6 Cryopreservation in Closed Bag Systems as an Alternative to Clean Rooms for Preparations of Peripheral Blood Stem Cells

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Abstract

Autologous and allogeneic stem cell transplantation (SCT) represents a therapeutic option widely used for hematopoietic malignancies. One important milestone in the development of this treatment strategy was the development of effective cryopreservation technologies resulting in a high quality with respect to cell viability as well as lack of contamination of the graft.

Stem cell preparations have been initially performed within standard laboratories as it is routinely still the case in many countries. With the emergence of cleanrooms, manufacturing of stem cell preparations within these facilities has become a new standard mandatory in Europe. However, due to high costs and laborious procedures, novel developments recently emerged using closed bag systems as reliable alternatives to conventional cleanrooms. Several hurdles needed to be overcome including the addition of the cryoprotectant dimethylsulfoxide (DMSO) as a relevant manipulation. As a result of the development, closed bag systems proved to be comparable in terms of product quality and patient outcome to cleanroom products. They also comply with the strict regulations of good manufacturing practice.

With closed systems being available, costs and efforts of a cleanroom facility may be substantially reduced in the future. The process can be easily extended for other cell preparations requiring minor modifications as donor lymphocyte preparations. Moreover, novel developments may provide solutions for the production of advanced-therapy medicinal products in closed systems.

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Keywords

Cryopreservation • Closed bag systems • Clean room facility • Peripheral blood stem cells

Abbreviations

ATMP	Advanced therapy medicinal products
CCPS	Closed cryo prep set
CCFPS Closed	cryo frozen prep set
CD	Cluster of differentiation
DLI	Donor lymphocyte infusions
DMSO	Dimethylsulfoxide
EMA	European Medicines Agency
EU	European Union
FACT	Foundation for the accreditation of cellular therapy
FDA	Food and Drug Administration
GMP	Good manufacturing process
HES	Hydroxyethyl Starch
HEPA	High-efficiency particulate air
LC	Langerhans cells
MNC	Mononuclear cells
PBSC	Peripheral blood stem cells
PVA	Polyvinyl alcohol
SCT	Stem cell transplantation (SCT)
SOP	Standard operating procedures
ULPA	Ultra-low particulate air

6.1 Introduction

Cryopreservation of peripheral blood stem cells (PBSC) collected by apheresis has become an essential procedure for many institutions worldwide as the number of autologous and allogeneic stem cell transplantations is constantly rising. Although cryopreservation of PBSC is in principle a simple procedure, it needs to ensure a high quality with respect to PBSC viability and lack of contamination being highly essential for successful transplantation of the immune-compromised patient.

Depending on the transplantation setting, PBSC are collected by apheresis from a patient or from an allogeneic donor following prior stem cell mobilization. Eventually, multiple apheresis procedures may be necessary to obtain the adequate PBSC numbers needed for one or multiple PBSC transplantations.

Cryopreservation of PBSC gives high flexibility with respect to repeated transplantations as well as patient specific schedules. In case of patients with multiple myeloma, the number of PBSC to be collected may be sufficient for up to four transplantations. Cleanrooms have been the standard for cryopreservation for many years – especially within Europe. Their construction and maintenance are strictly regulated by SOP (standard operating procedures) guidelines according to GMP (good manufacturing practice) regulating and controlling all procedures conducted in the cleanroom environment [1].

Although the advantages of a cleanroom facility ensuring the high quality of collected PBSC are predominant, cleanrooms are quite expensive. The initial fixed costs for the establishment of a class B clean room are approximately 1 million euro. The variable yearly costs are about 35,000–100,000 euros depending upon space and instrumentation. Thus, novel developments reducing the costs but maintaining high product quality are urgently needed.

6.2 Collection and Cryopreservation of PBSC

Since the first allogeneic transplantations being performed in the US in the 1950s and 1960s [2, 3] cell based therapies have become substantial in the treatment of hematopoietic malignant dis-

eases. Subsequently, novel therapeutic strategies using autologous stem cell transplantations developed [4]. To date, clinical indications for stem cell transplantation cover a broad range of hematopoietic malignancies but also solid tumors and non-malignant diseases [5–7]. Patients or healthy donors who donate PBSC undergo leukapheresis after stem cell mobilization. Apheresis is performed if the blood cell count contains a sufficient level of PBSC. The patients/donors blood is removed through a venous catheter and is transferred to a blood cell separator machine, where PBSC are separated by centrifugation. As anticoagulant, a citrate solution is commonly used. Once the required amount of PBSC is collected, DMSO is added to the cells at concentrations of 5–10 % and controlled rate freezing is started. The PBSC can be stored at temperatures below −130 °C for years. Before and after cell processing, cell viability and potential contamination is assessed as major aspects of quality control. When the cell product is required for an autologous or allogeneic transplantation, controlled thawing is performed prior to the transplantation and the cells are transfused to the patient.

6.3 Cryoprotectants

Cryoprotectant agents, controlled rate freezing and the usage of liquid nitrogen are the most important breakthrough developments enabling the storage of viable cells over a longer period of time. Cryoprotectants penetrate the cells and preserve their solute concentration, without being toxic to the cellular product. In 1948, Polge and colleagues accidently found that glycerol would enable fowl spermatozoa to survive freezing to −70 °C [8]. In 1959, Lovelock and Bishop [9] described that freezing damage could be prevented from cells by adding DMSO. Some years later, in 1965, Bouroncle et al. published the preservation of living cells in the presence of DMSO [10].

To date, DMSO is the most commonly used cryoprotectant. However, other substances are used for specific cell types or experimental purposes. Hydroxyethyl Starch (HES), a polymer with a high molecular weight, is used for cryopreservation of red blood cells [11]. Polyvinyl alcohol (PVA) [12] has been described as a new cryoprotectant and has also been applied for freezing of red blood cells. This agent might become an alternative to other cryoprotectants as DMSO and Glycerol in future. However, so far PVA was used only in experimental systems, thus, the potential clinical application needs to be further evaluated.

The addition of DMSO is, in fact, the only open manipulation during cryopreservation of PBSC. This has historically been performed without a cleanroom facility, as it is still performed outside of Europe. The DMSO concentration varies between different centers according to their internal standards and usually stays within a certain range of 5–10 % [13, 14]. The optimal concentration is still under debate although lower concentrations may result in reduced cell toxicity and side effects during and after transplantation [15]. It has been also published that the usage of 7.5 % DMSO was favorable compared to 10 % regarding engraftment of leukocytes, although platelet engraftment and transplantation-associated side effects did not seem to differ in both groups [16]. However, others report lack of statistical difference in therapeutic complications and engraftment for 5 % versus 10 % DMSO [17].

6.4 Controlled Freezing

With controlled-rate or slow freezing, biological materials are frozen down using programmable sequences. Slow freezing is protective for cellular products that have been pretreated with cryoprotectants such as glycerol or DMSO. A typical freezing program for PBSC would start at a temperature of about 2 °C. The temperature then decreases stepwise until a final temperature of −150 °C. The total freezing process takes about 1 h - in some protocols up to 2 h - and is supervised at all times and secured by a special alarm system. After the freezing process, the cellular products are transferred to Liquid Nitrogen (N_2) for further storage [18]. There are controversies about the need of a precooling step in the con-

trolled freezing protocol. According to Dijkstra-Tiekstra et al., precooling was not necessary for the viability of cryopreserved cells, however the freezing rate had an influence on the recovery of white blood cells: slow-rate freezing revealed a higher cell recovery than fast-rate freezing [19]. The optimal cooling rate may additionally depend on the cell type [20].

6.5 Differences in the Processing of PBSC Between the US and Europe According to GMP

GMP guidelines regulate the manufacturing process of hematopoietic stem cells for clinical use. GMP guidelines aim to secure a high product quality for routine manufacturing processes by implementation of high standards for quality control. They were first implemented by the FDA (Food and Drug Administration) in the US and later in Europe concluded by the EU directive 2003/94 [21]. In Europe, the European Medicines Agency (EMA) is responsible for the development of guidelines and standards for medicinal products. In addition, every medicinal product for human use has to be granted a marketing authorization delivered by a competent regional authority. In the United States, the Foundation for the accreditation of cellular therapy (FACT) and the Food and Drug Administration (FDA) promote standards for cellular therapies according to evidence-based requirements.

6.6 Legal Developments in the EU

In 1989, the first edition of the EU guideline to good manufacturing practice of medicinal products for human and veterinarian use was published including an annex of the manufacture of sterile medicinal products. In January 1992, a second edition was released implementing Commission Directives 91/356 and 91/412. Twelve additional annexes were included. The rules governing medicinal products in the European Union contain guidance for the interpretation of the principles and guidelines of good manufacturing practices. They were laid down in Commission Directives 91/356/EEC and were amended by Commission Directive 2003/94/EC, of 8 October 2003, combining the guidelines of good manufacturing practice in respect of medicinal products for human use.

In this directive, high standards for quality control securing the safety of medicinal products are described. The manufacturing and processing of cellular products in clean room facilities is regulated by EudraLex ("The rules govering medicinal products in the European Union"), Volume 4 (EU Guidelines to good manufacturing practice), Annex 1 2008 (Manufacture of Sterile Medicinal Products). The principle in Annex I defines the manufacture of sterile products as subject to special requirements in order to minimize risks of microbiological contamination and of particulate and pyrogen contamination. It therefore requires the manufacturing of sterile products in clean areas entry to which should be through airlocks for personnel and/or for equipment and materials. Clean areas should be maintained to an appropriate cleanliness standard and supplied with air which has passed through filters of an appropriate efficiency.

The goal of production in a cleanroom environment is to ensure a low and controlled level of contamination (e.g. airborne microbes, aerosol particles) as regulated in the EU GMP guidelines Annex I. Cleanrooms are classified in grades A-D, defined by the maximum permitted number of particles per m^3 at different sizes (0,5 µm or 5 µm) at rest or in operation and limits for microbial contamination (in cfu/m^3), either the air sample, the settle plate, the contact plate and the glove print. According to common standards, the air entering a cleanroom facility is filtered and the air inside is recirculated through high-efficiency particulate air (HEPA) and/or ultra-low particulate air (ULPA) filters.

There are different standards for clean rooms: the European GMP classification (A–D), the British BS 5295 (class 2–4) and the ISO 14644-1 cleanroom standards (ISO 1-9). The ISO 14644-1 designation 1-9 is classified by the number of particles (>0.1 until 1 particles/m^3) and the microbial active air action levels (1–100 cfu/m^3), with ISO 1 being the "cleanest environment and ISO 9 referring to common room air. ISO designations provide uniform particle concentration values for cleanrooms in multiple industries.

A class A cleanroom is defined by a maximum of 3520 particles of a size of 0.5 µm per m^3 at rest and in operation and 20 particles at 5 µm m^3 at rest and in operation. A class B cleanroom allows 3520 particles of 0.5 µm at rest (29 particles at 5 µm) and 352,000 particles at 0.5 µm at rest and 2900 at 5 µm in operation. A class C cleanroom is defined by 352,000 particles of 0.5 µm (2900 at 5 µm) at rest and 3,520,000 of 0.5 µm (29,000 at of 5 µm) in operation. A class D clean room is also defined at rest with 3,520,000 particles of 0.5 µm and 29,000 particles of 5 µm, no limiting values are defined for a cleanroom class D in operation.

Cleanrooms are routinely monitored in operation, in terms of particles and microbial counts (based on the temperature and humidity), and they are further monitored on formal risk analysis. The personnel has to be trained in regards to hygiene and is equipped with special protective clothing such as hoods, face masks, gloves, boots, and coveralls. Desinfectants/detergants have to be available and sterilization has to be performed using common principles as moist heat, ethylene oxide, UV radiation. Aseptical filling is performed using a filter with 0,22 µm. Integrity testing of the filter needs to take place before and after using the filter.

Regulations according to different cleanroom standards are essential to maintain a certain quality level of the cellular products leaving a cleanroom facility in terms of sterility and integrity. They help to provide international standards, especially for products being transferred from the EU to the US or vice versa.

6.7 Cryopreservation in the Closed Bag System

With the high costs associated with the establishment and maintenance of a cleanroom facility, alternatives to conventional clean rooms such as the closed bag systems for cryopreservation are of high interest. Closed bag systems have been proposed for the preparation of diverse cell products. They consist of tightly connected sterile bags for collection and cryopreservation of PBSC, without the need of reopening the system within the cryopreservation procedures. All steps from stem cell collection until the freezing procedure are performed in a "closed system".

Closed bag systems for collection and storage of previously collected cells have been primarily described in an experimental animal model in 1979 by Fliedner et al. [22]. In 1980, the closed bag system was proposed for collection and freezing of human blood-derived mononuclear cells (MNC) as well as thawing the cryopreserved cells and washing them free of DMSO before transfusion to a patient [23]. A closed bag system has also been described for umbilical cord blood, as it was described in 1997 by Ademokun et al. [24]. This publication is supported by the work of Armitage et al. in 1999, presenting also a triple bag system for cord blood collection [25]. Both publications show favorable recoveries of nucleated cell counts, total progenitors and CD34-positives, which is especially important for cord blood where cell counts are usually low and good recovery rates are essential. In 2006, closed bag systems have been applied for cryopreservation of human mesenchymal stem cells [26]. Closed bag systems have been also proposed for cell therapeutics with potential use in cancer immunotherapy [27, 28]. Genetically modified, biologically functional CD34+ hematopoietic progenitor cell-derived Langerhans cells (LC) were grown in hydrophobic, closed culture bags. Those LC were comparable in terms of their viability and functionality compared to other LC conventionally grown in

flasks or plates. However, in this system, the LC were used only experimentally and their clinical use needs to be further investigated [28].

Closed bag systems for cryopreservation of peripheral blood progenitor cells have been primarily described in 2007 in a preclinical study testing buffy coats and leukapheresis products of healthy donors [29]. In their study, Humpe et al. reported a closed system using a sterile filter to be equivalent to cleanroom-based methods in terms of quality of the cellular products and sterility [29]. The closed system described in this analysis consists of three cryopreservation bags, different tubing, a DMSO-resistant sterile filter, a tubing line for sterile connecting and a syringe for the removal of air.

The first functionally closed system involving routinely cryopreserved PBSC outside of a cleanroom facility for clinical use was published in 2015 [14]. This system has been accepted by legal authorities in the EU and is in process since 2010, so long-term results on a large patient cohort were available. Two systems for cryopreservation of peripheral blood stem cells (PBSC) within a functionally closed bag system were described: the "closed cryo prep set (CCPS) followed by a further development, the closed cryo frozen prep set (CCFPS) [14]. The CCPS consists of three components: two cryobags being connected to a tube system with a DMSO-resistant sterile filter, a syringe and a disposal bag. The system can be also used for manufacturing of donor lymphocyte infusions (DLI). In this context, the cell number can be individually adjusted by the treating clinician [14].

6.8 Comparison of Product Quality

Lack of contamination represents a crucial specification for product release. With respect to cleanroom facilities, divergent contamination rates are described. Donmez et al. found that in their setting, large-volume leukapheresis and high numbers of stem cell culture sampling were defined as risk factors for bacterial contamination [30]. Samples of the leukapheresis products were sent for bacterial culture from two time points: after processing and after thawing. Those samples were compared and microbial contamination was found in 5.7 % of the products after processing, and 3.66 % after thawing [30]. Kamble et al. found 4.5 % of their bone marrow and 3,9 % of their peripheral blood hematopoietic progenitor cell harvests to be contaminated. Both studies were conducted in cleanroom areas [31].

Few studies, in fact, compared contamination rates for manufacturing of PBSC in or outside of cleanroom facilities [32, 33]. In the study of Humpe et al., processing was performed according to a standard protocol under cleanroom class A conditions with surrounding class B conditions and appropriate monitoring (particulate and microbial). Outcome was compared to the processing in a normal laboratory without classified air class but with cryoprotective solution prepared under cleanroom conditions. Cellular integrity (viability) and cellular functionality did not differ substantially among both groups and no contaminations were reported. However, no clinical data are available, so no conclusion on clinical impact could be drawn in this context [29].

With respect to clinical application only two retrospective analyses compared PBSC cryopreservation in and outside the cleanroom. Cassens et al. reported in 2002 that there was no significant difference in contamination rates of clean benches (1.03 %) in comparison to clean rooms (1.32 %) [32]. This is in contrast to data published by Ritter et al. [33] demonstrating a contamination rate of 5.2 % in products from clean benches in contrast to 0.8 % in cleanroom derived compounds. Our group found only 1 out of 283 autologous and allogeneic cellular products being produced in the presented closed bag system outside of a cleanroom facility to be contaminated [14]. There are a number of factors potentially influencing the high contamination rate observed by Ritter et al. [33] including a high fluid volumes used for testing. In a study by

Weinstein et al., 5 ml versus 10 ml sample that was injected into blood culture flasks was compared and an increased rate of blood pathogens was detected when using a higher volume [34].

Of note, it seems that mostly dermal germs which usually do not cause bacteremia are the source of contamination of compounds as reported by diverse groups [31, 35]. Multiple factors can be associated with bacterial contamination including the presence of hair at the phlebotomy site [36] or desinfection of the involved skin [37] according to studies conducted by Perez et al. and Lee et al. demonstrating that bacterial contamination often occurs prior to the processing of the cellular product.

Thus, although it is undoubted that the risk should be minimized as best as possible, the role of infectious complications due to contaminated stem cell compounds seem to be overall of minor clinical significance independent of the usage of cleanroom facilities [38, 39]. Protective antibiotic treatment can be provided either by routine prophylaxis (sequential prophylaxis [40]) or according to previous sensitivity testing.

Apart from sterility, cell viability after cryopreservation is an important issue for cellular products which may depend on the defined processing conditions. Reduced viability of reinfused cells was shown to be associated with longer time to engraftment [41, 42] and viability post-thaw is important to assess in order to be able to predict potential delayed cell engraftment. In their study, Reich-Sloty and colleagues report that mostly granulocytes did not survive cryopreservation [43], other cell types as the CD34+ cells have clearly higher viability rates. We found in our previous study that cryopreservation of PBSC using a closed bag system was safe and effective with a high level of cellular viability and low contamination rates. When comparing the production in cleanroom areas and using closed bag systems, analysis of the viability of leukocytes and CD34+ stem cells and the recovery rate after manufacturing was found not to be compromised [14, 29].

6.9 Comparison of Transplantation Outcome

Cell products being manufactured in cleanroom facilities or with a closed bag system have been also analyzed in regard to their clinical outcome after transplantation. Mortality until neutrophil engraftment and duration until neutrophil/platelet engraftment after transplantation seem to be comparable for products being manufactured in a closed bag system compared to what is reported in the current literature [44–46]. However, there is few corresponding literature available and different factors may influence the outcome after transplantation which need to be taken into account as patient collective (older, heavily pretreated patients compared to younger ones without any significant comorbidities), antibiotic prophylaxis, the amount of transfused hematopoietic stem cells, remission status or conditioning treatment. Of note, mortality rates before engraftment seem to be comparable in the autologous as well as allogeneic setting using closed bag systems compared to centers using stem cell products having been prepared in cleanroom facilities [41, 42]. Future assessment will be necessary to further confirm these results.

6.10 Potential Use of Automatic Cell Manufacturing

GMP-compliant automatic cell manufacturing with the automat CliniMACS Prodigy [47] can be additionally used for a cleanroom-free processing of PBMC providing the advantage for additional major modifications. The automated system is able to save time of qualified staff, and more complex procedures as cell enrichment and depletion or final product formulation can be performed.

Hümmer et al. and Spohn et al. found that for this fully automatic system, recovery of target cells (e.g. CD34+ stem cells) after additional sorting was comparable to what was reported for

the semiautomatic method [47, 48]. However, another study conducted by Stroncek et al. found CD34+ recovery and CD3+ T cell depletion to be lower than with the semiautomated CliniMACS Plus method [49].

Both, closed bag and automatic cell manufacturing systems require highly trained professional staff to supervise and validate the procedure. The closed bag system has to undergo several manual tests, especially tightness tests, before the manufacturing process can be started. Automatic cell manufacturing systems require high technical support and close monitoring. However, the financial efforts are still inferior in comparison to the costs of establishing and maintenance of a cleanroom facility.

6.11 Conclusions/Outcome

Closed bag systems have been investigated for cryopreservation of PBSC and they were shown to be safe in terms of product quality and further clinical outcome after transplantation. One major advantage is that they are clearly cost-saving for the manufacturing of PBSC compared to the high costs necessary for establishment and maintenance of a cleanroom facility. These processes can be extended to other minimally manipulated processes such as donor lymphocyte infusions (DLI). Currently, closed manufacturing processes within automatic cell manufacturing systems are developed and validated to be used for more complex cellular products as advanced medicinal-therapy medicinal products (ATMP). With respect to limited resources within the health care systems, these developments may become an important alternative to the usage of cleanroom facilities and may facilitate the clinical application of cellular therapies including ATMPs.

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