Diploma Thesis

Cryopreservation and Thawing of Peripheral Blood Stem Cells

Submitted by

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Declaration of Authorship

I hereby declare that the work presented here is to the best of my knowledge and belief, original and the result of my own investigations, written without illegal help from others. I state that all passages taken out of publications either whole or in part, in words or ideas, have been indicated.

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1. Abstract

Background: Cryopreservation is the storage method of choice for autologous peripheral blood stem cells (PBSC) and allogeneic umbilical cord blood stem cells intended for haematopoietic stem cell transplantation. The number of haematopoietic stem cells ($CD34^+$) correlates with haematological engraftment. However, the viability of haematopoietic stem cells may be influenced by different variables during cryopreservation and thawing. The aim of the study was to investigate influence of dimethyl sulfoxide (DMSO), temperature and incubation time on different haematological cell types. To our knowledge, no commonly accepted algorithm for processing and short-time storage after thawing of cryopreserved PBSC exists.

Study Design and Methods: Frozen cryovials were thawed in a 37 °C water bath and subsequently DMSO was washed out either prior to (protocol I) or after an incubation period (protocol II). Cell samples were separately incubated at three different temperature stages (6 °C, 20 °C, 37 °C) and analysed after four different sampling delay periods (immediately at 0 min and after 30, 60 and 120 min) for cell counts, cell subtypes (FACS), viability (Annexin V/PI) and repopulating abilities (2nd CFU, LTC-IC). Statistical analysis was performed using a two-way analysis of variance method.

Results: Significant declines in cell counts were seen for some of T lymphocyte subpopulations ($CD3^+$, $CD3^+/4^+$, $CD3^+/8^+$) at 20 °C and 37 °C mainly if cells were incubated in the presence of DMSO; this decline was more pronounced with increasing incubation time. Although in this study protocol, $CD34^+$ numbers did not decrease, frequency of LTC-IC excessively decreased after 60 min (II) and 120 min (I) of incubation at 37 °C, respectively.

Conclusions: These results suggest that post-thaw processing and infusion of cryopreserved peripheral blood stem cell products should not take longer than 60 minutes in order to avoid cell loss and compromise of viability of haematopoietic stem cells. Immediate wash-out of DMSO additionally reduces detrimental effects on cell counts and repopulating potential. Thawed products should be infused at lowest temperatures possible, as warming-up to 37 °C body temperature negatively affects quality of graft.

2. Zusammenfassung

Hintergrund: Die Kryokonservierung ist die Lagerungsmethode der Wahl für Stammzellen aus autologem peripheren Blut und allogenem Nabelschnurblut, welche für eine hämatologische Stammzelltransplantation verwendet werden sollen. Die Anzahl der hämatopoetischen Stammzellen (CD34$^+$) korreliert mit der Geschwindigkeit des hämatologischen Engraftments. Das Überleben der Stammzellen kann von vielen Variablen im Prozess der Kryokonservierung und des Auftauens beeinflusst werden. Ziel der Studie war es, Einflüsse von Dimethylsulfoxid (DMSO), Temperatur und Inkubationszeit auf diverse hämatologische Zelltypen zu untersuchen. Nach unserem Wissensstand gibt es keinen allgemein akzeptierten Algorithmus für die Verarbeitung und Kurzzeit-Lagerung von kryokonservierten Stammzellen aus peripherem Blut nach dem Auftauen.

Studiendesign und Methoden: Kryo-Röhrchen wurden in einem Wasserbad (37 °C) aufgetaut. Anschließend wurde DMSO vor (Protokoll I) oder nach (Protokoll II) einer Inkubations-Periode ausgewaschen. Die Proben wurden separat auf drei verschiedenen Temperaturniveaus (6 °C, 20 °C, 37 °C) inkubiert und nach bestimmten Wartezeiten (sofort bzw. nach 30, 60 und 120 min) auf Zellzahlen, Zellsubtypen (FACS), Überleben (Annexin V/PI) und Fähigkeit zur Repopulation (2nd CFU, LTC-IC) hin untersucht. Unterschiede wurden mit Hilfe einer 2-Weg Varianzanalyse auf statistische Signifikanz überprüft.

Resultate: Zellzahlen von einigen T-Lymphozyten-Subtypen (CD3$^+$, CD3$^+$/4$^+$, CD3$^+$/8$^+$) waren bei 20 °C und 37 °C erniedrigt, hauptsächlich wenn die Inkubation zusammen mit DMSO erfolgte, und ausgeprägter bei längerer Inkubation. Obwohl die Zahl der CD34$^+$ Zellen nicht abnahm, sank die LTC-IC-Frequenz exzessiv nach Inkubation für 60 (II) bzw. 120 min (I) bei 37 °C.

Schlussfolgerung: Diese Ergebnisse legen nahe, dass die Verarbeitung und Infusion von kryokonservierten Stammzellen aus peripherem Blut nach dem Auftauen nicht länger als 60 min dauern sollte, um Zellverluste zu vermeiden, und das Überleben von hämatologischen Stammzellen nicht zu gefährden. Sofortiges Auswaschen von DMSO reduziert zusätzlich die nachteiligen Effekte auf Zellzahlen und Repopulation. Aufgetaute Produkte sollten bei möglichst niedriger Temperatur infundiert werden, und keinesfalls auf 37 °C aufgewärmt werden.

3. Introduction

3.1. Defining haematopoietic stem cells

Haematopoietic stem cells (HSC) are the most immature cells of the entire haematopoietic system. They are capable of self renewal as well as differentiation into more mature progenitor cells, both in a balanced state. In contrast, haematopoietic progenitor cells (HPC) sooner or later undergo terminal differentiation with a progressive loss of proliferative potential. [1]

Initiation of haematopoiesis is limited to a short period of time during embryogenesis. The first populations of haematopoietic cells are formed in the yolk sac; then they migrate to the fetal liver. Cells from the aortic-gonadal region around the great vessels replace cells derived from the yolk sac. Further migration leads to population of bone marrow and spleen. [2] Possibly HSC derive from two independent sites of origin, and some authors also point to a common precursor for haematopoietic and endothelial cells named haemangioblast. [3] Overall, a number of questions in the development of HSC during embryogenesis are still unanswered.

Pluripotent HSC are capable of forming ten different cell types (erythrocytes, neutrophils, eosinophils, basophils, monocytes/macrophages, megakaryocytes/platelets, B and T lymphocytes, mast cells, dendritic cells). In terms of reconstitution, long-term repopulating cells (LTRC, with lifelong repopulating ability regarding all cell lines), and short-term repopulating cells (STRC, with restricted proliferative potential regarding time and cell compartments) can be distinguished. [3]

HSC physiologically circulate and are capable of homing to marrow and other specific sites. Different growth factors and cell junctions to specific cells, such as stromal cells in the bone marrow, play key roles in proliferation and differentiation pathways of HSC. In terms of differentiation, migration from primary to secondary microenvironmental areas, with interactions between cells, extracellular matrix and soluble growth factor molecules may be an important step. [4]

If HSC maintain skills of self renewal may either be decided by a stochastic event or also be influenced by environmental factors. Notch receptor and its ligand seem to play an important role. Expression of Notch receptor is associated with differentiation, whereas expression of Notch ligand is responsible for self renewal. [3]

Several mechanisms which are involved in regulation of life, death, self renewal and differentiation of HSC assure haematopoietic homeostasis. Most HSC express telomerase, but telomere length cannot be kept constant during aging. Furthermore, DNA checkpoint controls and responses to damage are more precise in comparison to somatic cells. Several factors such as Cdk2, reactive oxygen species, Foxo3a, EPO, Flt3, Notch, Mcl-1 and others seem to influence regulation of HSC homeostasis.[1] Transforming growth factor-β1 (TGF-β1) inhibits proliferation of primitive progenitor populations. [5]

Haematopoietic stem and progenitor cells are characterised by analyzing expression of surface markers, allowing discrimination between different subtypes. Stem cells are identified by their expression of CD34 and Thy1, the absence of CD38, CD33 and HLA-DR and of so-called lineage markers normally present on mature blood cells. [1] The CD34 molecule, a transmembrane glycoprotein of 385 amino acids, is expressed on HSC and HPC but also on small vessel endothelial cells and a subset of fibroblasts. [6]

Lineage committed cells are characterized by their expression of CD38, CD33, HLA-DR and a greater proportion of cells in active cell cycle. Analysis of colony forming units (CFU) out of semi-solid cultures is performed to characterise cells morphologically. Whereas CFU-GEMM (granulocyte/erythrocyte/monocyte/megakaryocyte) are capable of multilineage development, more committed progenitors such as CFU-G (granulocyte), CFU-M (macrophage), BFU-E (burst-forming unit erythroid) and CFU-Meg (megakaryocyte) have only single lineage potential. [1]

There may be exceptions to the lineage restriction of committed progenitors. Switching between haematopoietic cell types has been reported. [3] A few HSC may also be able to convert to non-haematopoietic cell types, a feature called totipotency. [2-3]

3.2. Stem cell transplantation

3.2.1. History

The era of haematopoietic stem cell transplantation (HSCT) started with initial observations that either shielding of spleen or femur in mice or intravenous infusion of bone marrow could protect against lethal doses of ionizing radiation. [7]

Experiments in animal models led to advances in stem cell transplantation that allowed using it as a therapeutic tool in the treatment of patients with malignant diseases. The principle was to apply therapeutic radiation doses to eradicate cancer cells and then to rescue the patient’s irreversibly damaged bone marrow by infusion of haematopoietic cells from healthy donors. [7]

If genetically different (allogeneic) cells are infused, donor lymphocytes contained in the graft lead to immunologic reactions, the so-called graft-versus-host disease (GVHD). [8] All early attempts of stem cell transplantation in the 1950s and 1960s failed mostly due to graft failure or untreatable GVHD. Prevention and management of GVHD became possible with the discovery of histocompatibility matching in the 1960s and the introduction of post-transplant immunosuppression, which initially consisted of methotrexate. Results improved when combinations including cyclosporine or tacrolimus were used in later approaches. [7]

Findings of lower relapse rates in patients who developed GVHD let to the description of graft-versus-tumour/leukaemia effects of allogeneic HSCT. Development of newer chemotherapeutic agents such as cyclophosphamide or busulphan as well as monoclonal antibodies more and more replaced total body irradiation in the conditioning regimens. [7]

3.2.2. Pathophysiology

GVHD and graft-versus leukaemia/tumour effects are mediated by donor T-cells; graft rejection is caused by T-cells of the recipient. The intensity of these reactions correlates with the degree of HLA incompatibility between donor and recipient. It is strongest if there is a total mismatch of major histocompatibility antigens. Minor histocompatibility antigens, which are mostly small peptides, normally cause weaker responses. If minor antigens selectively expressed on haematopoietic cells are targeted, graft-versus leukaemia effect may occur without development of GVHD. [8]

GVHD is promoted by injury to the gastrointestinal mucosa due to the preparative regimens, resulting in attraction of T-cells. In addition, cytokines and chemokines involved in this attraction and also genetic polymorphisms play an important role in the development of GVHD. [8]

3.2.3. Preparative regimens

Myeloablative conditioning regimens offer the advantage of reduction of the malignant cell load combined with strong suppression of the autologous immune system which is a prerequisite for engraftment in the allogeneic setting. Furthermore, a release of tumour antigens may activate the immune response via antigen-presenting cells, resulting in a T-cell mediated graft-versus-tumour effect. [8] Additionally, alterations in bone marrow endothelium support homing of transplanted cells. [4]

Myeloablation and immunosuppression can be achieved by fractionated total-body irradiation (TBI) or radiation-free regimens such as busulphan combined with cyclophosphamide. [8]

Recently, non-myeloablative conditioning protocols with markedly reduced regimen-related toxicity have been introduced and allow stem cell transplantation to be performed in older patients and those with significant comorbidities. Therapeutical strategies will in the future concentrate on the improvement of graft-versus-tumour effects and will possibly be supported by customized vaccines or T-cell therapy. [7]

3.2.4. Stem cell sources, mobilization and harvesting

Hematopoietic stem cells can be harvested from bone marrow, peripheral blood and umbilical cord blood. [8]

Bone marrow was in the past the only source of stem cells for both autologous and allogeneic transplantation; it is harvested by multiple punctures of posterior iliac crests. [4, 8] Hip pain is one of the most common side effects after bone marrow aspiration, whereas fever, bleeding or local infections are rare. [9]

Peripheral blood stem cells (PBSC) are harvested after mobilization by leukapheresis. [8] The use of PBSC for allogeneic stem cell transplantation has increased over the past years, recently achieving rates of 50-60 %. [9] Compared to bone marrow, peripheral blood stem cells lead to more rapid haematopoietic reconstitution after transplantation, but also contain higher numbers of T-cells, thus causing higher incidences of GVHD. [8] In unstimulated peripheral blood, the fraction of $CD34^+$ cells among the total nucleated cells normally is 0.06 % compared to 1.1 % in bone marrow. After mobilization, yields of peripheral blood stem cell harvest may possibly be 2-4 times higher than the yield of $CD34^+$ cells collected with bone marrow aspiration. [9]

Mobilization of $CD34^+$ cells generally means recruitment of haematopoietic stem and progenitor cells to circulating blood as a result of previous chemotherapy or administration of cytokines. [10] Granulocyte colony-stimulating factor (G-CSF) is used as a standard mobilization agent; it is administered subcutaneously for 4-5 days and precedes large volume apheresis over 3-4 h on 1 or 2 days. Side effects associated with G-CSF are bone pain, headache and, rarely, an expansion of the spleen. [9] GM-CSF has lower mobilizing activity and higher incidence of side effects. Erythropoietin (EPO also has an ability to mobilize $CD34^+$ cells. Alternatively, stem cell factor (SCF) in combination with G-CSF can be used, but allergic reactions are common. In clinical trials, the binding inhibition of stromal derived factor (SDF-1a) to the receptor CXCR4 is under clinical investigation. [10]

Physiological consequences of $CD34^+$ mobilization are manifold, ranging from release of stem cell factor (SCF) and progenitor cell proliferation to modifications in adhesion molecules. An influence of stromal cell derived factor-1 (SDF-1) and its receptor CXCR4 has been confirmed in several studies. Released stem cells show increased homing back and can reengraft in bone marrow. [10]

Mobilization-related variables include age, preceding chemotherapy and mobilization regimen, platelet counts at time of mobilization and devices used for apheresis. If collection is started when $CD34^+$ counts in peripheral blood range from 8-20 cells/µl, the likelihood of collecting adequate numbers of at least 2.0-4.0 x 10^6

$CD34^+$ cells is increased. Peaks of $CD34^+$ stem cells appear about 3-6 hours after each G-CSF administration in peripheral blood; apheresis can therefore be timed to provide a maximum yield. [10]

Pegfilgrastim, a long-lasting and polyethylene glycol-conjugated form of recombinant human G-CSF may also be used for mobilization of PBSC. Because of increased serum half-life one single subcutaneous administration is sufficient. Fritsch et al. investigated the efficacy of Pegfilgrastim compared to Filgrastim mobilization in children. Fewer apheresis procedures were needed to harvest higher amounts of $CD34^+$ cells/kg body weight and earlier apheresis was possible within the Pegfilgrastim group. Interestingly peaks of leukocytes were 1.0×10^4 lower compared to Filgrastim stimulation. [11]

Hill et al. have investigated optimal timing of apheresis following administration of cyclophosphamide (1.5-3 g/m², day 0) and G-CSF (5 µg/kg, day 1 to completion of apheresis) in order to reach a minimum yield of 2.0×10^6 $CD34^+$ cells/kg. Patients attending first apheresis on day 9 required significantly fewer visits to achieve target yields. $CD34^+$ cell counts ≥ 10/µl in the peripheral blood predicted a desired yield by a single leukapheresis in 61 % of the patients. [12]

Furthermore, Fruehauf et al. found a strong correlation between the steady-state peripheral blood progenitor cells counts and harvest yields after mobilization. Patients not achieving critical steady state peripheral blood $CD34^+$ cell numbers of 0.4×10^6/l may therefore be recognized. [13]

Several studies have investigated differences between PBSC and BM transplantation with different results. Overall PBSC transplantation is associated with faster haematopoietic recovery, comparable rates of acute GVHD but increased rates of chronic GVHD. In adults overall survival of transplantation seems to be equal using PBSC compared to BM. This observation could not be confirmed in children. Higher relapse rates in patients after bone marrow transplantation may possibly be due to lower incidences of GVHD and graft-versus-leukaemia effects. [9]

A study by Elfenbein and Sackstein reviewed differences between mobilized PBSC, steady-state marrow and primed marrow in terms of outcome of stem cell transplantation. The evaluation of engraftment times, acute and chronic GVHD, relapse rates, overall and disease free survival showed equal results between mobilized blood stem cells and primed marrow collected after G-CSF treatment; both were superior to steady-state bone marrow. They hypothesize that G-CSF equally enhances engraftment potential of bone marrow and PBSC. [14]

Umbilical cord blood (UCB) contains higher numbers of stem cells, but is limited in volume. [8] After clamping and disinfection of the umbilical cord, veins are punctured with a sterile needle and cord blood is transferred to collection bags. [15] The advantages are easy collection without risk for mother or infant and low transmission rates of infections; less-stringent HLA matching is required; GVHD occurs less likely, in contrast graft-versus-leukaemia effects seem to be maintained. Disadvantages are the limited volume and therefore limited number of HSC that can be harvested; due to a slow haematological reconstitution, infections are common. [8, 16]

The immediate use of stored units after testing and HLA typing is possible. Volume reduction prior to storage is important in terms of economy. M-Reboredo et al. showed a feasible procedure separating UCB into buffy coat, red cells and plasma by two centrifugation steps using a closed triple bag system. Volume reduction up to 80.0 ± 5.8 % was achieved. [16]

3.2.5. Purification strategies

Purification of targeted cells can be achieved by different strategies: [4]

1. Density gradient separation
2. Pharmacologically by 5-fluorouracil (5-FU) as a cell cycle-specific agent eliminating proliferating cells and sparing stem cells in G_0 phase of cell cycle
3. Use of different binding abilities to cultured layers
4. Binding of specific antibodies coated with magnetic beads (Magnetic Cell Sorting, MACS)

Optimized purification of umbilical cord blood progenitors by immunomagnetic separation was investigated by Kekarainen et al. Enrichment of $CD34^+$ $CD133^+$ and Lin^- HSC from fresh and cryopreserved cord blood was done using an isolation kit modified with additional washing and labelling steps. Acceptable yields with purities > 90 % could be achieved. [17]

Methods for the depletion of red blood cells from umbilical cord blood were compared in a study by Schwinger et al. Although a two-step Ficoll gradient separation and a hydroxyethyl starch separation resulted in higher mononuclear cell (MNC) recoveries, no significant differences in $CD34^+$ recoveries after magnetic cell sorting could be found. [18]

Thawing methods prior to enrichment, growth factor and cytokine combinations and medium conditions may affect the outcome of expansion, sometimes showing preferential expansion of more mature (e.g. CFU-GM) or immature (e.g. LTC-IC) cells. Expansion of up to 50 % of the cord blood unit may possibly be an option to enhance stem cell numbers. [19]

3.2.6. Ex vivo expansion

Advantages of ex vivo expansion include shortened neutrophil or platelet recovery after transplantation and the possible use for retroviral infection of cells in terms of gene transfer. Anyway each manipulation step results in loss of cells, thus possibly reducing outcome. [20] Different protocols and combinations of cytokines for ex vivo expansion of haematopoietic stem and progenitor cells have been developed and tested in animal models. [21]

A study by Keil et al. demonstrated successful expansion of human marrow LTC-IC in suspension culture with addition of IL-10, IL-3 and stem cell factor (SCF). [20]

As investigated by Schwinger and co-workers, a basic cytokine combination of IL-3, SCF and Flt3 supplemented with MGDF increased the number of CFU-GEMM using umbilical cord blood in a short-term culture system. Further addition of G-CSF and EPO additionally increased number of colony forming cells, but gave rise to more differentiated progenitors (CFU-GM, BFU-E). [18]

Ex vivo expansion of human bone marrow long-term culture-initiating cells (LTC-IC) and extended LTC-IC (ELTC-IC) under various conditions was investigated by Ramsfjell et al. The authors concluded that a successful expansion of bone marrow ELTC-IC could be attained after exposure to c-kit ligand (KL), Flt3-ligand (FL) and megakaryocyte growth and development factor (MGDF) or interleukin-3 (IL-3). [22]

Lazzari and co-workers have investigated the effects of cryopreservation on ex vivo expansion of UCB stem and progenitor cells. A combination of thrombopoietin (TPO), FL (flt3-ligand), interleukin-6 (IL-6) and interleukin-11 (IL-11) in serum-free media induced expansion of primitive cord blood cells equally from both fresh and cryopreserved cells. [21]

Möbest et al. reported expansion of $CD34^+$ blood progenitor cells using SCF, FL and IL-3 in a serum-free culture medium. Successful engraftment in NOD-SCID mice was only seen if expansion period in culture was limited to 4 days. [23]

3.2.7. Current use and prospects

Various haematological and non-haematological diseases are indications for treatment by autologous or allogeneic stem cell transplantation. [8] Some of them have been summarized in Table 1.

Table 1: Diseases treatable by stem cell transplantation

malignant diseases
acute lymphoblastic leukaemia
acute/chronic myeloid leukaemia
Hodgkin's disease/Non-Hodgkin lymphoma
myelodysplastic syndrome
solid tumours

non-malignant diseases
a) inherited
immune deficiencies (severe combined immunodeficiency)
haematologic diseases (Fanconi anaemia, β-thalassaemia, Blackfan-Diamond anaemia, amyloidosis)
osteopetrosis (paroxysmal nocturnal haemoglobinuria)
metabolic disorders (mucolipidosis, adrenoleukodystrophy, Lesch-Nyhan syndrome)

b) acquired
aplastic anaemia
autoimmune diseases (multiple sclerosis, systemic lupus erythematosus, juvenile idiopathic arthritis)

In the future, the use of somatic as well as embryonic stem cells may be a therapeutical option in curing malignant but also genetic or degenerative diseases. Difficult challenges such as the directed differentiation of cells to therapeutical cells, cell-replacement strategies and overcoming of immune barriers have to be met.

An efficient engraftment of embryonic cells has been reported with limited success, and also nuclear reprogramming of cells turns out to be difficult. Reports dealing with transdifferentiation of somatic stem cells into tissues of other origin raise new questions. The use of stem cell transplantation for the treatment of myocardial infarction may also become important in the future. [24]

3.2.8. Outcome

Haematopoietic reconstitution after stem cell transplantation (SCT) not only depends on source, quantity and quality of stem cells. Also accessory cells and graft manipulation play an important role. Immunosuppression caused by different conditioning regimens, the degree of histocompatibility, cell composition and problems in the marrow microenvironment may lead to graft failure. Harvested stem cells may be affected by pre-transplantation procedures, but also be damaged subsequently during collection, separation, freezing and thawing procedures. [4]

An infusion of at least 2-2.5 x 10^6 $CD34^+$ cells/kg is usually associated with prompt haematopoietic engraftment. A study by Allan et al. investigated the relationship between the number of viable $CD34^+$ cells at reinfusion and engraftment kinetics. A shorter time to platelet engraftment was shown with higher numbers of $CD34^+$ cells, but no significance was reached for neutrophil engraftment. [25]

However, Specchia et al. found a correlation between total $CD34^+$ cell dose and early neutrophil engraftment. In terms of long-term engraftment, immature subsets of stem cells expressing $CD34^+/CD90^+$ and $CD34^+/CD38^-$ predicted neutrophil and platelet counts at 6 and 12 month. [26]

Transplantation with megadoses of matched unrelated stem cells may be an alternative for patients lacking a suitable HLA-matched family donor. In order to overcome the risk of graft rejection and GVHD, highly purified PBSC using magnetic cell sorting were successfully used for transplantation of children suffering from severe aplastic anaemia in a study by Schwinger et al. [27]

3.2.9. Complications of stem cell transplantation

Mucositis often occurs due to preparative regimens and prolonged myeloaplasia and may affect the whole gastrointestinal tract causing pain, nausea, cramping and diarrhoea.

Hepatic veno-occlusive disease is caused by obstruction of the hepatic circulation due to damage of sinusoidal endothelial cells; symptoms are painful hepatomegaly, jaundice and fluid retention.

Lung injuries as a result of TBI, certain chemotherapeutic drugs or GVHD occur within months after transplantation.

Infections are due to neutropenia, damage of several tissues and immunodeficiency caused by the transplantation procedure.

GVHD is the result of a T-cell mediated immune response and is now classified as acute and chronic GVHD and overlap syndrome. Acute GVHD with affection of skin, gut and liver can be treated with various immunosuppressive agents including corticosteroids. Serious chronic GVHD is associated with loss of self-tolerance and may manifest itself as bronchiolitis, keratoconjunctivitis sicca, malabsorption or general immunosuppression.

Fertility, ovulation and spermiogenesis can be compromised. Children have to be followed up for hormonal dysfunctions including growth and sex hormone deficiencies and should receive specific hormone substitution. Long-term follow up of stem cell transplant recipients is advised due to the increased risk of secondary malignancies. [8]

3.2.10. Infusion-related side effects

Cordoba and co-workers investigated adverse events after infusion of autologous peripheral blood stem cells attributed to the presence of dimethyl sulfoxide (DMSO) as a cryoprotectant. Thawed cells were infused after premedication of steroids, antihistamines and antiemetics. During infusion about 67 % of the patients developed adverse events, most commonly allergic reactions (rash, flushing, bronchospasm), gastrointestinal (nausea, vomiting, cramps) and respiratory symptoms (cough, dyspnoea). [28] Moreover, sedation, headache and dizziness are attributed to DMSO. [29]

Neurological events following the infusion of cryopreserved bone marrow or peripheral blood stem cells were also described by Hoyt et al. Out of 179 patients, one patient presented with transient global amnesia and two patients with cerebral infarction. Involvement of DMSO toxicity was supposed by the authors. [30]

Patient monitoring includes measurement of blood pressure, pulse and echocardiograms. Non-cryopreserved grafts are normally better tolerated, but risk of acute haemolysis is also present. [29]

3.3. Paediatric setting

A large retrospective study by Miano et al. analysed trends of haematopoietic stem cell transplantation (HSCT) in children between 1970 and 2002. Most frequently allogeneic transplantation was performed. About 70 % of transplantations were performed from HLA-matched siblings, but a trend towards alternative donors such as volunteer unrelated donors, partially matched family donors and cord blood was seen. Beyond 1996 the use of peripheral blood stem cells significantly increased. Indications in order of frequency were acute lymphoblastic leukaemia, solid tumours, non-malignant diseases, acute myeloblastic leukaemia, lymphomas, chronic myeloid leukaemia, myelodysplastic syndromes and autoimmune diseases. Transplantation-related mortality (TRM) after 2 years for allogeneic and autologous transplantation was 21 % and 9 %, respectively. Overall, an annual increase in HSCT of 5-10 % could be seen and was explained by an increasing number of transplant centres and broadened disease indications. [31]

Outcomes after allogeneic stem cell transplantation were analysed by Ferry et al. At 10 years, overall survival was 75 ± 5 %, cumulative incidence of chronic GVHD (cGVHD) was 36 ± 4 %; 31 ± 4 % of the patients had a first episode of infections within this period. Pulmonary complications were frequently associated with cGVHD. Thyroid dysfunction, growth retardation and ovarian or spermatogenesis dysfunction were present in 36 ± 4 %, 75.7 % and 41 %, respectively. Secondary malignancies were diagnosed in 7.1 %. Half of the patients developed psychological impairments like depression, sleep disturbance or anorexia. The authors underline that long-term complications increase with time and are mainly associated with total body irradiation. [32]

A situation requiring expert management is harvesting of peripheral blood stem cells in children weighing less than 10 kg. Because of low body weight, the volume of the separation set can exceed 25 % of the total blood volume. Vascular access may be difficult to assure, flow capacity of tunnelled central venous catheters may be inadequate and the use of packed red blood cells for priming of the selection device might be necessary. Sensitivity to hypocalcaemia can also be a problem. [33]

3.4. Cryopreservation

3.4.1. Background

Cryopreservation is the storage method of choice for all types of collected stem cells and other tissues (e.g. haematopoietic stem cells, ovarian tissue, semen). Routinely, autologous PBSC and allogeneic UCB stem cells are cryopreserved in the clinical setting of haematopoietic stem cell transplantation. Recently it has been shown that cryopreservation of PBSC in allogeneic settings might be necessary and feasible in some cases. [34]

The process includes pre-transplantation preparation of the graft, addition of cryoprotectants to different stem cell resources, freezing and thawing procedures and viability assessment. A minimal cell dose for transplantation of 2.5-5.0 x 10^6 $CD34^+$ cells/kg body weight is considered to be standard. [35]

Storage temperatures vary from -196 °C in liquid phase of nitrogen to -156 °C in vapour phase of nitrogen and -80 °C in a mechanical freezer. Also suprafreezing storage at 4°C was examined by several studies. [35]

In a standard controlled-rate freezing procedure, stem cells are frozen at a rate of 1-2 °C/min until a temperature of -40 °C is reached and afterwards the speed is accelerated to 3-5 °C/min until -120 °C. This method is time consuming and also needs qualified staff. The main advantage of this procedure is credited to a possible better compensation of liberating fusion heat, which otherwise may be detrimental to stem cells. However, uncontrolled-rate freezing using a mechanical freezer at -80 °C after cooling down the specimen to -4 °C has also given comparable results in some studies. [35] Rapid freezing of saline cell solutions results in a formation of intracellular ice crystals, whereas slow freezing can cause extracellular ice crystal formation and osmotic dehydration of cells. [36]

In the course of cryopreservation, more immature haematopoietic stem cells seem to be better preserved compared to BFU-E and CFU-GM [35]. Apoptosis and necrosis of frozen-thawed cells may occur due to cryopreservation-induced loss of adhesion molecules but also depend on the cooling rate and the concentration of cryoprotectants. Cryopreservation of granulocytes gave poor results. Clumping of platelets and/or granulocytes can increase infusion-related toxicity. [36] A study suggested that cryopreservation may also preferentially destruct T-cells during the freeze-thaw process, thus lowering the incidence of GVHD. [37]

The addition of cryoprotectants to stem cell concentrates is necessary to avoid intra- and extracellular ice crystal formation resulting in cell death. Dimethyl sulfoxide (DMSO) at a concentration of 10 % combined with saline and serum albumin is the standard cryoprotectant. Hydroxyethyl starch (HES) in combination with DMSO has also successfully been used. Furthermore propylene glycol, trehalose and a combination of alpha tocopherol, catalase and ascorbic acid are sometimes used. [35]

Usually frozen specimens are thawed in a 37 °C water bath until ice crystals disappear. Alternatively 37 °C warm gel pads were used in a study. Time and temperature of incubation after thawing have been investigated by several authors. Rapid processing in order to avoid cell clumping and exposure of cells to DMSO is necessary. [35]

Moreover, washing procedures in order to reduce DMSO-related side effects have been applied. A two step dilution with 2.5 % human serum albumin (HSA) and 5 % dextran 40 followed by centrifugation reduced DMSO concentration to 1.7 %. However, the procedure is associated with loss of cells. [35]

The avoidance of microbial contaminations is essential for cryopreservation of stem cell grafts. Contamination rates vary from 0-4.5 %. Bone marrow cryounits are associated with higher contamination rates due to more invasive harvesting procedures. Also post-thaw procedures bear risk of contamination. Surprisingly the stability and spread of viruses in liquid nitrogen has been reported. Clean processing, microbial monitoring through all stages of procedure and screening of donors may prevent infectious complications. [35]

Various functional assays are available for the investigation of donor grafts. Cell counters and flow cytometric investigation of $CD34^+$ cells are used for quantification. Viability can be assayed by trypan blue exclusion, 7-amino actinomycin D and propidium iodide. Clonogenic assays include CFU-GM, CFU-GEMM, BFU-E and LTC-IC. Engraftment in NOD/SCID mice (non-obese-diabetic/severe combined immunodeficient) is furthermore used to evaluate quality of stem cells. [35]

3.4.2. Physical basics

Freezing starts with ice-like configuration of some water molecules until they reach a critical size; afterwards favourable energetic circumstances allow further crystal growth. Extracellular ice formation usually occurs before intracellular freezing. Osmotic pressure forces water to leave the cell and macromolecule-rich domains are excluded due to dehydration. [38]

During freezing an equilibrium phase transition between liquid and solid phase liberates fusion heat. This process generally represents a balanced state between the lower enthalpy of the solid phase and higher entropy of the liquid phase. At a specific temperature (T), phase transition occurs with change in entropy (Δ S) and heat of fusion (L) is liberated according to the following formula. [38] A prolonged transition period from liquid to solid phase resulted in higher degrees of cell destruction. [39]

$$\boxed{\boldsymbol{L = T\,\Delta\,S}}$$

High viscosity and sufficiently quick cooling can create a stable non-equilibrium and amorphous phase and also hinder formation of nuclei and ice crystals, a process called vitrification. Vitrification protects membranes from dehydration or diffusion of solutes and lowers the probability of crystallization. [38]

In order to limit cell death during the process of freezing and thawing cryoprotectants like dimethyl sulfoxide (DMSO) are added. DMSO, a colourless organic and water-affine liquid, freezes at 18.5 °C. It crosses cell membranes and therefore creates high osmolarity both in- and outside the cells, thus offsetting salt gradients and preventing from intracellular ice crystal formation. [36]

The osmolarity of a cryoprotectant solution containing 10 % DMSO is about 1.4 Osm compared to 270-300 mOsm for isotonic solutions. Rapid efflux of water in order to balance the chemical potential follows, resulting in volume change and possible cell lysis. Because expansion is more critical to produce lysis, attention has to be paid to post-thaw protocols. [40]

Temperature dependent toxicity of cryoprotectants and osmotic excursions may also damage cells during the first equilibrating phase until cells return to normal size when the cryoprotectant has penetrated through the membranes. Finally further osmotic excursions occur during thawing and elution of cryoprotectants. [41]

Investigations on response of cord blood CD34$^+$ cells showed ideal osmometric response from 160-1800 mOsmol/kg. Hypotonic conditions were less well tolerated than hypertonic exposure. Membrane damage was significantly increased when cells were exposed to an osmolality of 1800 mOsmol/kg. Colony forming capacity was also impaired below 160 and at 1500 mOsmol/kg. After exposure to the cryoprotectant, CD34$^+$ cells differently shrank to approximately 55-60 % of their isotonic volume depending on temperature. The authors recommend the introduction of equilibration periods after addition of the cryoprotectant and before cooling is started. Also phased addition of the diluent in the thawing process reduced cell swelling. [41]

An evaluation of direct DMSO-related toxicity showed no significant loss of cells or functional impairment if cells were exposed to concentrations of 5-25 %. Also exposition through longer time periods at 2 °C did not affect membrane integrity or function, only a 60 min exposure to 25 % DMSO at 20 °C reduced survival. Cooling rates from 1-2.5 °C/min were found to be optimal. [42]

3.4.3. Cryoprotectants

Cryoprotectants can be divided into two groups by their ability to enter cells. While dimethyl sulfoxide (DMSO), glycerol, sucrose, trehalose, methanol, glucose, proline, glycine betaine, fructose, galactose and lactose permeate cell membranes, others like hydroxyl ethyl starch (HES), dextran and polyvinylpyrrolidine are not able to do so. [43]

Wide variations in cryopreservation techniques among different centres exist regarding the final concentration of DMSO, content of plasma proteins, cooling equipment and storage conditions. [44]

Solutions normally used contain either 10 % DMSO or 5 % DMSO in combination with 6 % hydroxyethyl starch (HES). A single-blinded, randomized study by Rowley and co-workers compared these two protocols. Patients receiving DMSO/HES showed significantly faster white blood count and absolute neutrophil count recovery, however differences were small. [44]

Varying concentrations of DMSO were investigated by Windrum et al. Final concentrations mainly used were 10 %, 5 % and 7.5 %. DMSO-related toxicity was seen in 1.5 % with 10 % DMSO but only in 0.3 % when lower concentrations were used or washing strategies were performed. [45]

Abrahamsen and co-workers compared cryopreservation using 5 % or 10 % DMSO with regard to cell viability, apoptosis and necrosis. Higher amounts of viable, non-apoptotic, non-necrotic CD34$^+$ cells were seen in the 5-%-DMSO-group. Median cell recoveries were 88 % and 78 % for 5 % and 10 % DMSO, respectively. The authors conclude that cryopreservation with 5 % DMSO alone is feasible [46]. A study by Akkök et al. retrospectively compared clinical outcome in patients receiving grafts previously cryopreserved in 5 % and 10 % DMSO, respectively. No significant differences in neutrophil or platelet engraftment, transfusion requirements or hospital stay were found. [47]

Cryopreservation with 5 % DMSO and 3 % HES in a -80 °C mechanical freezer resulted in higher viability and CFU-GM activity compared to 10 % DMSO and use of rate-controlled freezing. [48]

A study by Limaye and Kale investigated the addition of different membrane stabilizers and bioantioxidants to conventional freezing medium containing 10 % DMSO. Addition of the disaccharide trehalose at a concentration of 25 µg/ml resulted in higher CFU counts both at -196 °C and -80°C without lineage specificity, thus confirming membrane stabilizing effects. Addition of taurine also positively affected clonogenic outcome. Bioantioxidants like ascorbic acid, catalase or α-tocopherol have also been added in studies, but only catalase had significant cryoprotective effects. Additional effects were seen when combining catalase with trehalose. [49]

Cryopreservation using trehalose and sucrose was further investigated by Rodrigues et al. In comparison to a standard protocol using 10 % DMSO, addition of 30 mmol/l trehalose with 2.5 % DMSO or 60 mmol/l with 5 % DMSO gave similar results in terms of CD34$^+$ count and clonogenic capacity. The authors suggest that a reduction of the concentration of DMSO is therefore possible. [50]

Successful cryopreservation of purified $CD34^+$ cells in 7.5 % DMSO has been reported by Beaujean et al. [51]

3.4.4. Cell concentrations

The effect of pre-freeze cell concentration on recovery of cells after thawing was studied by Rowley et al. Cells were cryopreserved at an average cell concentration of $3.7 \pm 1.9 \times 10^8$ cells per ml. No detrimental effects on cells or engraftment kinetics could be shown. Cryopreservation at high cell concentrations may therefore reduce volume and also limit required amount of DMSO. [52]

Meyer and co-workers investigated optimal cryopreservation protocols for umbilical cord blood stem and progenitor cells. Best results were obtained with 10 % DMSO and 2 % human serum albumin (HSA) at high cell concentrations. Fast addition and removal of DMSO further improved outcome. [15]

Cryopreservation of fetal human liver-derived haematopoietic progenitor cells was evaluated by Zhao et al. Optimal recovery was reported using 5 % or 10 % DMSO in combination with 20% or 70 % fetal bovine serum (FBS). Rate-controlled freezing at concentrations of 1×10^6 cells per ml was further recommended. [53]

3.4.5. Controlled vs. uncontrolled-rate freezing

A model using an optimized freezing curve to improve cryopreservation outcome was introduced by Tijssen and co-workers. A theoretical model was used to define optimal conditions, giving a slow cooling rate of approximately -1 °C/min at high subzero temperatures, increasing in speed at -20 °C and finally decreasing again. Post thaw viability was significantly increased if this optimized freezing protocol was used in combination with 10 % DMSO but not with 5 % DMSO. [54]

Cost reduction and facilitation are major benefits of uncontrolled-rate freezing. Freezing at -80 ° for 24-48 hours prior to storage in liquid nitrogen did not affect either recovery of $CD34^+$ cells, LTC-IC or committed progenitors and had no impacts on long-term engraftment. [55]

Another uncontrolled-rate freezing protocol was investigated by Cilloni et al. Cells were frozen at -80 °C for 24-48 hours and subsequently stored in a mechanical freezer at -140 °C. Although a significant reduction in the absolute number of nucleated cells was recognized, committed progenitors and primitive LTC-IC were mainly unaffected. [56]

Freezing to -80 °C using immersion in a methanol bath showed that obtained cooling rates were constant around 1.2 °C/min, regardless of the bag volume. Standardization of unprogrammed-freezing may therefore be possible using this technique. [57]

Similar viabilities after one month of storage were observed in a study by Ratajczak et al. comparing controlled-rate freezing and unprogrammed mechanical freezing. [58]

3.4.6. Short-term storage before cryopreservation

It has been shown that short-time storage of peripheral blood stem cells is possible without negative effects on cell viability or engraftment potential, but decreased viability was seen when cells were stored for 24-72 h. A retrospective study by Parkins and co-workers compared immediate cryopreservation and cryopreservation after overnight storage at 2-6 °C. Viability of stored cells was identical before and after overnight storage and also no impacts on engraftment potentials were found. [59]

In terms of overnight storage, Petzer et al. investigated effects on primitive progenitor cells such as LTC-IC. Different conditions were tested. Mean viability values were similar regardless of the protocol used. Optimal recoveries of 99.9 ± 8.5 % were seen at 4 °C with addition of autologous plasma and the possibility of gas exchange both for colony forming cells (CFC) and long-term culture initiating cells (LTC-IC). [60]

A study by Matsumoto et al. investigated storage conditions at subzero non-freezing temperatures. Subzero non-freezing temperatures range from 0 °C to the specific freezing point of a solution. Supercooling slowly reduces temperature below the freezing point, but liquids remain unfrozen. However storage at 4 °C resulted in higher survival of CFU-GM. Also optimal storage at supercooled temperatures (-2 °C) without cryoprotectants for up to 72 hours was reported. [61]

Liquid storage and shipment of cord blood was analysed by Hubel et al. Original and diluted samples were stored and shipped via overnight courier for 24 or 72 h and cryopreserved afterwards. Analysis of thermal history showed that precooling the samples had positive effects on temperature changes during shipment. Storage and shipment durations beyond 24 hours resulted in decreased viable cell recovery after thawing. [62]

3.4.7. Storage of cryopreserved units

Long-term storage of autologous stem cell grafts has been evaluated by Liseth and co-workers. Peripheral blood progenitor cells were cryopreserved in various concentrations of DMSO and stored for at least 5 years. Viability of $CD34^+$ cells and different T- and natural killer (NK)-cell subsets was analysed. Higher viability for $CD34^+$ cells was reported when cells were cryopreserved in 4 % or 5 % DMSO compared to 2 % and 10 %. This was also true for lymphocytes in general, but the different subsets were differentially protected depending on DMSO concentrations used. No major impact on NK-cells could be seen. In terms of engraftment potential after a median storage time of 42 months comparing the use of 5 % or 10 % DMSO, no differences have been reported. The authors conclude that cryopreservation using 5 % DMSO as a cryoprotectant is superior in terms of long-term storage. [63]

Storage is recommended for stem cell grafts in view of autologous stem cell transplantation in a paediatric setting. Because mobilization may be compromised due to pre-treatment modalities, stored grafts can therefore be used in case of relapse or occurrence of a secondary malignancy. [64] Due to difficulties in harvesting stem cells in time of relapse, some centres cryopreserve stem cells in sufficient quantities for two autologous stem cell transplantations. [63]

3.4.8. Microbial contamination

Microbial contamination of cells during storage is a major problem in the course of cryopreservation. Blood bags are frequently used to store cells and as a result of extremely low temperatures bag may fracture resulting in contamination. Mismatch of expansion and contraction due to thermal changes between bag and stem cell medium may cause these brittle fractures. Furthermore increased pressure within the bag due to gas expansion (e.g. CO_2) can stress the polymer. Geometrical properties of the bag and optimized welding procedures can reduce stresses. [65]

Contamination may occur due to an infection of the apheresis venous line and venous access manipulation, but also during ex vivo processing, cryopreservation, thawing and infusion. Testing for bacterial contamination at four different points in the course of cryopreservation was done by Larrea and co-workers. Positive cultures were seen in 6.9 % after PBSC collection, 7.2 % before cryopreservation, 5.4 % after thawing and in 2.3 % after washing. Coagulase-negative staphylococcus was most often isolated. [66]

3.4.9. Reconstitution potentials after cryopreservation

Reich-Slotky and co-workers have investigated viability and recovery of frozen-thawed $CD34^+$ cells. They found a good correlation between post-thaw viability and non-granulocytic white blood cells, indicating rather high viability of immature stem cells and lymphocytes after cryopreservation. [67]

A study describing long-term reconstitution and clinical outcome after cryopreservation was performed by Galmes et al. A method using 5 % or 10 % DMSO as a sole cryoprotectant and uncontrolled-rate freezing at -80 °C was evaluated over a twelve-year period. No differences in long-term haematologic recovery have been reported. The 5 %-DMSO-group showed a faster recovery of platelets. [68]

Recovery after autologous stem cell transplantation in lymphoma patients and influence of number of $CD34^+$ cells was investigated by Mounier and co-workers. A significant correlation between pre-freeze and post-thaw $CD34^+$ cell counts has been described. $CD34^+$ counts were predictive for both granulocyte and platelet recovery and values above 5×10^6 cells/kg predicted normal blood counts one year after transplantation. [69]

3.5. Thawing

3.5.1. Methods

Frozen blood products are usually thawed using a 37 °C water bath with sterile distilled water until total disappearance of ice crystals. Others use gel pads or heated plates at a temperature of 37 °C. Alternatively, thawing by keeping the blood products at room temperature is possible.

3.5.2. Post-thaw incubation

The effects of incubation time and temperature on post-thaw viability of haematopoietic progenitor cells were studied by Yang et al. Results of different viability assays were compared. Incubation at 0 °C significantly reduced the loss of viable cells. In contrast to membrane integrity assays, no effects for incubation time were seen in recovery of CFU-GM. [70]

3.5.3. Wash-out of DMSO

In order to avoid infusion-related side-effects, reduction of the amount of DMSO before infusion of grafts can be necessary (e.g. by washing procedures). [71]

Akkök et al. have studied the impact of manual DMSO depletion for PBPC autografts on engraftment kinetics in a total of 53 consecutive patients. The reduction procedure resulted in extracellular DMSO concentrations of less than 1 %, but also significantly reduced CD34$^+$ cell counts and number of neutrophils. No difference in neutrophil engraftment was seen, and time to platelet engraftment was prolonged only from 12 to 14 days when DMSO was washed out. As expected, the wash-out procedure reduced adverse effects. [71]

Nagamura-Inoue and co-workers investigated the influence of DMSO wash-out on clinical outcome of cord blood transplantations. A two-step dilution method reduced DMSO concentration to less than 1.7 %. No effects either on neutrophil or platelet recovery were seen comparing the washed and unwashed group, respectively. [72] A study by Foïs et al. evaluated clinical toxicity of thawed and washed peripheral blood stem cell grafts. Only 19 % of the patients developed infusion-related adverse events, mostly of low grade. [73]

3.6. Quantity, viability and quality

3.6.1. Flow cytometry

Various clinical applications have been developed using fluorescence activated cell sorting (FACS). Fluorescent molecules are excited by lasers at a specific range of wavelengths and they therefore emit light of specific ranges which can be measured by different detectors (channels). Fluorochrome-coupled monoclonal antibodies or various fluorescent reagents are used. In order to avoid measurement of light in more than one channel (spill-over), corrections for fluorescence light compensation have to be applied. Control samples and gating path strategies are necessary to define boundaries between subsets of cells under investigation. [74]

Two different strategies are available for the enumeration of white blood cells (WBC). Dual-platform assays combine a per cent enumeration of the investigated cell subset by flow cytometry with the calculation of the white blood cell count by a cell analyser. In contrast, single-platform assays use a known number of fluorescent beads within the assay to calculate cell counts. Variations in leukocyte counts and the need of a second instrument can be avoided. [75] Protocols like Pro-COUNT^{TM}, single and dual-platform ISHAGE and Milan/Mulhouse have been described and compared by several studies. [75-78]

In the course of cryopreservation, this technology is important to quantify CD34$^+$ cells in grafts in order to estimate engraftment potential. [79]

3.6.2. Membrane integrity

Determination of viable cells after thawing remains challenging due to a lack of validated and accurate assays. Assessment of membrane integrity, CD34$^+$ cell enumeration and clonogenic assays are mostly used. Trypan blue (TB) is a membrane integrity stain colouring damaged cells blue, but absorption of TB can vary. Fluorescence methods using acridine orange (AO) or Syto in combination with propidium iodide (PI) can also evaluate membrane integrity. While Syto penetrates viable cells and stains nucleic acids, PI can only penetrate cells with damaged membranes. [70]

Apoptosis of cells can be determined morphologically by nuclear fragmentation and formation of apoptotic bodies but also by detection of DNA degradation. Plasma membrane alterations and exposure of phosphaditył serine (PS) on outer plasma membranes are also associated with apoptosis. Fluorescent vital stains like acridine orange or fluorescein diacetate are able to enter cells and stain DNA, RNA or mitochondria. Stains like 7-amino actinomycin D (7-AAD) or propidium iodide (PI) are only able to enter cells if membrane permeability is increased. [80]

Annexin V is able to selectively bind to PS and can therefore be used to detect apoptosis in cells. [81] Viable cells remain unstained by annexin V-fluorescein isothiocyanate (FITC). [82]

Variations in quality of cryopreserved UCB grafts may be caused by early apoptotic CD34$^+$cells and therefore impacts on outcome after transplantation are possible. A flow cytometric assay for Annexin V binding was used by Shim et al. to evaluate viability of CD34$^+$ cells. [83]

De Boer and co-workers analysed early apoptosis of cryopreserved and thawed CD34$^+$ cells with the vital stain Syto16 in combination with the permeability marker 7-AAD. They found that 58 % of the CD34$^+$ cells in the investigated samples were apoptotic or secondary necrotic; the authors therefore conclude that lower threshold doses of CD34$^+$ cells are necessary in terms of haematologic recovery. [84]

Another study using Syto16 and 7-AAD in combination with a P-glycoprotein inhibitor (PSC833) to identify apoptotic cells was performed by Schuurhuis et al. Using this method, numbers of non-apoptotic viable cells were lower in contrast to viability evaluation using trypan blue or Annexin V. The authors suggest that cells with lower Syto16 fluorescence reflect early apoptotic cells. [80]

3.6.3. Culture assays

Appropriate assays should specifically and precisely measure the cells of interest, their quantity and proliferative potential. Unfortunately conclusions from the phenotype of a cell to its function cannot be drawn. Samples contain a heterogeneous mixture of cells, which additionally changes during proliferation and self renewal. Assays measure the number of cells and lineages representing progeny. While stem and progenitor cells need many divisions and a period of at least 5 weeks to produce differentiated cells, lineage-committed cells will do so in approximately 3 weeks. Influence of many growth factors on proliferation and differentiation has been described, but little is known on additional regulation of accessory cells and molecules. [85]

Colony assays or single-cell cultures selectively allow proliferation of a single lineage and therefore allow inferences on proliferative potential of a single progenitor. In contrast, if heterogeneous cell populations are assayed for example in liquid cultures, these conclusions are impossible. [85]

Short-term clonogenic assays are suitable for measuring quantitative changes in different cell types, evaluation of growth factor responsiveness or regulation of differentiation. An addition of nutrients and growth factors is necessary. [4] They identify and quantify lineage-restricted progenitors using viscous semi-solid media like methylcellulose, agar or plasma clot. Thus erythroid, granulocytic, macrophagic and megakaryocytic progenitors can form colonies of different levels of maturity. Due to a restricted lifespan of the medium, self-renewal of stem cells cannot be adequately detected. [85]

Long-term in vitro assays are able to identify these immature progenitors by extending time periods beyond 3-5 weeks and therefore allow complete differentiation. [85] Long-term culture systems moreover offer possibilities of investigating self-renewal of different stem and progenitor cell types. After culturing a feeding layer out of stromal cells, addition of haematopoietic cells leads to migration to the stromal layer, proliferation and release of CFU-GM to the supernatant medium. In weekly intervals, half of the supernatant is harvested and cultured in short-term assays, providing proliferation activity measurements. After 4-5 weeks of culture and disappearance of pre-existing CFU-GM, further production of CFU-GM can be credited to primitive cells, so called long-term culture-initiating cells (LTC-IC). [4]

Limiting dilution experiments using Poisson’s statistics are necessary to evaluate frequency of LTC-IC. The output of colony-forming cells in culture is retrospectively analysed. [85]

A production of myeloid progenitors in long-term culture assays of more than 60 days was credited to Extended LTC-IC (ELTC-IC), possibly reflecting a more primitive population of stem cells. Further investigations showed that ELTC-IC were only present in the $CD34^+$ $CD38^-$ population whereas LTC-IC could also be found in the less primitive $CD34^+$ $CD38^+$ population of stem cells. [22] However, ELTC-IC may remain in a prolonged quiescent state. [85]

In a study by Summers et al. cord blood cells were comparatively selected for the glycoprotein $AC133^+$ or $CD34^+$ and analysed in culture for up to 30 weeks. Furthermore G_0 and G_1 phases of cell cycle were analysed. An LTC-IC incidence of 1 in 4.2 cells was seen in the $AC133^+$ G_0 cell fraction. It has also been reported that $AC133^+$ $CD34^-$ cells were capable of generating $CD34^+$ cells in culture, thus maybe reflecting a more primitive and ancestral group of cells. [86]

Moezzi et al. evaluated effects of cryopreservation on cord blood progenitor cells. Although viability decreased, no differences in $CD34^+$ $CD38^-$ numbers, clonogenic and in vitro expansion potential were seen. [87] Repeated freeze-thaw procedures resulted in a decrease of more differentiated progenitors while LTC-IC remained unchanged. [88]

Other assays characterize primitive cells by their ability to form cell crowds similar to cobblestone areas (cobblestone area-forming cells, CAFC). [4]

In vivo assays are mostly done in the murine system and assay longevity, multipotentiality and ability to home to the bone marrow. In competitive repopulating unit assays (CRU) decreasing numbers of distinguishable test cells are injected and the proportion of donor-derived populations with at least ≥ 1 % cells is measured. Immune-deficient mice with several genetic manipulations have been raised for the development of new assays (e.g. SCID, NOD-SCID). Within 2 weeks short-term repopulating cells can be detected whereas 8-10 weeks are necessary to evaluate long-term repopulating cells. Problems include a decrease of primitive progenitors with time, fluctuations in homing abilities but also the development of thymic lymphomas in NOD-SCID mice. [85]

Cord blood stem and progenitor cells were tested after 15 years of cryopreservation for colony-forming potential, ex vivo expansion and engraftment capability by Broxmeyer and co-workers. Extensive proliferation ability and engraftment in NOD/SCID mice was reported. [89]

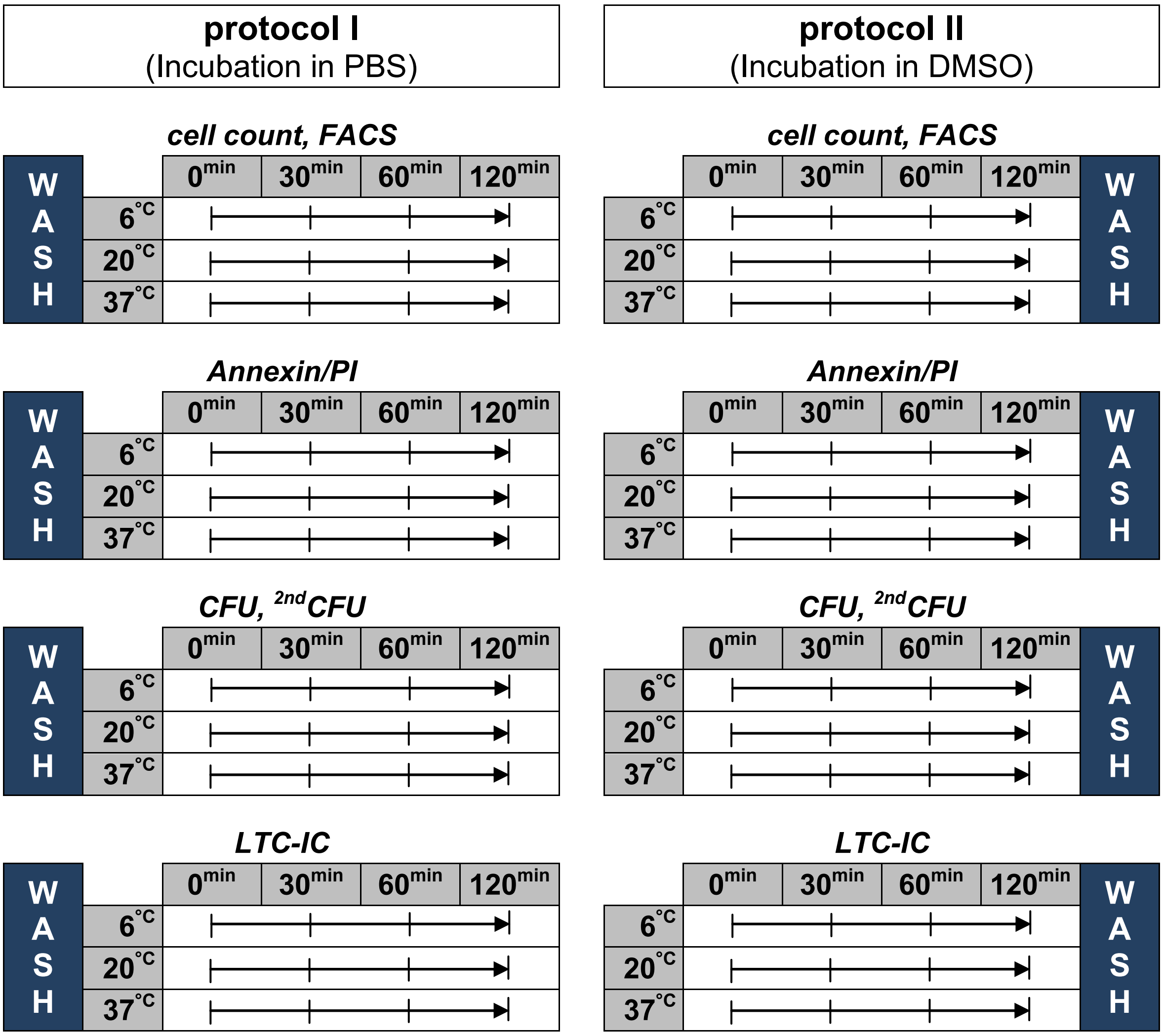
4. Materials and Methods

4.1. Study protocol and assays

4.1.1. Study protocol

Frozen peripheral blood stem cells were processed based on two different protocols. After thawing, a washing step in order to remove DMSO was either performed prior to (protocol I) or after an incubation period (protocol II).

Cells therefore were incubated with DMSO (II) or without DMSO (I) and analysed after 4 different sampling delay periods (0 min, 30 min, 60 min, 120 min). Additionally, incubation periods were performed separately at 6 °C; 20 °C and 37 °C for both protocols. Cell counts, viability and proliferative potential were determined by several assays.



4.1.2. Cells and pre-freeze assays

Peripheral blood stem cells from a single patient were obtained by leukapheresis after informed consent and used for all experiments. 4.1 ml were withdrawn from the apheresis product, which was later used for autologous stem cell transplantation.

A cell counter (Sysmex SE-9500, Sysmex, Germany) was used for determination of cell counts (WBC 244.7 x 10^9/l, absolute WBC count 1.0 x 10^9 cells, 69 % MNC). Subsequently the raw material was diluted with PBS (4.1 ml cell suspension + 45.9 ml PBS). Samples of 1 ml for FACS analysis and 1 ml for CFU assays were drawn. FACS analysis revealed 0.65 % $CD34^+$ within the raw material. Diluted cell suspension was placed on ice prior to further processing, in order to reduce temperature to 0 °C.

4.1.3. Cryopreservation

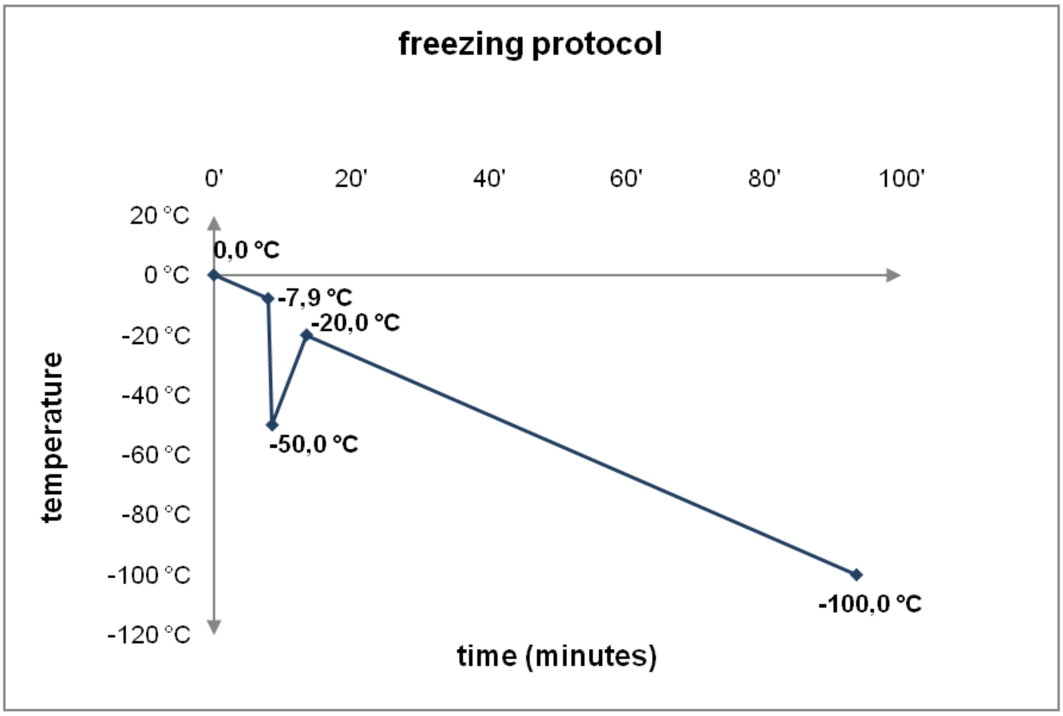
The cryopreservation solution consisted of 70 % Modified Eagle Medium (MEM), 20 % DMSO and 10 % human serum albumin (HSA) and was also precooled by placement on ice. An equal volume of the cryopreservation solution was added to the cell suspension (50 ml diluted cell suspension + 35 ml MEM + 10 ml DMSO + 5 ml HSA), gently mixed and placed on ice again. Final concentrations of 1.0 x 10^7 cells/ml, 10 % DMSO and 5 % HSA were obtained.

Aliquots of 1 ml each were transferred to precooled cryovials and immediately placed in a controlled-rate freezer (IceCube 1810, SY-LAB, Neupurkersdorf, Austria), which was prepared by an equilibration period of 10 min at 0 °C.

The controlled-rate freezing programme was then started and cells were frozen with a rate of -1 °C/min until a temperature of -7.9 °C was reached. Rapid freezing to -50 °C was then performed in order to avoid cell damage by liberation of fusion heat. The local transition point for the laboratory was estimated at -8.2 °C.

Temperature was then raised up to -20 °C again before slow freezing to -100 °C again at a rate of -1 °C/min started (Figure 1). Finally, vials were stored in the liquid phase of nitrogen.

Figure 1: controlled-rate freezing protocol using a SY-LAB Ice Cube 1810



4.1.4. Thawing

Cryovials were taken out from the storage container, brought to the stem cell laboratory and thawed in a 37 °C water bath using sterile distilled water until ice crystals disappeared and content was fully liquefied.

4.1.5. Wash-out of DMSO

After splitting each specimen into 4 portions of 200 µl and transfer to falcon tubes (15 ml), each portion was equally diluted with PBS/2 % HSA (200 µl specimen + 200 µl dilution solution) and centrifuged at 1.200 rpm (320 g) for 10 min at room temperature (20 °C). The supernatant was removed and the pellet was resuspended in 1 ml of PBS/2 % HSA. This step was either performed before (protocol I) or after (protocol II) the incubation period. Wash-out was always performed prior to further analysis.

4.1.6. Cell counts

A CASY® TT Cell Counter (Innovatis AG, Bielefeld, Germany) was used to determine cell counts directly after thawing and incubation periods. With this technology, values are measured by electronic pulse area analysis. Only absolute number of white blood cells (WBC) was used for further analysis.

4.1.7. FACS immune status

Flow cytometric analysis was performed using a FACS Calibur Flow Cytometer (Becton Dickinson, Heidelberg, Germany). The following surface antigens were included: CD3, CD4, CD5, CD8, CD14, CD19, CD34, CD45, CD56 and HLA-DR. A dual-platform procedure was performed combining the absolute number of white blood cells (WBC) measured by a CASY® TT Cell Counter with percentages of subtypes determined by flow cytometry.

$$\boxed{\boldsymbol{absolute\ number}\ (\boldsymbol{cells/\mu l}) = \boldsymbol{WBC} \times \boldsymbol{subtype}\ (\%)}$$

Fluorescein isothiocyanate (FITC), phycoerythrin (PE) and PerCP (peridinin-chlorophyll protein complex) were used as fluorescent dyes. Most of the conjugated antibodies were purchased from Becton Dickinson (Heidelberg, Germany) except for CD45 antibodies (Beckman Coulter, Krefeld, Germany), CD19 and CD14 antibodies (Dako, Vienna, Austria). Tubes were set up according to the following panel (Table 2).

Table 2: Pipetting scheme for FACS analysis

tube	FITC	PE	PerCP
# 1	-	-	-
# 2	mIgG1 (5 µl)	mIgG2a (2,5 µl)	mIgG1 (5 µl)
# 3	-	CD45 (2 µl)	-
# 4	CD45 (5µl)	-	-
# 5	-	-	CD45 (5 µl)
# 6	CD19 (2 µl)	CD3 (2 µl)	CD45 (5 µl)
# 7	-	CD19 (5 µl)	-
# 8	CD8 (5 µl)	CD4 (5 µl)	CD3 (5 µl)
# 9	CD3 (1 µl)	-	HLA-DR (5 µl)
# 10	-	CD56 (5 µl)	CD3 (5 µl)
# 11	CD14 (1 µl)	CD34 (5 µl)	CD45 (5 µl)

50 µl of the cell suspension was transferred to each antibody-containing tube and incubated for 20 min at 6 °C. Subsequently 1 ml of buffer (consisting of PBS, BSA

and NaN_3) was added followed by centrifugation at 13.000 rpm for 30 sec, removal of supernatant and analysis. 5 µl were withdrawn for determination of cell counts.

A CD45/SSC gating strategy was used as described by Barnett et al. [90] Briefly, a CD45/SSC plot was used to set a so-called lymphomononuclear gate. Lymphocytes are determined both by the gate and carriage of appropriate surface antibodies.

4.1.8. Viability (annexin V/propidium iodide)

Apoptotic or necrotic cells were additionally determined using an annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining procedure combined with a CD3/14/19-allophycocyanin (APC) counterstain (Becton Dickinson, Heidelberg, Germany). Tubes were prepared as described in Table 3.

Table 3: Pipetting scheme for viability analysis

tube	CD3-APC	CD14-APC	CD19-APC
# 1	-	-	-
# 2	5 µl	-	-
# 3	-	5 µl	-
# 4	-	-	5 µl

100 µl of the cell suspension was transferred to each antibody-containing tube and incubated for 20 min at 4 °C. After the addition of 1 ml of annexin-binding-buffer, a centrifugation step with 13.000 rpm for 30 sec followed. The supernatant was removed and annexin V/PI was added (Table 4).

Table 4: Addition of annexin V and PI

tube	annexin V-FITC	PI
# 1	-	-
# 2	5 µl	5 µl
# 3	5 µl	5 µl
# 4	5 µl	5 µl

Tubes were then incubated for 15 min at 20 °C protected from light using a polystyrene box prior to analysis. Vital cells were defined to be annexin V negative and PI negative, apoptotic cells to be annexin V positive and PI negative and necrotic cells to be positively stained by annexin V and PI.

4.1.9. CFU and ^{2nd}CFU-assays

Quality of myeloid progenitor cells was evaluated by performing colony-forming unit assays (primary CFU-GM). A semisolid methylcellulose-based culture medium (Methocult H4534, StemCell Technologies, Vancouver, Canada) was used. Thawed cells at a concentration of 1.5 x 10^5 cells/ml were mixed with 500 µl of the medium and transferred to 12-well flat-bottom suspension culture plates (Greiner Bio One, Kremsmünster, Austria). Wells were then incubated for 14 days at 37 °C in humidified air containing 5 % CO_2. Colonies were scored if containing more than 50 cells and frequency was calculated.

In order to evaluate proliferative potential of myeloid progenitor cells, secondary CFU-GM assays were performed by picking 120 primary colonies and transferring them to separate wells of a 48-well flat-bottom microtitre plate. Single cell suspensions were prepared by dispersing each single colony in alpha medium (Gibco, Invitrogen, Austria) with 15 % FCS and mixing with methylcellulose culture medium. Well plates were then incubated under the same conditions for another 14 days and finally secondary colonies were again scored.

4.1.10. LTC-IC

Frozen fibroblast layer cells (M2-10B4, kindly provided by StemCell Technologies, Vancouver, Canada) were thawed in a 37 °C water bath, diluted 1:10 with PBS and centrifuged at 1.000 rpm for 10 min. The supernatant was removed and the pellet resuspended in 10 ml culture medium consisting of RPMI (Gibco, Invitrogen, Austria), 10 % FCS (fetal calf serum) and 1 % PS (Penicillin Streptomycin solution). Cell suspension was then transferred to a culture flask and fed as required by partial exchange of medium.

Passage of layer cells was performed after removal of culture medium and rinsing with PBS. Cells were coated with Trypsin (2 ml per 250 ml culture bottle)/EDTA to release cells from ground and incubated for 5 min. Culture medium was added to stop the reaction. After a centrifugation step (1.000 rpm for 10 min) the supernatant was removed and the pellet was resuspended in culture medium and transferred to a 50 ml culture flask.

In order to seed layer cells, the harvesting procedure was performed as described above. After centrifugation, cells were resuspended in LTC-IC medium (consisting of 100 ml Myelocult + 1 ml of 0,1 mM Hydrocortison) followed by irradiation (80 Gy, 25.5 min). Cell count was adjusted to 1.0 x 10^4 cells/ml. A 48-well microtitre plate was set up with 500 µl of cell suspension per well resulting in a final layer cell concentration of 0.5 x 10^4 cells/well.

Frozen vials were thawed in duplicate as described above. Aliquots were handled according to the study protocol. In terms of washing out DMSO, 4 portions each with 450 µl were transferred to 15 ml falcons and equally diluted with PBS/2 % HSA (450 µl cell suspension + 450 µl PBS/2 % HSA). After centrifugation at 1.200 rpm for 10 min at room temperature, the supernatant was removed and the pellet was resuspended in 900 µl of LTC-IC medium. A dilution series was set up, the negative control consisted of LTC-IC medium alone (Table 5).

Table 5: LTC-IC dilution series

well	pipetting scheme	$CD34^+$/100 µl
# 1	900 µl raw material	3.250 x 10^3
# 2	500 µl (# 1) + 500 µl LTC-IC medium	1.625 x 10^3
# 3	500 µl (# 2) + 500 µl LTC-IC medium	0.813 x 10^3
# 4	500 µl (# 3) + 500 µl LTC-IC medium	0.406 x 10^3
# 5	500 µl (# 4) + 500 µl LTC-IC medium	0.203 x 10^3
# 6	500 µl (# 5) + 500 µl LTC-IC medium	0.102 x 10^3
# 7	500 µl (# 6) + 500 µl LTC-IC medium	0.051 x 10^3
# 8	negative control (500 µl LTC-IC medium)	0.000 x 10^3

100 µl of every dilution were transferred to prepared wells in triplicate. Cells were fed twice a week by exchange of 200 µl of LTC-IC medium and kept for 5 weeks.

After 5 weeks, well plates were decanted, 200 µl of PBS was added and another decantation step followed. 1 bead of trypsin was added per well to release cells and the plate was incubated for 3-5 min at 37 °C. Subsequently 1 bead of FCS was added to stop the reaction. After addition of 300 µl of MEM, cells were harvested by repeated pipetting and transferred to a 15 ml falcon. Two further steps with addition of 200 µl of MEM and harvesting followed. The cell suspension was then centrifuged at 1.200 rpm for 10 min and the supernatant was removed except for 300 µl.

CFU-assays were subsequently performed and statistically evaluated in terms of limiting dilution analysis. Briefly, 300 µl of cell suspension were mixed with 3 ml of methylcellulose-based medium (Methocult GF H4434, Stemcell Technologies, Vancouver, Canada) and kept untouched for 5 min to allow escape of gas bubbles. Subsequently, 1.1 ml were transferred to 35-mm culture dishes in duplicate and incubated for 14 days (37 °C, 5 % CO_2, 95 % H_2O). Presence of a single colony (> 50 cells) was scored and interpreted in terms of limiting dilution analysis using L-Calc software (L-Calc 1.0, Stemcell Technologies, Vancouver, Canada) and frequency of LTC-IC was calculated using Poisson's statistics.

4.2. Statistics

Within each temperature stage data were analysed using a two-way analysis of variance method (ANOVA). A p-value < 0.05 was accepted to denote statistical significance. Protocols (PBS vs. DMSO incubation) and sampling delay periods (0 min, 30 min, 60 min and 120 min) were defined as independent variables (factors). Main effects are credited to a single factor (protocol or time). Interaction effects consist of influences of both factors and reflect dependence between one factor and the level of another factor. Values express mean ± standard deviation for both protocols at 0 and 120 min.

One specimen was totally excluded from further analysis (specimen 4, 6 °C, 0 min, DMSO group) because values exceeded threefold standard deviation distance of maximum from mean (3.7, 4.8 and 4.1 standard deviations from mean for $CD3^+$, $CD56^+/3^+$ and $CD56^+/3^-$ cells, respectively).

Box-and-whisker plots were used to visualize cell counts and viability. The box contains 50 % of the values (range from 25 % to 75 %). The median is expressed by a horizontal line within the box. Whiskers are marking all values within 1.5 times the interquartil range (IQR). Values outside this range are marked as outliers (O, < 3.0 IQR) or extremes (*, > 3.0 IQR).

Secondary CFU assays were analysed using the number of secondary CFU-GM produced by each primary CFU-GMA as raw data. A maximum of 100 colonies was counted. Secondary replating capacity was calculated by adding 1 to the mean of the log 2 of colony numbers and differences were examined by using a one-way ANOVA model with significance assigned at the 5 % level.

5. Results

Cryopreserved and thawed peripheral blood stem cells from a single patient were analysed in terms of cell count, viability and repopulating ability under certain conditions based on the study protocol.

Post-thaw incubation of cells was done at 3 different temperature levels (6 °C, 20 °C and 37 °C). Two protocols of incubation (PBS, DMSO) were used with 4 different sampling delay periods (0 min, 30 min, 60 min, 120 min) resulting in 8 different incubation conditions per temperature stage. Absolute counts of cells/µl were calculated by multiplying the absolute white blood count estimated with a cell counter by percentage of white blood cell subtypes estimated using a flow cytometer.

Based on flow cytometric specifications, white blood cell subtypes were classified as listed in Table 6.

Table 6: Classification of white blood cell subtypes by flow cytometry

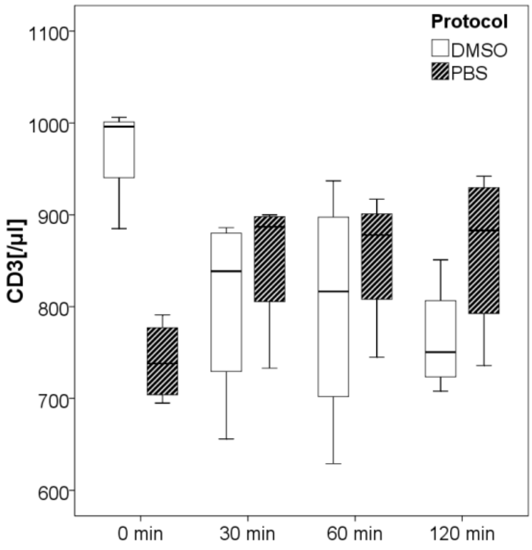
characteristics	cell subtype
$CD3^+$	T lymphocytes
$CD3^+/4^+$	T helper cells
$CD3^+/8^+$	T suppressor cells
$CD3^+$/HLA-DR^+	activated T cells
$CD56^+/3^+$	cytotoxic T cells
$CD56^+/3^-$	natural killer cells
$CD14^+$	monocytes/macrophages
$CD19^+$	B lymphocytes
$CD34^+$	haematopoietic stem cells

Cell counts were visualized using box-and-whisker plots. Incubation in PBS (protocol I) and DMSO (protocol II) is reflected by shaded and white boxes, respectively.

5.1. Incubation at 6 °C

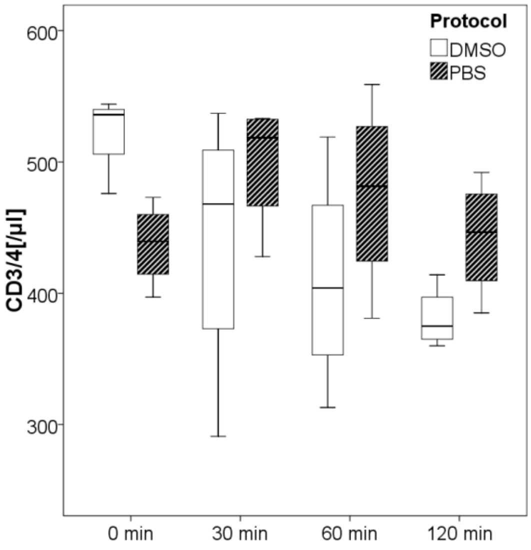
At 6 °C no remarkable differences were seen with regard to protocols or incubation times. In detail, T lymphocytes in general ($CD3^+$) and T helper cells ($CD3^+/4^+$) did not show significant declines in cell counts with increasing incubation time (Figures 2 and 3).

Figure 2: $CD3^+$ cells at 6 °C



cells/µl	***0 min***	***120 min***
PBS	*740.5 ± 44.3*	*861.0 ± 92.1*
DMSO	*962.3 ± 67.2*	*765.0 ± 61.5*

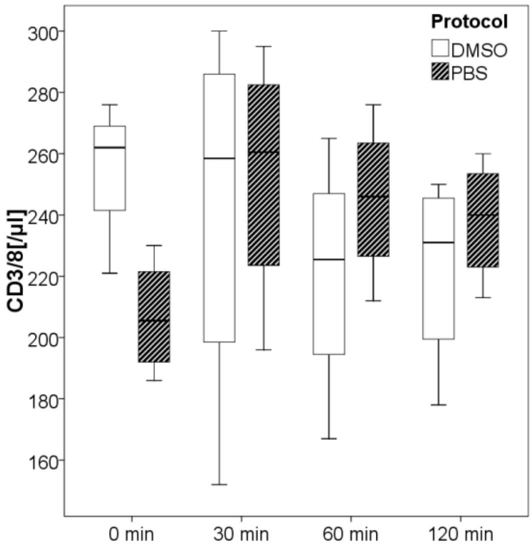
Figure 3: $CD3^+/4^+$ cells at 6 °C



cells/µl	***0 min***	***120 min***
PBS	437.3 ± 31.7	442.5 ± 45.1
DMSO	518.7 ± 37.2	381.0 ± 23.5

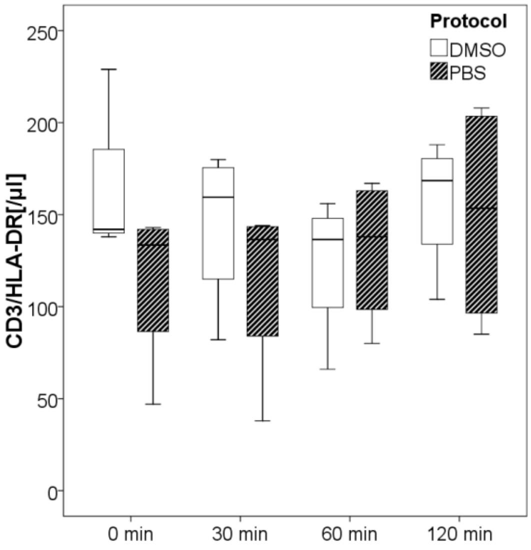
No statistically significant differences between protocols or variation in time could be seen for CD3$^+$/8$^+$ and CD3$^+$/HLA-DR$^+$ cells, either. (Figures 4 and 5)

Figure 4: CD3$^+$/8$^+$ cells at 6 °C



cells/µl	***0 min***	***120 min***
PBS	206.8 ± 19.0	238.3 ± 20.1
DMSO	253.0 ± 28.6	222.5 ± 32.0

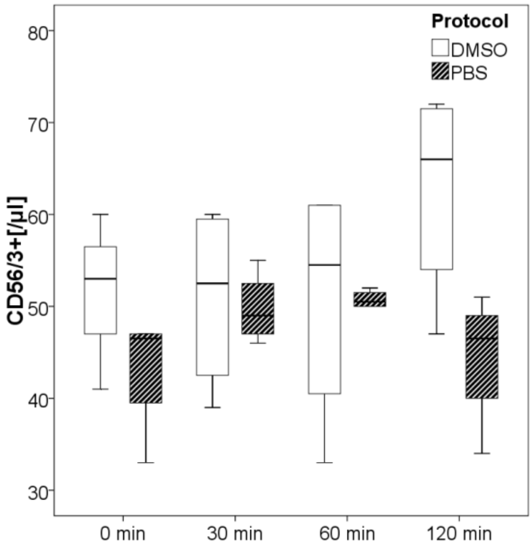
Figure 5: CD3$^+$/HLA-DR$^+$ cells at 6 °C



cells/µl	***0 min***	***120 min***
PBS	114.3 ± 45.5	150.0 ± 62.6
DMSO	169.7 ± 51.4	157.3 ± 36.9

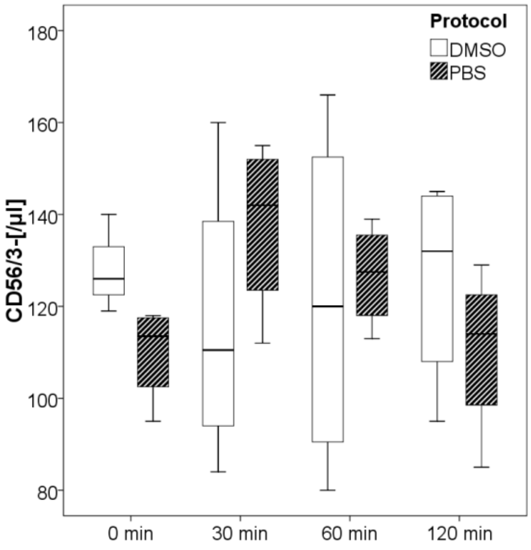
No differences in cell counts were seen for cytotoxic T cells (CD56$^+$/3$^+$) and natural killer cells (CD56$^+$/3$^-$) at 6 °C (Figures 6 and 7).

Figure 6: CD56$^+$/3$^+$ cells at 6 °C



cells/µl	***0 min***	***120 min***
PBS	*43.3 ± 6.9*	*44.5 ± 7.3*
DMSO	*51.3 ± 9.6*	*62.8 ± 11.6*

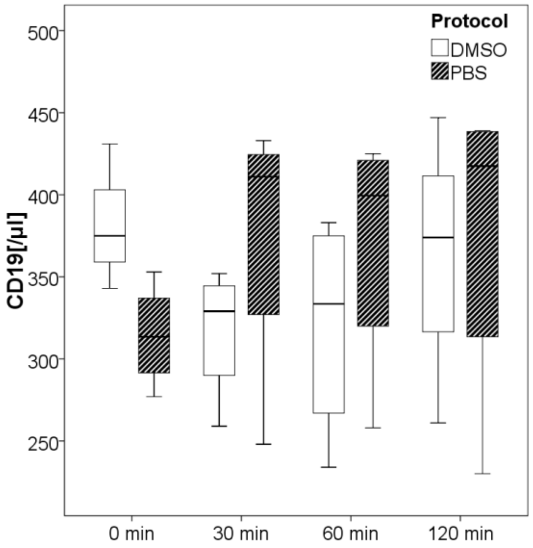
Figure 7: CD56$^+$/3$^-$ cells at 6 °C



cells/µl	***0 min***	***120 min***
PBS	*110.0 ± 10.6*	*110.5 ± 18.5*
DMSO	*128.3 ± 10.7*	*126.0 ± 23.4*

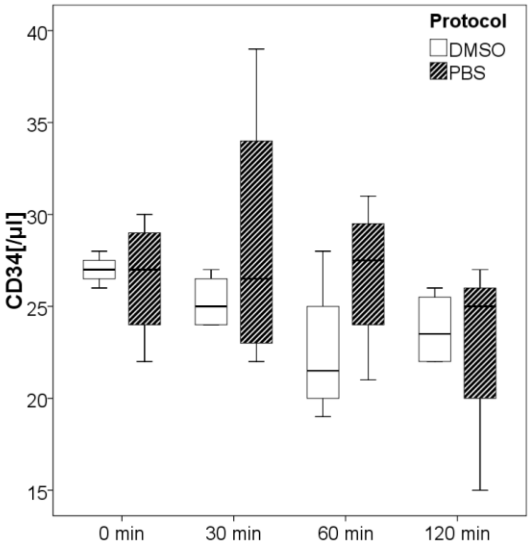
B lymphocyte (CD19$^+$) counts did not significantly vary comparing the protocols (Figure 8). Counts of CD34$^+$ stem cells also remained stable comparing values at 0 and 120 min for both protocols (Figure 9).

Figure 8: CD19$^+$ cells at 6 °C



cells/µl	*0 min*	*120 min*
PBS	*314.3 ± 31.6*	*367.0 ± 99.3*
DMSO	*383.0 ± 44.5*	*364.0 ± 76.8*

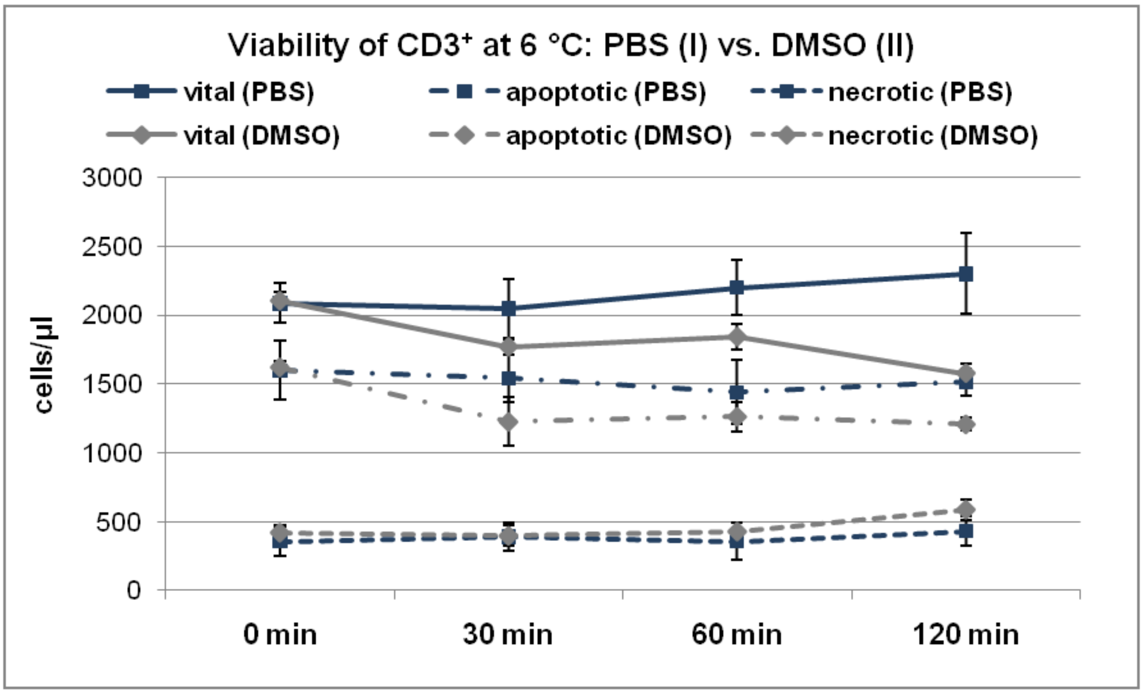
Figure 9: CD34$^+$ cells at 6 °C



cells/µl	*0 min*	*120 min*
PBS	26.5 ± 3.4	23.0 ± 5.4
DMSO	27.0 ± 1.0	23.8 ± 2.1

Viability of T lymphocytes (CD3$^+$) tended to decrease in the DMSO group, but difference did not reach significance (Figure 10). Although levels of apoptotic CD3$^+$ cells tended to be lower in the DMSO group, this decrease in viability may be explained by an increase of necrosis of cells incubated with DMSO for 120 min. Overall, no statistical significance was reached.

Figure 10: Viability of T lymphocytes (CD3$^+$) at 6 °C

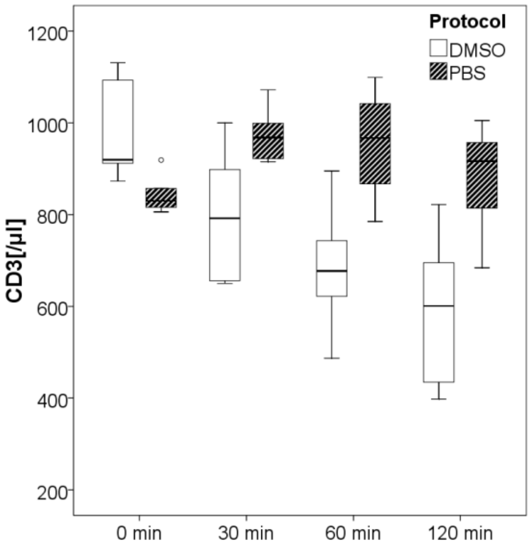


5.2. Incubation at 20 °C

T lymphocyte ($CD3^+$) counts showed a continuous decline from 0 to 120 min if cells were incubated in the presence of DMSO with a highly significant interaction effect ($p < 0.001$, Figure 11). In contrast, counts of cells incubated in PBS remained stable.

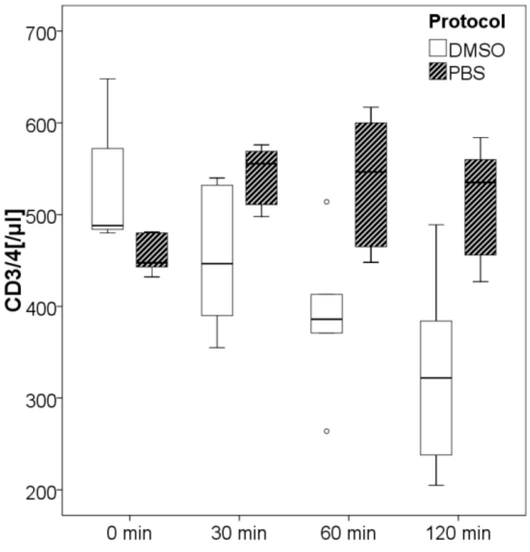
Similar results were seen for $CD3^+/4^+$ cells (Figure 12). Again a significant downward tendency was seen in the DMSO group (interaction $p < 0.001$) while cell counts slightly increased in the PBS group.

Figure 11: $CD3^+$ cells at 20 °C



cells/µl	*0 min*	*120 min*
PBS	843.0 ± 41.7	882.2 ± 115.9
DMSO	974.7 ± 108.5	592.0 ± 160.8

Figure 12: $CD3^+/4^+$ cells at 20 °C

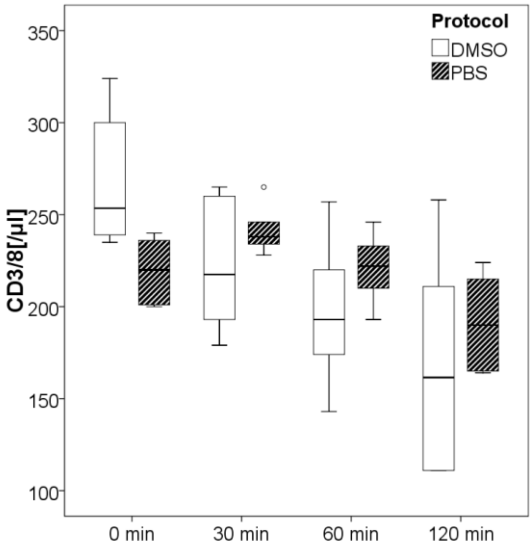


cells/µl	*0 min*	*120 min*
PBS	455.2 ± 20.4	516.2 ± 61.4
DMSO	526.7 ± 69.0	326.7 ± 105.7

A drop-off with incubation time ($p < 0.001$) was also seen for CD3$^+$/8$^+$ cells but both for DMSO and PBS incubation (Figure 13). A significant interaction ($p = 0.033$) was only found at 0 min explained by higher initial values in the DMSO group.

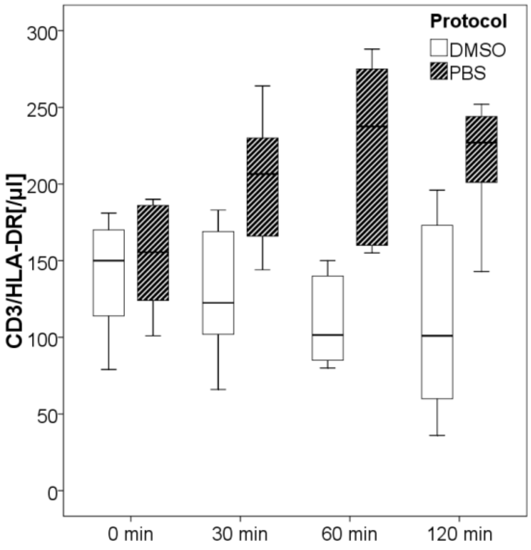
A significant difference between both protocols was found for activated T lymphocytes (CD3$^+$/HLA-DR$^+$, interaction $p = 0.033$) from 30 to 120 min of incubation. This finding can be explained by an increase in cell counts within the PBS group (Figure 14).

Figure 13: CD3$^+$/8$^+$ cells at 20 °C



cells/µl	***0 min***	***120 min***
PBS	219.5 ± 17.8	191.3 ± 25.2
DMSO	267.5 ± 36.1	169.0 ± 59.5

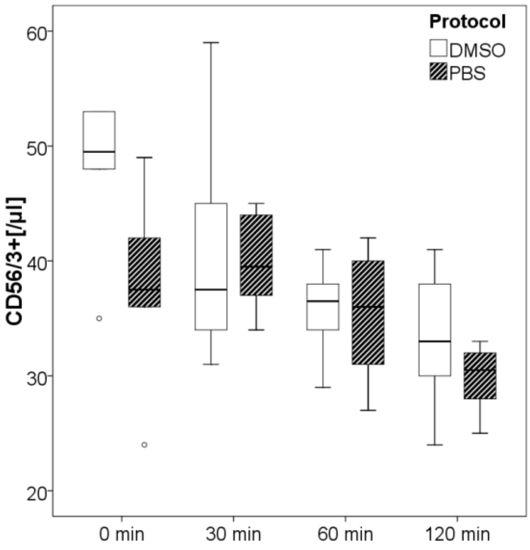
Figure 14: CD3$^+$/HLA-DR$^+$ cells at 20 °C



cells/µl	***0 min***	***120 min***
PBS	*152.0 ± 80.0*	*215.7 ± 40.2*
DMSO	*140.7 ± 37.9*	*111.2 ± 63.7*

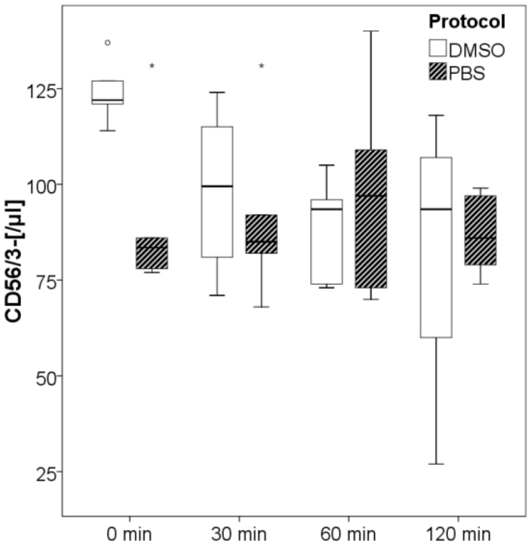
Counts of cytotoxic T cells (CD56$^+$/3$^+$) significantly dropped with increasing incubation time (p = 0.001), independently from protocol handling (Figure 15). No remarkable differences were seen for natural killer cells (CD56$^+$/3$^-$, Figure 16).

Figure 15: CD56$^+$/3$^+$ cells at 20 °C



cells/µl	*0 min*	*120 min*
PBS	*37.7 ± 8.3*	*29.8 ± 2.9*
DMSO	*48.0 ± 6.7*	*33.2 ± 6.3*

Figure 16: CD56$^+$/3$^-$ cells at 20 °C

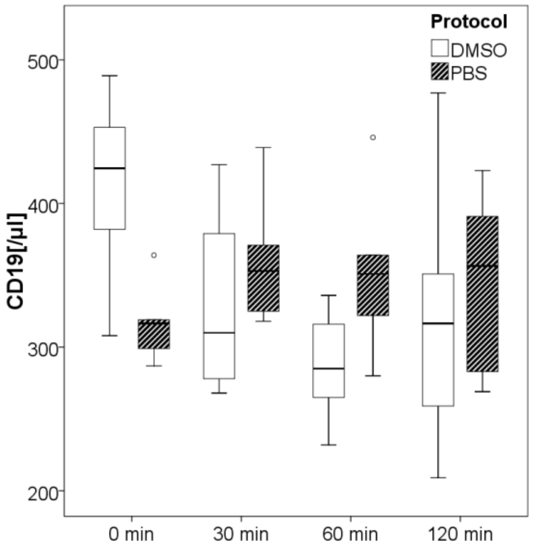


cells/µl	*0 min*	*120 min*
PBS	*89.8 ± 20.5*	*86.8 ± 10.1*
DMSO	*123.8 ± 7.7*	*83.2 ± 34.8*

A significant interaction effect was found for B lymphocytes at 0 and 60 min (p = 0.009), which could partially be explained by higher initial values in the DMSO group (Figure 17).

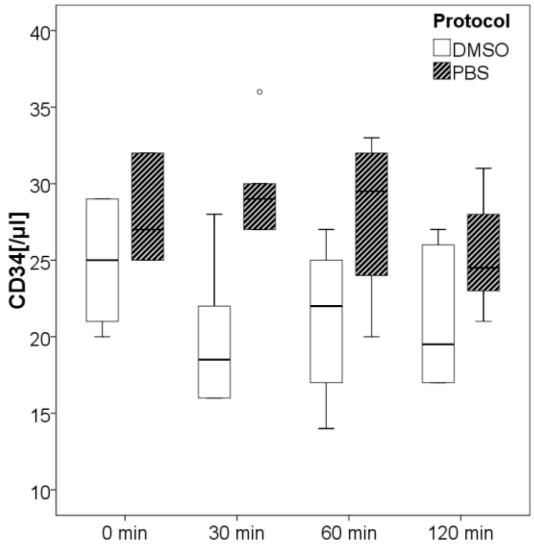
Consistent with results at 6 °C, counts of $CD34^+$ stem cells did not show a significant decline after 120 min for both protocols (Figure 18). Although interaction was not significant, values within the PBS group remained on a significantly higher level (p < 0.001) than those in the DMSO group.

Figure 17: $CD19^+$ cells at 20 °C



cells/µl	*0 min*	*120 min*
PBS	*317.0 ± 26.2*	*346.5 ± 60.2*
DMSO	*413.5 ± 63.2*	*321.5 ± 93.4*

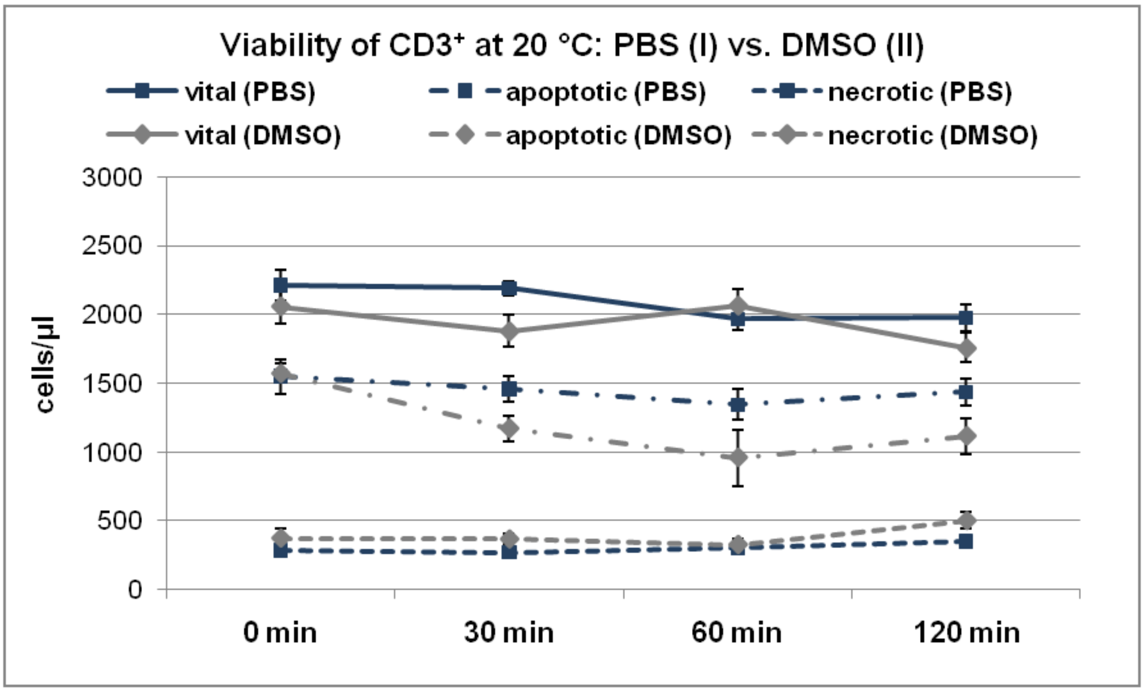
Figure 18: $CD34^+$ cells at 20 °C



cells/µl	*0 min*	*120 min*
PBS	*28.0 ± 3.2*	*25.3 ± 3.7*
DMSO	*24.8 ± 3.9*	*21.0 ± 4.4*

Viability of T lymphocytes (CD3$^+$) was not remarkably affected by different protocols or incubation time (Figure 19). Although there was a tendency towards lower values of apoptotic cells in the DMSO group, this observation did not reach significance.

Figure 19: Viability of T lymphocytes (CD3$^+$) at 20 °C

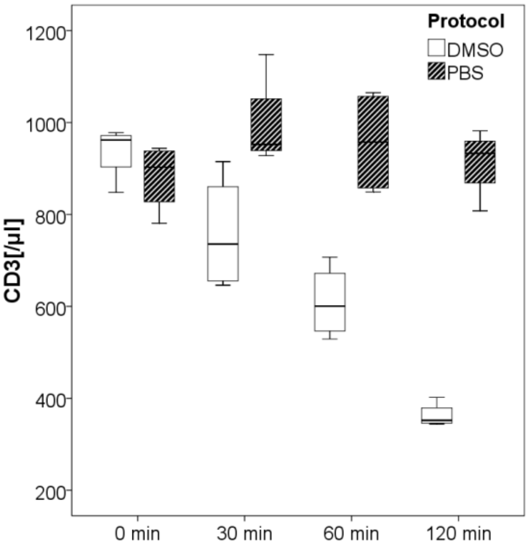


5.3. Incubation at 37 °C

At 37 °C, changes in counts of T lymphocyte subtypes were similar to those seen at 20 °C. Again, some T lymphocyte subtypes showed downward tendencies mainly in DMSO groups.

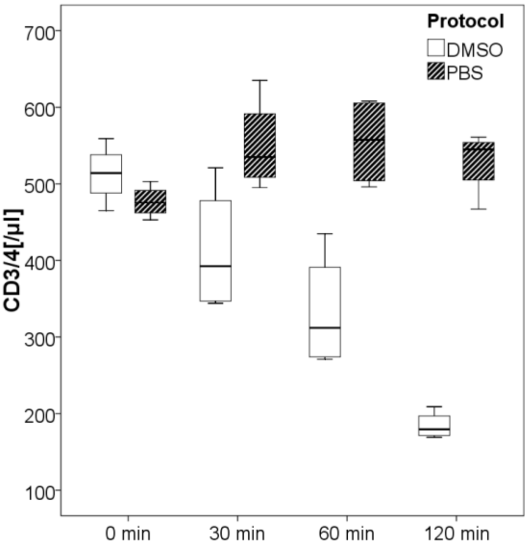
A highly significant and continuous decline (interaction $p < 0.001$) was seen if CD3$^+$ and CD3$^+$/4$^+$ cells were incubated according to protocol II (DMSO) in contrast to protocol I (PBS, Figures 20 and 21). While basic values did not differ significantly, differences reached statistical significance after 30 min, 60 min and 120 min of incubation.

Figure 20: CD3$^+$ cells at 37 °C



cells/µl	*0 min*	*120 min*
PBS	*882.8 ± 74.4*	*914.0 ± 74.4*
DMSO	*937.5 ± 60.2*	*362.5 ± 26.8*

Figure 21: CD3$^+$/4$^+$ cells at 37 °C

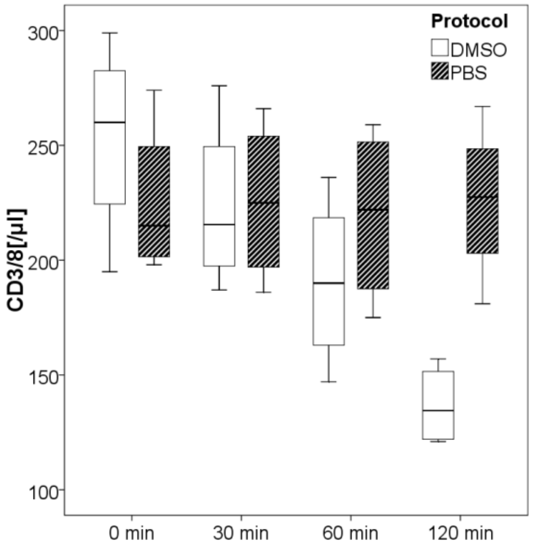


cells/µl	*0 min*	*120 min*
PBS	*476.8 ± 20.8*	*529.5 ± 42.4*
DMSO	*513.0 ± 38.5*	*184.3 ± 17.8*

In terms of CD3$^+$/8$^+$ cells, interaction effect was statistically significant only at 120 min of incubation (interaction p = 0.022, Figure 22).

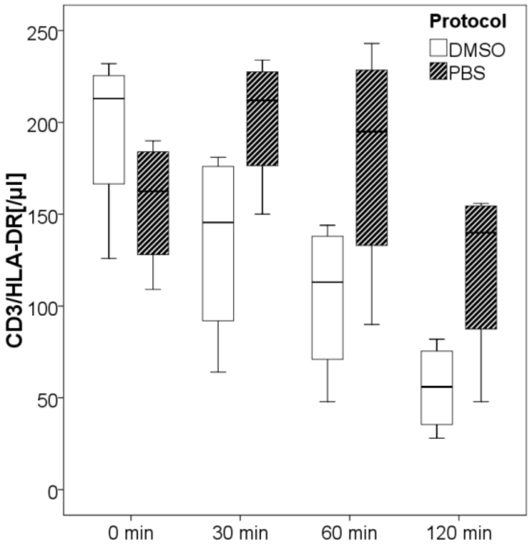
Counts of CD3$^+$/HLA-DR$^+$ cells significantly declined with incubation time (p = 0.004) and dependent on protocol handling (p = 0.016) without reaching significant interaction (Figure 23).

Figure 22: CD3$^+$/8$^+$ cells at 37 °C



cells/µl	*0 min*	*120 min*
PBS	*225.5 ± 34.3*	*225.8 ± 35.2*
DMSO	*253.5 ± 43.4*	*136.8 ± 17.6*

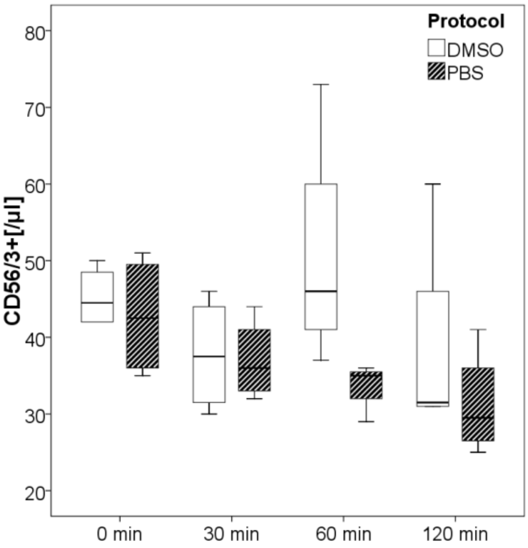
Figure 23: CD3$^+$/HLA-DR$^+$ cells at 37 °C



cells/µl	*0 min*	*120 min*
PBS	*156.0 ± 36.2*	*121.0 ± 50.4*
DMSO	*196.0 ± 47.8*	*55.5 ± 24.5*

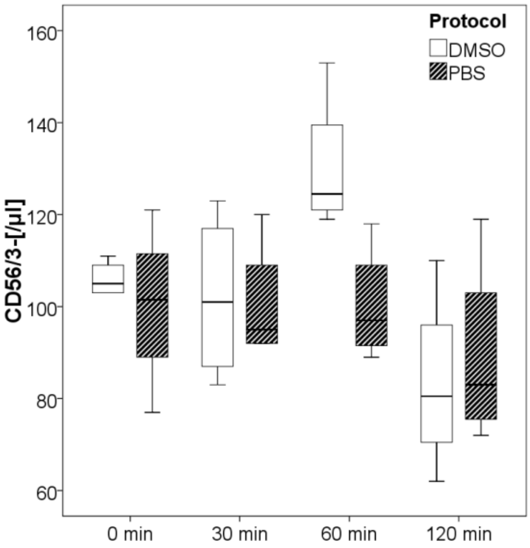
While no significant results were found for CD56$^+$/3$^+$ cells, a general decline in cell counts was seen for CD56$^+$/3$^-$ cells at 37 °C (Figures 24 and 25). Overall, no significant interaction effects were found.

Figure 24: CD56$^+$/3$^+$ cells at 37 °C



cells/µl	*0 min*	*120 min*
PBS	*42.8 ± 7.9*	*31.3 ± 6.9*
DMSO	*45.3 ± 3.9*	*38.5 ± 14.3*

Figure 25: CD56$^+$/3$^-$ cells at 37 °C

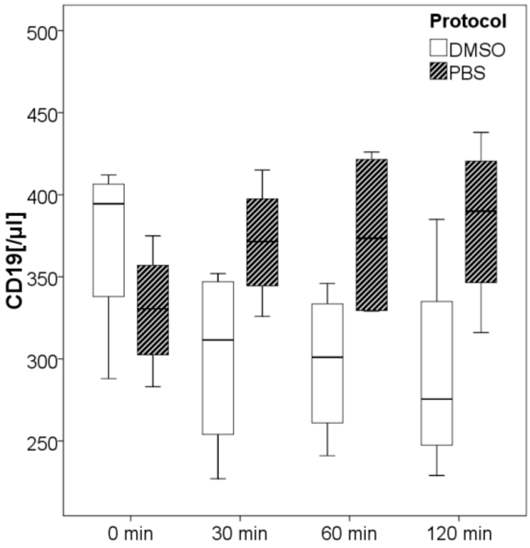


cells/µl	*0 min*	*120 min*
PBS	*100.3 ± 18.0*	*89.3 ± 20.8*
DMSO	*106.0 ± 3.8*	*83.3 ± 19.9*

In terms of B lymphocytes (CD19$^+$), differences between the two methods reached borderline statistical significance (p = 0.057, Figure 26).

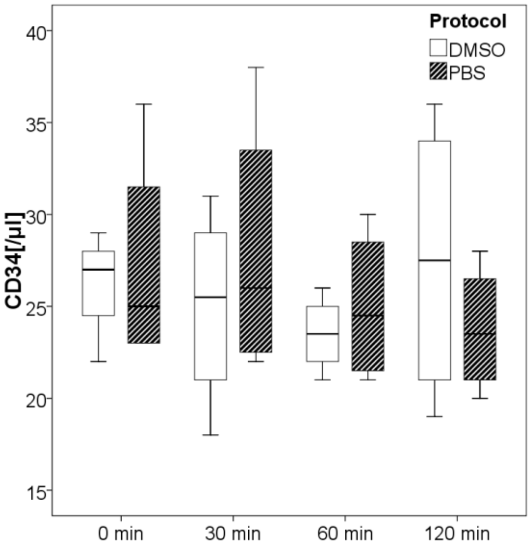
Counts of CD34$^+$ stem cells were neither impaired by DMSO incubation nor incubation time (Figure 27).

Figure 26: CD19$^+$ cells at 37 °C



cells/µl	*0 min*	*120 min*
PBS	*329.8 ± 38.2*	*383.5 ± 51.5*
DMSO	*372.3 ± 57.0*	*291.3 ± 66.7*

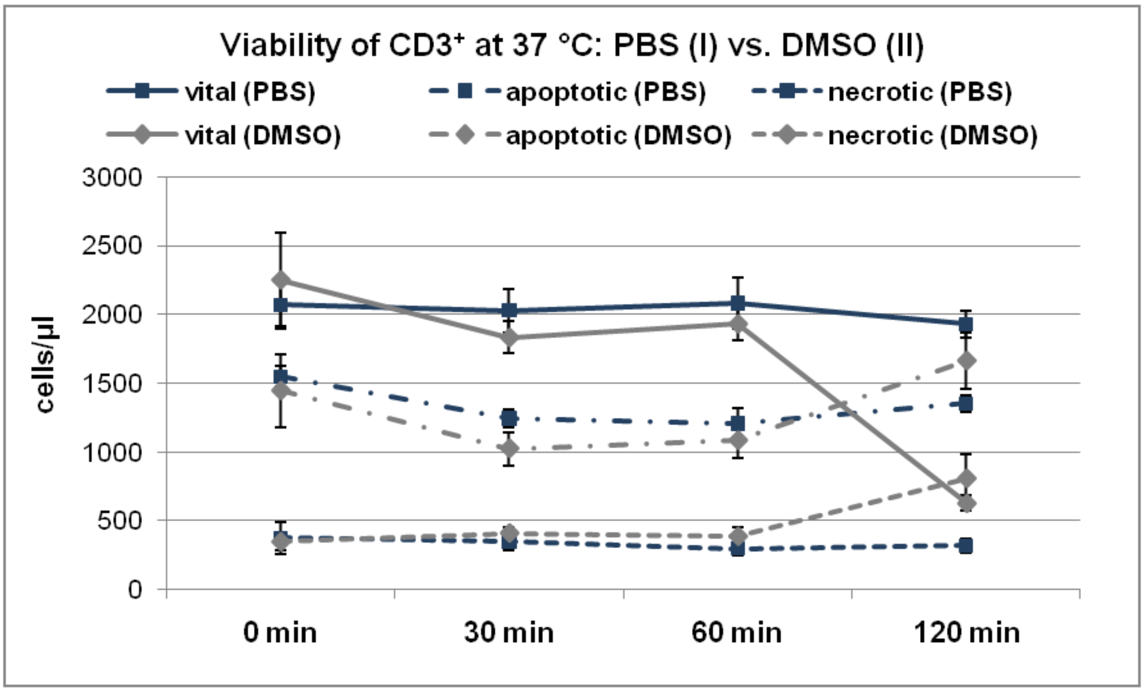
Figure 27: CD34$^+$ cells at 37 °C



cells/µl	*0 min*	*120 min*
PBS	*27.3 ± 6.1*	*23.8 ± 3.5*
DMSO	*26.3 ± 3.0*	*27.5 ± 7.9*

Although differences were not statistically significant, viability of T lymphocytes remained relatively constant for 60 min in both groups and dropped to a low level at 120 min in the DMSO group (Figure 28). This trend may be explained both by an increase in apoptotic and necrotic cells after 120 min of incubation time.

Figure 28: Viability of T lymphocytes (CD3$^+$) at 37 °C



5.4. Summary

None of the cell types showed significant changes in cell counts at 6 °C, whereas different cell types showed significant declines in numbers at higher temperatures.

Interestingly and similar for 20 °C and 37 °C, highly significant declines in cell counts were seen for T lymphocytes in general ($CD3^+$) and $CD3^+/4^+$ cells in particular, if these cells were incubated in the presence of DMSO. Effects were more intense if sampling delay periods were longer.

While $CD3^+/8^+$ cells equally dropped at 20 °C regardless of the protocol used, a significant decrease was seen after 120 min of incubation at 37 °C in the DMSO group.

No remarkable trends were seen for other cell subtypes ($CD3^+$/HLA-DR^+, $CD56^+/3^+$, $CD56^+/3^+$ and $CD19^+$ cells) either comparing protocols, incubation time, or temperatures.

Overall, counts of stem cells ($CD34^+$) were not impaired by any conditions.

In terms of viability, no significant differences were found for $CD3^+$ cells, although viability tended to decrease after 120 min at 37 °C in the DMSO group.

DMSO incubation was never superior in order to maintain cell counts compared to incubation in PBS after DMSO wash-out. While no negative impacts were seen at low temperatures (6 °C), effects were more distinctive at 20 °C and 37 °C. As expected, shorter incubation times were also superior in contrast to longer sampling delays.

5.4.1. Primary and secondary CFU

At 20 °C, the number of primary CFU-GM colonies significantly decreased from 0 to 120 min equally for both protocols ($p < 0.001$, Figure 29). Proliferative capacity investigated by secondary CFU assays declined independently from protocols, although an increase after 60 min of incubation was seen in the DMSO group (Figure 30).

Figure 29: CFU-GM at 20 °C

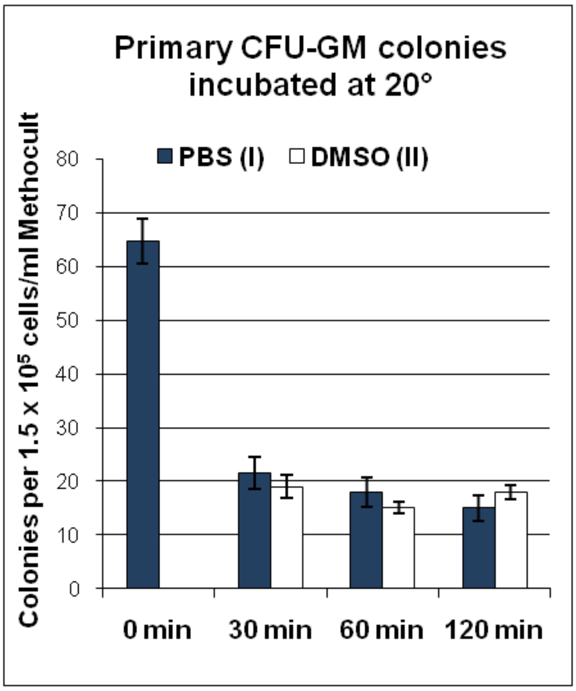
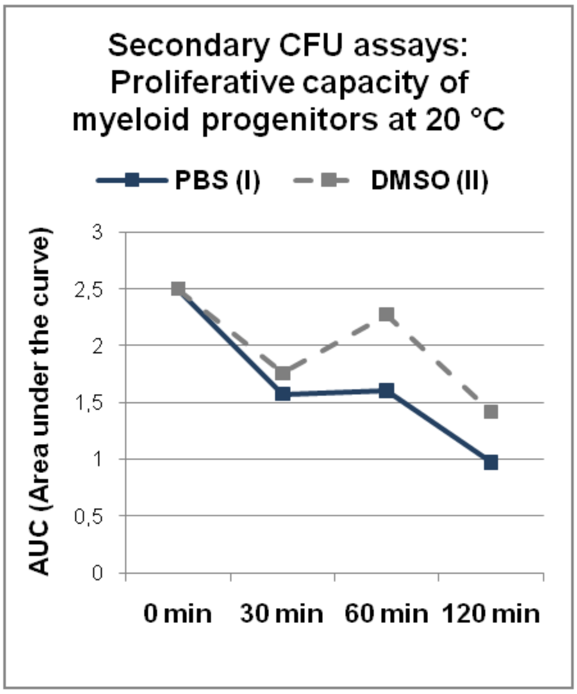


Figure 30: 2nd CFU at 20 °C



At 37 °C, number of primary CFU-GM colonies again declined after longer incubation and independent from protocols ($p < 0.001$, Figure 31). Additionally, colony counts at 120 min in the DMSO group significantly differed from values at 30 min (DMSO) and 60 min (PBS). Proliferative capacity at 37 °C again decreased to lowest values at 120 min (Figure 32). It has to be noted, that only $n = 11$ colonies were picked at 120 min in the DMSO group due to decrease of primary CFU.

Figure 31: CFU-GM at 37 °C

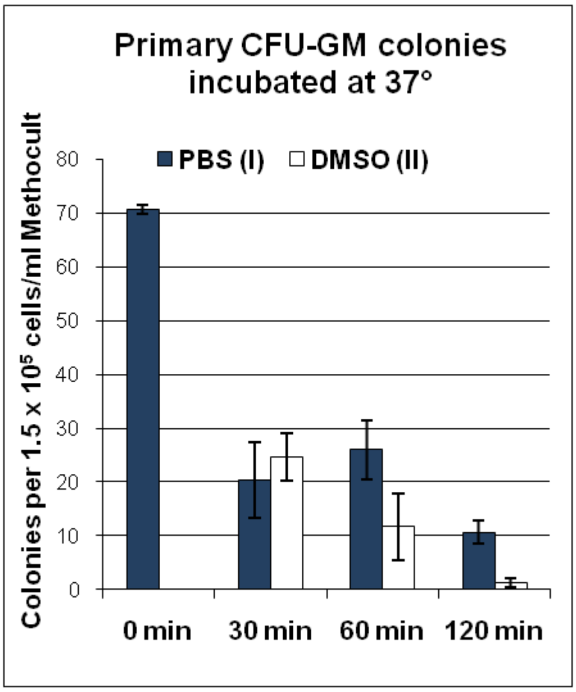
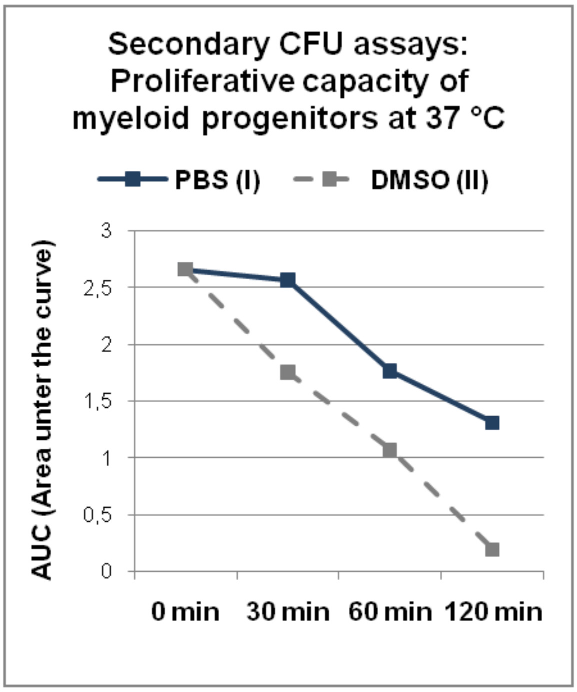


Figure 32: 2nd CFU at 37 °C



5.4.2. LTC-IC

Frequency of long-term culture-initiating cells (LTC-IC) was determined in order to visualize possible detrimental effects on repopulating ability caused by study protocol conditions. No major differences or increase in frequency were seen neither at 6 °C (1:414 in PBS vs. 1:288 in DMSO at 120 min, Figure 33) nor 20 °C (1:417 in PBS vs. 1:391 in DMSO, Figure 34).

Figure 33: LTC-IC at 6 °C

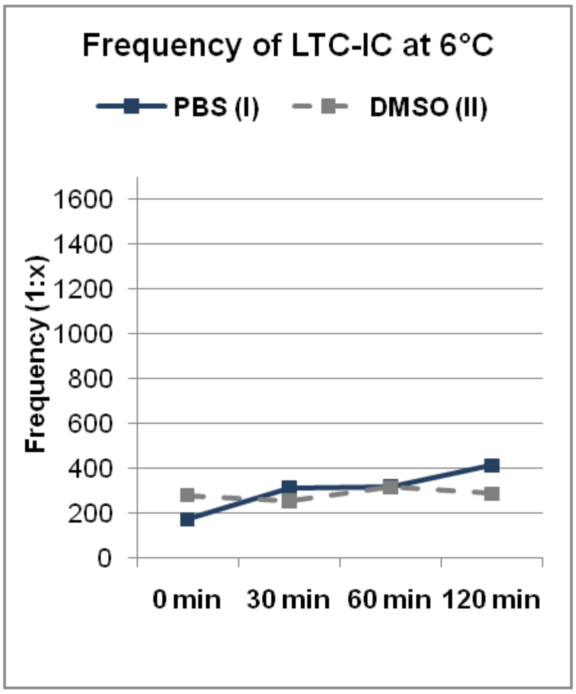
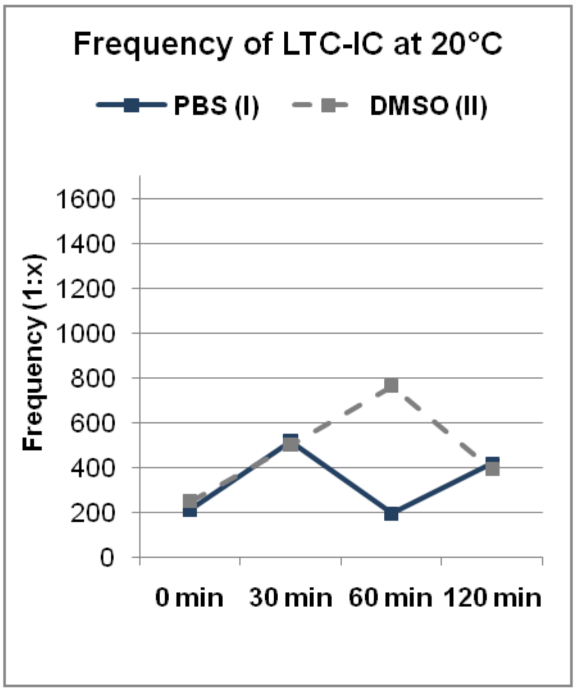


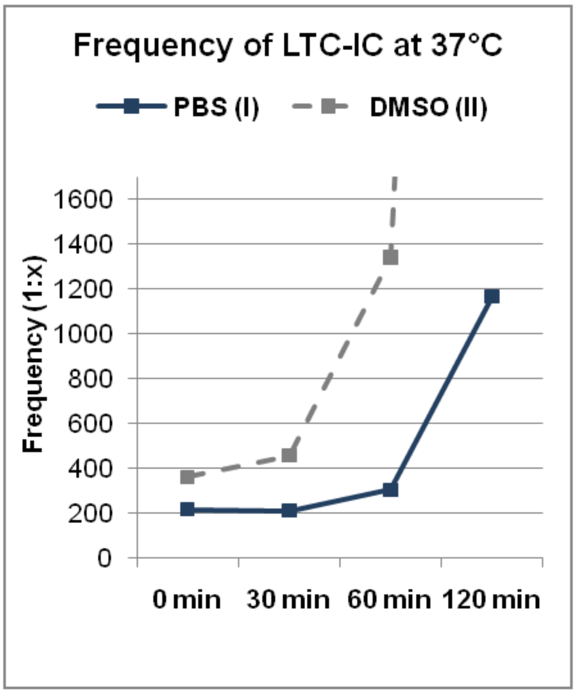
Figure 34: LTC-IC at 20 °C



At 37 °C, incubation in the presence of DMSO resulted in an excessive loss of LTC-IC (Figure 35). Frequency dropped from 1:362 at 0 min to 1:1.343 at 60 min and tended to infinity at 120 min.

Similar results were seen for PBS incubation, the frequency dropped from 1:305 at 60 min to 1:1.166 at 120 min of incubation.

Figure 35: LTC-IC at 37 °C



6. Discussion

Use of haematopoietic stem cell transplantation in children continuously increased over the last years. Cryopreservation of grafts offers the possibility of long-term storage without detrimental effects on graft quality and engraftment potential. In multimodal therapy regimens grafts can therefore be used on demand in terms of transplantation, thus facilitating the transplantation procedure.

Although various cryopreservation protocols and cryoprotectants have been studied and used with different outcomes, only few data are available on optimal post-thaw processing, temperature and infusion rate. This study was designed to investigate effects of DMSO wash-out, short-term storage after thawing, graft temperature and time to completed infusion in order to minimize detrimental effects on stem cells and maintain their ability to sufficiently restore the haematopoietic system.

Numbers of 2.5-5.0 x 10^6 $CD34^+$ cells/kg body weight have been determined to predict a secure and sustained haematopoietic engraftment. [25, 35] Our own results clearly demonstrate that higher numbers of transplanted stem cells resulted in an earlier haematopoietic recovery and therefore reduced transplantation-related risks. Short- and long-term stem cell assays are the method of choice to evaluate the long-term haematopoietic reconstitution after stem cell transplantation by visualizing colony forming potential. [85] Therefore this investigation focused on counts of $CD34^+$ cells, primary and secondary CFU assays and frequency of LTC-IC.

Our results indicate that detrimental effects on the quantity of different cells increase with rising temperature and prolonged sampling delay periods. These findings did not affect all cell subtypes equally. Incubation for 30 or 60 min was more favourable compared to 120 min incubation in most cases, indicating advantages of rapid processing and infusion. Although management of cryopreserved blood products is time consuming, additional delays should be avoided and highest possible infusion speeds should be applied.

Compared to 20 °C or 37 °C, DMSO incubation at 6 °C was more favourable with respect to quantity and quality of $CD3^+$, $CD3^+/4^+$ and $CD3^+/8^+$cells. Counts of $CD3^+$

and $CD3^+/4^+$ cells preferentially decreased within the DMSO group at higher temperatures, while no impacts on counts of $CD34^+$ cells were seen under any conditions. Overall viability of $CD3^+$ cells did not indicate remarkable detrimental effects. Evaluation of samples applying primary and secondary CFU assays showed similar decreases of primary colonies and proliferative capacity from 0 to 120 min of incubation at 20 °C and 37 °C, thus indicating that longer delays at higher temperatures are not advantageous. Incubation at 37 °C negatively affected quantity of long-term repopulating cells shown in a dramatic decrease in frequency of LTC-IC after 60 (I) and 120 min (II), respectively.

Incubation of cells in DMSO resulted in a decrease in cell counts for some of the lymphocyte subpopulations. Numbers of $CD34^+$ cells remained stable in both protocols. Frequencies of LTC-IC decreased at 37 °C within shorter periods of sampling delays compared to PBS incubation. Protocols did not remarkably differ in terms of CFU assays.

Longer incubation time reduced cell counts of T cell subpopulations ($CD3^+$, $CD3^+/4^+$, $CD3^+/8^+$) without any effects on stem cells at 20 °C. Incubation for 120 min at 20 °C in the presence of DMSO may therefore be an option to safely deplete grafts from T cells simply by implementing sampling delays or performing slow infusion. If desired, reduction of the incidence of GVHD in an allogeneic setting may therefore be possible.

In a paediatric setting, total volume of infusion, time to completed infusion and temperature of the graft have to be adjusted due to low body weight of the patient. The maximal volume of infusion is limited with regard to the patient's blood volume. Toxicity is associated with the amount of DMSO infused and temperature of blood products also contributes to the maximal tolerable infusion rate. Infusion rates of 5-20 ml/min (10 % DMSO), 20-50 ml (5 % DMSO) and fractionated infusion of products have been reported. Some authors suggest that cardiac side effects partially depend on infusion rate but can also occur due to acute volume expansion and coldness of the product caused by vagal responses. In studies, dose of DMSO infused ranged from 0.2 to 1 g/kg body weight. [29]

Optimized conditions for infusion of frozen-thawed peripheral blood stem cells and demands of patients are summarized in Table 7.

Table 7: Different demands of PBSC grafts and patients regarding an optimized transplantation procedure

parameters	graft	patient
1. temperature	6 °C	37 °C
2. volume	high	low
3. rate of infusion	high	low
4. DMSO	equal	wash-out

As shown by our results, incubation at 6 °C was superior to higher temperatures in order to preserve graft functionality. On the other hand, infusion of a 6 °C cold stem cell product will not be practicable, even less in a paediatric setting. Infusion at 37 °C body temperature would be optimal especially for children.

Reduction in volume may facilitate infusion and also reduce the amount of DMSO needed. This procedure is limited as cell concentrations should not exceed 2.0×10^8 cells/ml in order to avoid clumping.

In terms of preservation of cells with repopulating abilities, detrimental effects of DMSO were mostly small and only increased at 37 °C and with longer sampling delays. Therefore decision if wash-out in order to reduce amount of DMSO is performed or not must be weighed against unavoidable loss of cells during the washing procedure. Especially with paediatric patients it can be necessary to eliminate DMSO in order to limit toxicity and side effects.

Schlegel et al. have limited infusion of cryopreserved stem cell products to a maximum of 4 ml/kg body weight/day containing 10 % DMSO to reduce risk of DMSO dependent neurotoxicity. Furthermore, splitting infusion to subsequent days is recommended if necessary. [91]

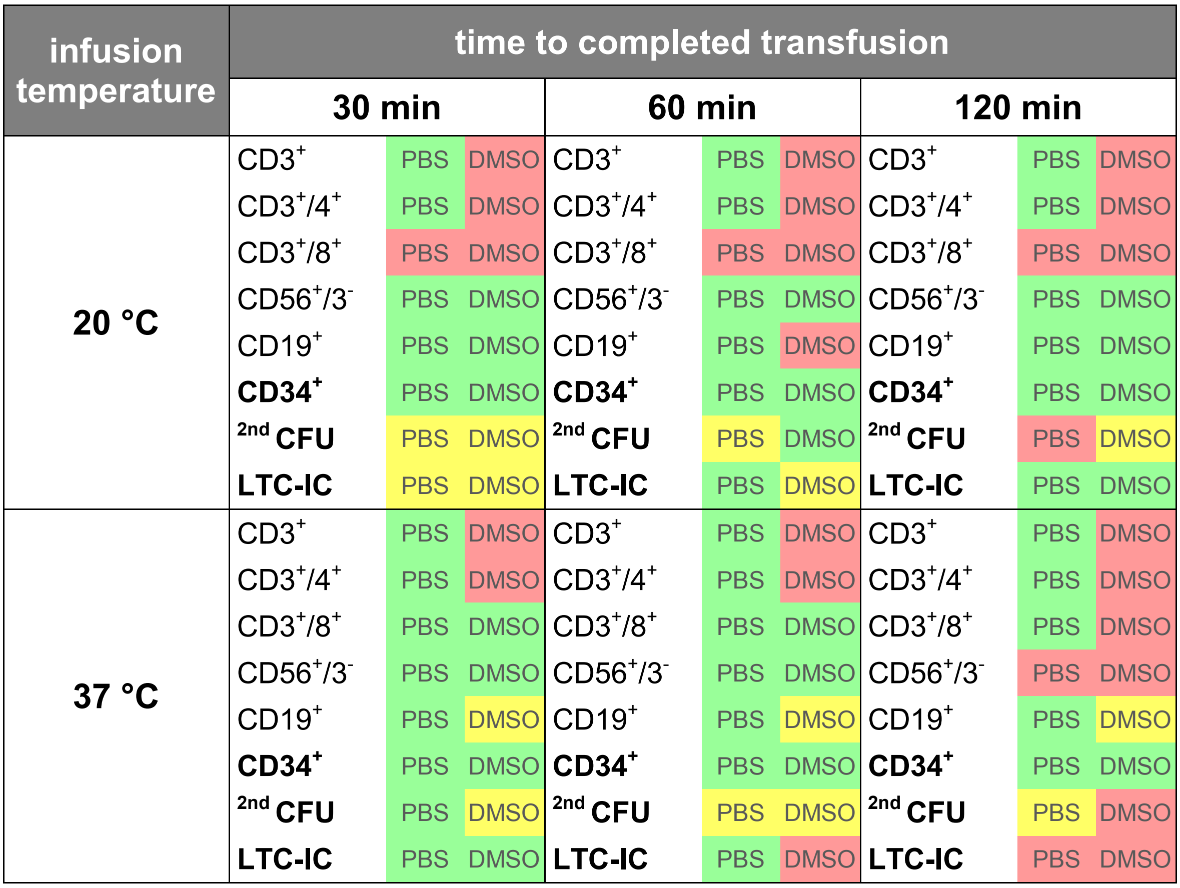
Summarizing all data, we conclude that infusion of frozen-thawed peripheral blood stem cells at 20 °C (room temperature) within 60 min after thawing seems practicable and maintains stable amounts of $CD34^+$ cells and LTC-IC regardless of DMSO wash-out.

Therefore infusion rate can individually be adjusted to complete infusion within 60 min from thawing if total volume, temperature and amount of DMSO can be tolerated by the patient. A washing procedure should be performed if total amount of DMSO exceeds estimated thresholds. Fractionated infusion on subsequent days may be feasible, but possible negative impacts on graft quality after overnight storage have to be taken into account. If fractionated infusion seems essential due to low body weight of the patient pre-transplantation, splitting the apheresis product before cryopreservation may be practicable.

We conclude that an algorithm for post-thaw processing of cryopreserved PBSC grafts seems not practicable as patients have individual risk profiles regarding age, body weight and pre-existing organ toxicities. Therefore we tried to establish a simple scheme (Table 8) which illustrates low (green), intermediate (yellow) and high risk (red) of cell loss or impaired repopulating potentials. Risks can be weighed up against specific patient demands and post-thaw processing can be individually adjusted.

Results obtained at 6 °C and 0 min (bolus injection) have been excluded from illustration, both forms of application are not feasible in paediatric patients. Only selected data are shown. Significance in reduction of cell counts ($CD3^+$, $CD3^+/4^+$, $CD3^+/8^+$, $CD56^+/3^-$, $CD19^+$, $CD34^+$), area under the curve (AUC) based on secondary repopulating abilities (2^{nd} CFU) and frequency (LTC-IC) were taken for categorization.

Table 8: Scheme illustrating risk of cell loss or impaired repopulating potential



infusion temperature	time to completed transfusion								
	30 min			60 min			120 min		
20 °C	CD3$^+$	🟩 PBS	🟥 DMSO	CD3$^+$	🟩 PBS	🟥 DMSO	CD3$^+$	🟩 PBS	🟥 DMSO
	CD3$^+$/4$^+$	🟩 PBS	🟥 DMSO	CD3$^+$/4$^+$	🟩 PBS	🟥 DMSO	CD3$^+$/4$^+$	🟩 PBS	🟥 DMSO
	CD3$^+$/8$^+$	🟥 PBS	🟥 DMSO	CD3$^+$/8$^+$	🟥 PBS	🟥 DMSO	CD3$^+$/8$^+$	🟥 PBS	🟥 DMSO
	CD56$^+$/3$^-$	🟩 PBS	🟩 DMSO	CD56$^+$/3$^-$	🟩 PBS	🟩 DMSO	CD56$^+$/3$^-$	🟩 PBS	🟩 DMSO
	CD19$^+$	🟩 PBS	🟩 DMSO	CD19$^+$	🟩 PBS	🟥 DMSO	CD19$^+$	🟩 PBS	🟩 DMSO
	CD34$^+$	🟩 PBS	🟩 DMSO	**CD34$^+$**	🟩 PBS	🟩 DMSO	**CD34$^+$**	🟩 PBS	🟩 DMSO
	^{2nd} CFU	🟨 PBS	🟨 DMSO	**^{2nd} CFU**	🟨 PBS	🟩 DMSO	**^{2nd} CFU**	🟥 PBS	🟨 DMSO
	LTC-IC	🟨 PBS	🟨 DMSO	**LTC-IC**	🟩 PBS	🟨 DMSO	**LTC-IC**	🟩 PBS	🟩 DMSO
37 °C	CD3$^+$	🟩 PBS	🟥 DMSO	CD3$^+$	🟩 PBS	🟥 DMSO	CD3$^+$	🟩 PBS	🟥 DMSO
	CD3$^+$/4$^+$	🟩 PBS	🟥 DMSO	CD3$^+$/4$^+$	🟩 PBS	🟥 DMSO	CD3$^+$/4$^+$	🟩 PBS	🟥 DMSO
	CD3$^+$/8$^+$	🟩 PBS	🟩 DMSO	CD3$^+$/8$^+$	🟩 PBS	🟩 DMSO	CD3$^+$/8$^+$	🟩 PBS	🟥 DMSO
	CD56$^+$/3$^-$	🟩 PBS	🟩 DMSO	CD56$^+$/3$^-$	🟩 PBS	🟩 DMSO	CD56$^+$/3$^-$	🟥 PBS	🟥 DMSO
	CD19$^+$	🟩 PBS	🟨 DMSO	CD19$^+$	🟩 PBS	🟨 DMSO	CD19$^+$	🟩 PBS	🟨 DMSO
	CD34$^+$	🟩 PBS	🟩 DMSO	**CD34$^+$**	🟩 PBS	🟩 DMSO	**CD34$^+$**	🟩 PBS	🟩 DMSO
	^{2nd} CFU	🟩 PBS	🟨 DMSO	**^{2nd} CFU**	🟨 PBS	🟨 DMSO	**^{2nd} CFU**	🟨 PBS	🟥 DMSO
	LTC-IC	🟩 PBS	🟩 DMSO	**LTC-IC**	🟩 PBS	🟥 DMSO	**LTC-IC**	🟥 PBS	🟥 DMSO

🟩 **low risk:**
no significant reduction of cell count ($p > 0.06$)
or high proliferative capacity of 2nd CFU (AUC > 2.0)
or high frequency of LTC-IC (> 1:500)

🟨 **intermediate risk:**
borderline significant reduction of cell count ($p = 0.04$-0.06)
or medium proliferative capacity of 2nd CFU (AUC = 2.0-1.0)
or medium frequency of LTC-IC (1:500-1:1,000)

🟥 **high risk:**
highly significant reduction of cell count ($p < 0.04$)
or low proliferative capacity of 2nd CFU (AUC < 1.0)
or low frequency of LTC-IC (< 1:1,000)

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8. Appendix

8.1. List of abbreviations

5-FU	5-fluorouracil
7-AAD	7-amino actinomycin D
AO	acridine orange
APC	allophycocyanin
ATP	adenosine triphosphate
B cells	B lymphocytes
BFU-E	burst-forming units erythroid
BM	bone marrow
BSA	bovine serum albumin
CAFC	cobblestone area-forming cells
CD	cluster of differentiation
CFC	colony forming cells
CFU	colony forming units
CFU-GEMM	CFU granulocyte/erythrocyte/monocyte/megakaryocyte
CFU-GM	CFU granulocyte/macrophage
CFU-Meg	CFU megakaryocyte
cGVHD	chronic graft-versus-host disease
CRU	competitive repopulating unit assays
CXCR4	chemokine receptor
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELTC-IC	extended long-term culture-initiating cells
EPO	erythropoietin
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FL	Flt3-ligand
G-CSF	granulocyte colony-stimulating factor
GVHD	graft-versus-host disease
HES	hydroxyethyl starch
HLA	human leukocyte antigen
HPC	haematopoietic progenitor cells
HSA	human serum albumin
HSC	haematopoietic stem cells
HSCT	haematopoietic stem cell transplantation
IL-10	interleukin-10
IL-11	interleukin-11
IL-3	interleukin-3

IL-6	interleukin-6
IQR	interquartil range
KL	c-kit ligand
LTC-IC	long-term culture-initiating cells
LTRC	long-term repopulating cells
MACS	magnetic cell sorting
MEM	modified Eagle medium
MGDF	megakaryocyte growth and development factor
MNC	mononuclear cells
NK	natural killer cells
NOD-SCID	non-obese-diabetic/severe combined immunodeficient
PBPC	peripheral blood progenitor cells
PBS	buffer
PBSC	peripheral blood stem cells
PE	phycoerythrin
PerCP	peridinin-chlorophyll protein complex
PI	propidium iodide
PS	phosphatidyl serine
PS	Penicillin Streptomycin solution
SCF	stem cell factor
SCT	stem cell transplantation
SDF-1	stromal cell derived factor-1
STRC	short-term repopulating cells
T cells	T lymphocytes
TB	trypan blue
TBI	total body irradiation
TPO	thrombopoietin
TRM	transplantation-related mortality
UCB	umbilical cord blood
WBC	white blood count

8.2. List of Figures

8.3. List of Tables

8.4. Curriculum Vitae

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Work experience

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