2 main sources: the serum of animal or human origin and the facilities where the cells are cultivated in an open system, whose contamination can come from the operator of the process[7]. Performing the mycoplasma test on the final product is essential to detect possible contamination.

The PCR test for the detection of mycoplasma species is the most recommended, but rapid detection assays can be performed since providing that they demonstrate adequate sensitivity and specificity.

- Endotoxin

The endotoxin test detects a lipopolysaccharide in the cell membrane of gram-negative bacteria; endotoxin is released in the environment after bacterial cells death and it can cause troubling effects such as fever, septic shock and, even death[8]. According to the Brazilian Pharmacopoeia section 5.5.2.2, bacterial endotoxin test is used to detect or quantify endotoxins from gram negative bacteria[9]. The aqueous extract of circulating amebocytes from Limulus polyphemus or Tachypleus tridentatus is used.

There are two techniques with different sensitivity for this test:

- Gel Coagulation Method: based on clot or gel formation (semi-quantitative method);
- Photometric Methods: that is a quantitative method and could be divided in 2 types - Turbidimetric Method or Chromogenic Method.

The validation of the chosen method should be performed and some parameters such as maximum valid dilution as well as potential inhibitors factors must be carried out.

- Number of copies of the transgene/lentivirus replication

Replication Competent Virus (RCV) testing is performed to confirm the absence of RCV (using validated and sensitive assays) in the starting material after use of the viral vector and to exclude RCV formation during the manufacturing of genetically modified cells. In this case, a risk assessment must be submitted to address the potential generation of RCVs during the manufacturing process. Whenever possible, retention samples should be stored for future analysis.

FDA has guidelines recommendations regarding the replication competent virus (Testing of Retrovi-

ral Vector-Based Human Gene Therapy Products for Replication Competent Retrovirus During Product Manufacture and Patient Follow-up); according to these guidelines, the possible pathogenicity of the retroviruses must be evaluated to monitor replicating particles in the final product10. When manufacturing cells to be transduced using retroviruses, agency recommends the test to be done in the cells including the ones cultivated for 4 days or less.

In ex vivo genetic modification of cells, the RCV in the vector should be at minimum level, however, the manufacturing procedure can provide a favorable environment for RCV amplification so the test should be performed for each lot of cells regardless the lenght if time the cells were cultivated. This chapter also presents some assays for RCV analysis.

Regarding patient monitoring, FDA recommends that samples from the patient should be analyzed at some specific time-points: pre-treatment, testing after 3, 6 and 12-months after the gene therapy, and yearly for up to 15 years.

- Purity

Purity of advanced cell therapy is related to cell type and transduction efficiency; purity must be defined considering the nature and use of its production consistency, the production method and also the degree of production process. Purity criteria must be determined and be within specified limits.

When a viral vector is used for transduction, the level of replicating particles in the final product must be determined and kept below a justified threshold. When using transposon vectors, it must be shown that the final cell population is free of transposase activity.

In the case of genome editing, the persistence of gene editing tools in cells must be assessed; ideally, they are no longer present when the cells are released for clinical use.

- Identity

Flow cytometry is one of the most used techniques to identify cells and protein biomarkers. Detection is performed on cell samples from an incident laser beam that allows measurement of the scattering and fluorescence of the reflected laser. In this way, it is possible to obtain fast and accurate information with the identification of numerous intrinsic and extrinsic characteristics contained in the cells, accurately recognize the size and granularity by reading the intensity of the fluorescence reflected in cells previously stained with fluorescent antibodies.

In this context, flow cytometry fits as a technique used for the purpose of releasing advanced therapy product from CAR-T cells. On the day of product release, two immunophenotyping panels are required for accurate detection and quantification of cells present in the final product. Panel A has markers that allow the evaluation of all immune components present in the final composition of the product, while panel B has markers for the quantification of positive CAR cells (Table 3), allowing a reliable calculation of the dose to be infused in the patient.

TABLE 3 - Immunophenotypic profile of CAR T cells

Panel A	**Panel B**
Immune Composition Profile	**Transduction Profile**
CD45+	CD45+
CD4+	CD4+
CD8+	CD8+
CD3+	CD3+
CD56/CD16	CAR+
CD19	7-AAD
7-AAD	

- Cytogenetics

According to RDC No. 508/2021, performing cytogenetics is mandatory in case of extensive manipulation and applies to the release of the CAR-T cells product. In this context, the G-band karyotype is performed to detect clonal and non-clonal changes in each sample[2]. Ten metaphases are analyzed and, in case of alteration, a total of 20 metaphases must be evaluated. If confirmation of the change occurs, a FISH analysis (fluorescence in situ hybridization) is requested for safer detection of any possible change.

- Potency

Potency of genetically modified cells must be assessed to determine the functionality of the cells; this test should provide quantitative information about the function of the cells and the transgene product. Whenever possible, a reference lot of cells with assigned potency should be established and used to calibrate tests[11].

Potency testing should not be limited to cell functionality but also include other relevant tests such as cell viability. Potency test for products containing genetically modified T cells against tumor cells is preferably based on the cytotoxic potential of the T cells. Tests on the potential of CAR-T cells can be performed with the analysis of the release of cytokines and/or cytotoxic molecules or the expression of T cells activation markers, providing the data of tumor cells death[12].

- Advanced Therapy Product Release Template

General Information	
Grant:	Patient ID:
Patient weight: kg	Dose:
Cultivation data	
Culture Start date: / / /	Duration of cultivation: days
Transduction Percentage: %	Cell Count Starting Material:
Cell Count Final Product:	CART Cell Count:
Cell Viability:	
Results Exams Release	
Karyotype	Normal ( ) Alteration ( ) Type de alteration:
Immunophenotype	CD3+ ( ) CD4+ ( ) CD8+ ( ) CD45+ ( )
Microbiological analysis	Negative ( ) Positive ( )
Mycoplasma Analysis	Negative ( ) Positive ( )
Endotoxin Analysis	Negative ( ) Positive ( ) _____EU/mL
Potency Test	% Citotoxicity _____
Product release	
Product: Released ( ) Conditionally Released ( ) Blocked ( )	
checked by: _____ Date ____/____/____	
Released by: _____ Date ____/____/____	

- Frequency of training or competence assessment

Initial training for all employees involved in the process of releasing the product containing CAR-T cells. Annual retraining required.

- Critical Points and Risks

Critical Points:

- Refinement of acceptability criteria for effective detection of positive or negative from advanced therapy product release tests;
- Preventive maintenance of equipment used in the release of advanced therapy products;
- Validation and qualification of reagents used in advanced therapy product release assays.

Risks:

- Associated with the type of vector used;
- False positive or false negative results.

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THAWING AND INFUSION OF CAR-T CELL PRODUCTS

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ABSTRACT

Some patients with hematologic malignancies may benefit from chimeric antigen receptor-T (CAR-T) cell treatment. Usually, the manufacture of this cells is carried out by centralized and specialized cell therapy laboratories that cryopreserve the CAR-T cells in bags and send it for infusion in the patient treatment center. In this context, the thawing and infusion of the CAR-T cell products are critical steps in this custody chain. This article aims to discuss the most relevant points related with the CAR-T cell thawing and infusion and proposes a kind of standard operational protocol (SOP) for these processes.

Keywords: CAR-T cells; thawing; infusion.

OBJECTIVES

Describe steps in the process of thawing CAR-T cell products, avoiding damage of product and loss of viability or sample contamination.

Describe check list steps before its infusion for correct administration.

Describe pre-infusion measures, including premedication use.

INTRODUCTION

The thawing and infusion procedure are critical steps to ensure the safety and effectiveness of the chimeric antigen receptor-T (CAR-T) cell treatment. The background with progenitor cell infusion provides a basis for infusion of other products such as CAR-T cells. Even though they are similar procedures, a validation process is suggested for each product, determining correct handling of them. General guidelines for handling CAR-T cells products must be established at each institution. Products manufactured by industry have specific guidelines and must be included in operational procedures. Procedures and measures adopted during thawing and infusion contribute to prevent, identify and minimize possible complications.

CAR-T cells can be fresh infused or cryopreserved.[1,2] Advantages of fresh product are associated with lower toxicity, due to the absence of cryoprotectant solution, but it requires infusion without conclusion of quality control tests. Cryopreservation allows conclusion of all quality controls prior to infusion, expands availability of product to centers far from manufacturing, but requires an infrastructure for storage until its administration.[1]

Fresh products manufactured in another institution must be transported refrigerated, with a temperature between 2-8ºC and cryopreserved products must be transported at temperatures below minus140ºC.[1] Transport of genetically modified organisms must follow national rules according to risk assessment of each vector used to manufacture CAR-T cells and procedures and measures to protect environmental and human health.[3]

Indication and contraindication

All patients in a CAR-T cells treatment protocol, with fresh-infused or cryopreserved products must follow recommendations, with no contraindication for this protocol.

Minimum requirements:

- Initial training of all employees involved in the process of thawing and infusion of CAR-T cells. The annual competence of all staff is required.
- Product release report approved by the Director of Cell Therapy Lab and the quality manager.
- In case of exceptional release – report and release approved by Medical Director of Cell Therapy Lab.
- Equipment for thawing products calibrated and cleaned with 70º alcohol prior to use.
- Transport container validated.
- Medical prescription for the infusion.

Material:

- Blood transfusion set
- Infusion set Y-Type, dual spike (optional)
- Sterile plastic bag or zip lock
- Saline in a 50 or 100 mL bag
- Water bath at 37ºC with sterile water or saline solution or dry bath
- Transport container (dry shipper or suitcase)

Pre and post-procedure guidance:

- Prior to procedure, medical prescription for infusion should be checked and premedication administered.
- Carry out a double-check of product with the nurse team, ensuring that correct product will be administered to correct patient.
- Perform pre-infusion care checks.
- Educate patient about the infusion process and possible post-infusion adverse effects.
- Perform vital signs control periodically during infusion.

PROCEDURE

Product receipt:

All centers must adopt strategies to check the integrity of the product, its identification and for products manufactured in other institutions, the transportation conditions, as described in Annex I. The centers must have facilities and equipment for storage of genetically manipulated products according to their risk assessment. It is important to check the bags and cassettes size and configuration prior their receipt and verify if they will fit in the nitrogen tank rack.[4]

The process to check and transfer cryopreserved units in storage tank must be carried out carefully and quickly, thus avoiding heating of products and the possibility of reaching critical temperatures, which can lead to loss of viability.[5,6]

Patient evaluation before infusion:

Before infusion, patient should be assessed. Changes in his clinical condition may postpone product infusion, as described in table 1.

TABLE 1 - Clinical conditions and its recommendations before CAR-T cell infusion. Adapted.[4]

Clinical condition	Recommendation	Comments
Active infection	Contraindication for infusion	Infection must be controlled or treated prior to infusion
Cardiac arrhythmia	Specialist release	Individual risk-benefit assessment
Hypotension requiring vasopressor	Contraindication for infusion	Infusion should be delayed until complete resolution of hypotension
Non-hematological clinical worsening with new comorbidity or worsening of previous comorbidity	Identify cause	Individual risk-benefit assessment
Disease progression	Identify cause	Individual risk-benefit assessment

Previous use of corticosteroids may impair the effectiveness of treatment, and its use is suggested only in life-threatening cases. Some clinical studies also advise washout intervals for drugs that could affect the cells therapeutic effect or be associated with increased risk to the patient, such as monoclonal antibody, antiproliferative therapy including oral and intrathecal chemotherapy, as described in table 2. Generally, recommended washout times are 2 weeks for systemic chemotherapy, 4 weeks for pegylated l-asparaginase, and 72 hours for steroids.[7] A check list should be applied to guarantee that all clinical conditions were evaluated before infusion, as described in Annex I.

TABLE 2 – Washout drugs before CAR-T infusion

Type of therapy	Recommendations
Corticosteroid	Stop steroids therapeutic dose 3 days prior to infusion[7,8]
Chemotherapy (including low dose maintenance therapy)	Stop ≥ 2 weeks before infusion[7,8]
Short-acting drugs used to treat leukemia or Lymphoma (hydroxyurea, tyrosine kinase inhibitors [TKI])	TKIs and hydroxyurea must be stopped ≥ 3 days before infusion[8]
Immunomodulatory drugs	Stop antibodies 4 weeks before infusion[8]
Prior radiation therapy	Radiation must be completed > 2 weeks before CAR-T infusion[8]
Intrathecal chemotherapy	Central nervous system prophylaxis must be stopped ≥1 week before[8]
Graft-vs-host disease therapies (e.g. calcineurin inhibitors)	Stop > 2 weeks before CAR-T infusion to confirm that GvHD recurrence is not observed[9]
Immunosuppressive therapy	Stop ≥ 2 weeks before infusion[8]

Thawing CAR-T cells product:

Before thawing product, a double check must be performed by trained employee to verify identifiers and their match in all documentation. Identifiers include patient name, record number or date of birth.

Although bone marrow transplant team is used to thaw progenitor cells, some precautions are important to avoid bacterial contamination and ensure the best viability.

Water baths are devices commonly used, although literature shows dry equipment as an alternatives in this process, without impacting viability and reducing risks of contamination.[4,10,11]

Temperature for thawing should be programmed to 37ºC (± 2ºC) and must be checked before thawing each aliquot. Thawing product in higher temperatures will impair its viability. The use of a secondary plastic bag during thawing is suggested to facilitate the cell recuperation in case of bag rupture and to reduce the risk of product bacterial contamination.[4]

After thawing, the product must be immediately infused as the cryoprotectant solution can damage the cells at room temperature. Thus, it is suggested to conduct thawing procedure quickly, homogenizing sample, to avoid temperature gradients in product and remove it from the thawing device once the process is completed. If more than one bag will be infused, wait to thaw next bag until it is determined that previous bag has been safely infused.

Steps for thawing product:

- Make sure water bath or dry thaw equipment is cleaned with germicidal wipes and sterile alcohol.

- Fill it with sterile water, according to institutional procedure.

- Make sure the water bath has been turned on and is ready for thawing the CAR-T cells infusion bag in advance.

- Heat the water bath at least 30 minutes before the thaw start time to allow the equipment to reach the temperature of 37°C.

- Remove the CAR-T cells product from the transport container using cryogloves.

- Remove the CAR-T cells infusion bag from its cassette.

- Examine the infusion bag for breaks or cracks

- Place the infusion bag in a secondary bag to avoid direct contact between the bag and the water.

- Immerse the bag in water bath and homogenize the product with care.

- Remove the bag from the water bath as soon as the process is completed.

Product infusion

Premedication

To prevent acute adverse effects of T cell infusions patient should be premedicated with antipyretic

and diphenhydramine or another H1 antihistamine approximately 30 to 60 minutes before the infusion. These medications should be repeated every 6 hours as needed.[4,7]

Infusion

A transfusion set is required for cell infusion. It is important to emphasize that infusion fluids set, bacterial filter or transfusion set with leukocyte depletion filter are unacceptable. Some manufacturers recommend using unfiltered tubing set, however Brazilian standards for infusion device require macroaggregate filter in all sets.[4,12]

The line to use for CAR-T cells infusion must be clearly designated. No simultaneous drugs can be administered in this line during the infusion.

Infusion should start as soon as product has been thawed, usually no longer than 30 minutes.

Dimethyl sulfoxide, used as a cryoprotectant, can be toxic to progenitor cell products. Although some studies with mononuclear cells show less toxicity, it seems prudent to infuse it as soon as possible after thawing.[13] CAR-T cells product has small volumes that allow rapid infusion.

Transfusion set should be primed using 0.9% sodium chloride before CAR-T cells bag is connected to device. At the end of infusion, transfusion set line should be washed with saline. In Y-type set, it is possible to wash the CAR-T cells bag with saline connected on other spike, before washing line.

Infusion bag and set must be discarded in accordance with specific institutional policies and regulations of genetically modified organism waste.

CRITICAL POINTS AND RISKS

For early detection of adverse event, vital signs should be monitored prior to, during and immediately after infusion, then every 15min for the 1st hour and every 30min for 2nd hours or until the signs are satisfactory and stable.

Prior to infusion, 2 doses of tocilizumab must be confirmed as available for the management of cytokine release syndrome and its related adverse events.

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ANNEX

ANNEX I - Receipt of CAR-T product

Patient identification	
Name:	Medical record number:
Date of birth:	Protocol Nº:

Affix patient identification tag

Product Identification:			
Donor identification number (DIN):			
Product:			
( ) HPC(CBU)	( ) HPC, APHERESIS	( ) HPC, MARROW	( ) MNC, APHERESIS
( ) HPC, CD34 APHERISIS Enriched	( ) MSC, MARROW	( ) CAR-T	( ) _____ _____
Number of bags:		Number of tubes:	

DOCUMENTS:	
Product report ( ) yes ( ) no	( ) yes ( ) no
Release and product report	( ) yes ( ) no
Signature and date:	
PRODUCT TRANSPORT AND PACKAGING	
Box/dry shipper	( ) yes ( ) no
Box/dry shipper sealed	( ) yes – seal number_____ ( ) no
Temperature	_____ºC
Transport time	_____ hours and _____ minutes
Signature and date and hour:	

PRODUCT AND SAMPLES	
Correct bag identification	( ) yes ( ) no
Patient ID is correct	( ) yes ( ) no
All tubes are identified with the same product number	( ) yes ( ) no
Time to storage product (from box/dewar until its storage in nitrogen tank):	_____ min (cryopreserved product should be storage in 2 minutes)
Integrity	( ) yes ( ) no
Visual inspection	( ) Ok ( ) no _____
Comments: ( ) not applicable	
Signature and date:	Signature and date:

ANNEX II – Patient assessment for CAR-T infusion

Patient identification	
Name:	Medical record number:
Date of birth:	Protocol Nº:

Affix patient identification tag

Clinical Condition	
Signs of active infection last 48 hours	( ) yes - contraindication to administer ( ) no
Cardiac arrhythmia	( ) yes – specialist assessment: ( ) Released by _____ ( ) Not released ( ) no
Hypotension requiring vasopressor	( ) yes - contraindication to administer ( ) no
Clinical worsening	( ) yes – assessment: ( ) Released by _____ ( ) Not released ( ) no
Disease progression	( ) yes – assessment: ( ) Released by _____ ( ) Not released ( ) no
Drugs	
( ) Corticosteroid: _____ dose_____ last dose date _____ ( ) released by _____ ( ) not released	
( ) monoclonal antibody: _____ dose_____ date last dose_____ ( ) released by _____ ( ) not released	
( ) chemotherapy: _____ dose_____ date last dose_____ ( ) released by _____ ( ) not released	
( ) Radiotherapy: _____ dose_____ date last dose_____ ( ) released by _____ ( ) not released	