NTNU – Trondheim
Norwegian University of
Science and Technology

The Effect of Platelet Lysate as a Medium Supplement in Cultures of Human Multiple Myeloma

Ingrid Engum Bruvoll

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Supervisor: Berit Johansen, IBI
Co-supervisor: Magne Børset, Institutt for kreftforskning og molekylær medisin

Norwegian University of Science and Technology
Department of Biology

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Ingrid Engum Bruvoll

Abstract

Multiple myeloma (MM) is a cancer of antibody-producing plasma cells in the bone marrow. It is the second most frequent hematological malignancy and comprises 1 % of all cancer. Most human myeloma cell lines (hMCLs) derived from MM patients are cultured in animal serum. However, the hMCLs KJON, OH-2 and IH-1 established at the Department of Cancer Research and Molecular Medicine in Trondheim are cultured in a growth medium supplemented with human serum (HS), since it has been impossible to keep them alive for extended periods in animal serum. HS is provided by the blood bank at St. Olavs University Hospital in Trondheim, but the access is intermittently poor and the use of HS is otherwise economically and ethically unfavorable.
The aim of this thesis was to study the effect of platelet lysate (PL) as an alternative medium supplement to HS in cultures with the MM cell lines KJON, OH-2 and IH-1. Platelet lysate is known to contain large amount of various growth factors and has been shown to be effective in stimulating proliferation of other cell types.

Flow cytometry and CellTiter-Glo assay were performed to study apoptosis and proliferation respectively in various conditions with PL and HS. Long-term cultivation of cells was performed to test the proliferative effect of PL over time. Long-term cultivation showed that IH-1 cells could utilize PL as a medium supplement to some extent, but proliferation was too slow to consider PL as a good alternative to HS. OH-2 cells did not proliferate in PL over extended periods of time, and the cells seem to be impossible to wean from their dependence on HS. KJON cells had a slower proliferation rate in PL compared to HS. Apoptosis assay showed in general small differences in the percentage of viability among the cells cultured in HS and PL, while proliferation assay revealed that HS is more effective in stimulating proliferation of the cells of the respective cell lines.

If HS is to be replaced by an alternative medium supplement, this type of medium should give a stable and high proliferative effect on the cells and support growth and survival at the same extent as HS. PL cannot substitute HS because it does not contribute to optimal growth and proliferation of the cell lines IH-1, KJON and OH-2. The processing of PL was also time-consuming and less practical than for HS.

Norwegian abstract

Myelomatose (MM), også kalt beinmargskreft, er en krefttype som skyldes ukontrollert vekst av antistoffproduserende plasmaceller i beinmargen. Myelomatose er den nest vanligste hematologiske kreftvarianten og utgjør 1 % av alle krefttyper. De fleste cellelinjene som er etablert fra pasienter med beinmargskreft dyrkes i et vekstmedium som er tilsatt animalsk serum. Cellelinjene KJON, OH-2 og IH-1 er myelomcellelinjer som har blitt etablert på Instituttet for kreftforskning og molekylær medisin på St. Olavs hospital i Trondheim. Disse cellelinjene dyrkes i humant serum (HS). Instituttet har tilgang til humant serum via blodbanken på St. Olavs hospital, men tilgangen er periodevis dårlig. I tillegg til de etiske spørsmål som blir reist i sammenheng med bruk av humant serum, anses serumet også som lite økonomisk gunstig i forskning. Målet med denne masteroppgaven var å teste platelysat (PL) som et alternativt vekstmedium til HS for de tre ovennevnte cellelinjene. Platelysat inneholder mange vekstfaktorer og forskning viser at det er et godt vekstmedium for andre celletyper.

Apoptose- og proliferasjonsstudier med ulike betingelser av PL og HS ble utført med henholdsvis Flow Cytometry og CellTiter-Glo assay. Videre ble det utført langtidsdyrkning av disse cellene for å teste virkningene av PL over tid. Resultatene fra langtidsdyrkningene viste at IH-1-celler kan utnytte PL i noen grad. Proliferasjonen var derimot for langsom til at PL kan anses som et bedre alternativ til HS. OH-2-cellene var ikke mulig å dyrke lengre enn 10 dager i PL, da de sluttet å proliferere. Hos KJON-celler gav PL en lavere prolifererende effekt sammenlignet med HS. Apoptoseassay viste generelt små forskjeller mellom HS og PL i levedyktighet. Proliferasjonsanalyser viste derimot at HS stimulerer til proliferasjon i større grad enn PL.

For at HS skal kunne erstattes av et alternativt vekstmedium, må dette mediumet stimulere til proliferasjon og støtte vekst og overlevelse i samme grad som HS. PL kan ikke erstatte HS fordi det ikke bidrar til optimal vekst blant cellelinjene KJON, IH-1 og OH-2. Forberedelsene til bruk av PL var også noe mer tidkrevende og mindre praktisk sammenlignet med for HS.

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Abbreviations

ATP	Adenine triphosphate
bFGF	Basic fibroblast growth factor
BMSC	Bone marrow stromal cells
CCL5	Chemokine (C-C motif) ligand 5
DNA	Deoxyribonucleic acid
ECGF	Epithelial growth factor
EGF	Epidermal growth factor
EMM	Extramedullary
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
HGF	Hepatocyte growth factor
hMCL	Human myeloma cell line
HRD	Hyperdiploid
HS	Human serum
IGF-1	Insulin-like growth factor-1
IGH	Immunoglobulin heavy chain
IL	Interleukin
IU	International units
MIP	Macrophage inflammatory protein
MM	Multiple myeloma
MSC	Mesenchymal stem cells
NRDH	Non-hyperdiploid

PAS	Platelet-additive solution
PCL	Plasma cell leukemia
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PL	Platelet lysate
PL-PPC	Pooled platelet concentrates
PS	Phosphatidylserine
RLU	Relative luciferase units
RNA	Ribonucleic acid
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor

1. Introduction

Finding the optimal cell culture conditions is an essential part of working with cells for a variety of researchers. The type of culture supplement will affect the cell preparations and have both disadvantages and advantages (Hemeda, Giebel, & Wagner, 2014). Animal serum, such as fetal calf serum (FCS) or fetal bovine serum (FBS), is widely used as a cell culture medium supplement because it contains a number of different growth factors that promote cell proliferation and survival in cultures. Human serum (HS) is more rarely used because it needs to be provided by a blood bank and screened for hepatitis B and other viruses. All use of human material in research must also be approved by an ethical committee (Freshney, 2010). Platelet lysate (PL) has been shown to be promising as a medium supplement. PL is obtained by freezing and thawing platelets. It is known to be a rich source of growth factors, easy to obtain and handle, and economically and ethically favorable (Chiara Barsotti et al., 2013; Jonsdottir-Buch, Lieder, & Sigurjonsson, 2013; Naaijkens et al., 2012).

Multiple myeloma (MM) is a cancer of antibody-producing plasma cells (Goldsby, Kindt, Osborne, & Kuby, 2003). Many questions about the cancer still remain to be answered. Department of Cancer Research and Molecular Medicine in Trondheim consists of a research group for MM. Several human MM cell lines (hMCLs) are established at the respective department. The cell lines are either cultured in medium supplemented with FCS or HS provided by the blood bank at the hospital. Some of these cell lines are completely dependent on HS and do not survive in FCS. This is true for the hMCLs named IH-1, OH-2 and KJON. However, the availability of HS provided by the blood bank is intermittently poor, as HS is reserved for patients at the hospital. The use of HS in research is also considered economically unfavorable, and ethical questions can be raised against the use of HS in research. It would thus be appreciable to find an alternative growth medium.

The aim of this thesis was to study the proliferative effect of PL as a medium supplement in cultures with the cell lines IH-1, KJON and OH-2. The majority of research that includes PL as a medium supplement is related to mesenchymal stem cells (MSC). Little is known about PL as a medium supplement in other cell cultures. No reports were found that compare the use of PL and HS or test the effect of PL on hMCLs. If PL can serve as a medium supplement

in cultures with MM cell lines, PL would be more advantageous than HS since it is a surplus product and easier to access. This forms the basis of this thesis: can PL fully replace HS as a medium supplement in cultures of the hMCLs IH-1, KJON and OH-2?

1.1 The basics of a cell's life

1.1.1 Cell proliferation

Cell proliferation and death is a highly regulated mechanism in an organism. Cell proliferation refers to an increase of the number of cells (Hughes & Mehmet, 2003). Cell division and cell proliferation are fundamental throughout the life of all eukaryotes. The reproduction of a cell is organized in a sequence of events in which the cell duplicates and divides in two (Lodish et al., 2008). These events constitute the cell cycle, which is defined into four different phases: G_1 phase, S phase, G_2 phase and M phase. The first phase (G_1) is a gap phase in which the cells grow and double their mass of proteins and organelles. This phase is the longest and ensures that the environmental conditions are suitable. At the end of the G_1 phase, the cells must pass a restriction point to continue the cell cycle. After the cells have passed this restriction point they enter the S phase, where DNA is synthesized and the chromosomes are duplicated. In G_2 phase the cells prepare for chromosome segregation and cell division that happen in M phase (Lodish et al., 2008).

1.1.1.1 Cancer cells proliferate uncontrolled

Cancer cells are defined by two properties. First, they divide uncontrolled and do not respond to normal restraints on cell growth and division. Secondly, they are able to invade other tissues and areas. A tumor, or *neoplasm*, is a result of uncontrolled proliferation and growth of a cell. A malignant tumor has the ability to escape and invade other tissues, which also defines the term cancer (Alberts et al., 2008). The cells forming a malignant tumor grow and divide faster than normal cells. This uncontrolled proliferation is a result of an accumulation of changes in a single precursor cell that give rise to the cancer (Lodish et al., 2008).

1.1.2 Apoptosis

Cell death is another crucially important mechanism in animals and plants. Cell death occurs of billions of cells every hour in a human being (Lodish et al., 2008). Programmed cell death happens when cells activate their own death program in a controlled way. The most common form of programmed cell death is referred to as *apoptosis*. This process is characterized by morphological changes: shrinking and condensing of the cells, collapse of the cytoskeleton, disassembling of the nuclear envelope and fragmentation of the chromatins (Lodish et al., 2008). Apoptosis is dependent on a family of proteases called caspases (c for cysteine and asp for aspartic acid). These proteins are synthesized as inactive precursors called procaspases. The procaspases can be activated by proteolytic cleavage on specific aspartic acids. Cleavage and activation of procaspases result in an amplifying proteolytic cascade (Lodish et al., 2008).

The morphological changes that happen during a cell's apoptosis are recognizable. In live cells the negatively charged phospholipid *phosphatidylserine* is located in the inner leaflet of the lipid bilayer of the plasma membrane. During apoptosis, phosphatidylserine flips to the outer leaflet. There it serves as a marker of apoptotic cells which can be visualized with a labeled form of the protein *Annexin V* that binds to the phosphatidylserine (Lodish et al., 2008).

1.2 Multiple myeloma

1.2.1 Characteristics of the disease

Multiple myeloma (MM) is a cancer of antibody-producing plasma cells (Goldsby et al., 2003). It is the second most frequent hematological malignancy and comprises about 1 % of all cancers. In 2008 it was estimated 103,000 people worldwide diagnosed with MM. Incidence increases above an age of 55-59 and is highest for 85 + years. The disease is incurable, but it is considerable variation in lifespan from the time of diagnosis (Cancer Research UK, n.d).

In healthy humans the plasma cells secrete a single molecular species of antibody and eventually die. MM is characterized by clonal expansion of malignant plasma cells in the

bone marrow. The clone of plasma cells divides uncontrolled without requiring activation by antigen to induce proliferation (Goldsby et al., 2003; Roodman, 2002). The malignancy of the cells typically occurs in the bone marrow probably due to external stimuli by the surrounding microenvironment (Fagerlig et al., 2011). The clone of plasma cells will eventually invade adjacent bone, resulting in bone pain and pathological fractures. Bone pain in spine and chest and anaemia are present at diagnosis in two of three patients. Other common characteristics at diagnosis are detection of a paraprotein called mono-clonal protein (M-protein) in the serum or urine, hypercalcemia and renal insufficiency (Katzel, Hari, & Vesole, 2007; Roodman, 2002; The International Myeloma Working Group, 2003).

1.2.2 Human myeloma cell lines

A cell line is a population of cells that has the ability to grow and divide continuously (Lodish et al., 2008; Olsen, 2012). The first hMCLs, RPMI 8266 and U-266, were established in 1966 and 1968, respectively. Most hMCLs are derived from pleural fluid or peripheral blood from patients with MM, or patients suffering from plasma cell leukemia (PCL) (Drexler & Matsuo, 2000). Establishment of hMCLs is difficult; the cells have extended doubling time and limited availability. Since plasma cells are mature B cells, MM cell lines have longer expected doubling time than other hematopoietic cell lines (Drexler & Matsuo, 2000).

In 2000 it was registered 112 established MM cell lines. The majority of the cell lines is not described regarding features such as clinical and cell culture data, cytogenetic and functional features. Nearly all of the established MM cell lines are grown in RMPI 1640. The cells grow mostly in suspension as single cells or sometimes in cluster. 27 of 112 cell lines are reported to be IL-6 dependent (Drexler & Matsuo, 2000).

MM tumors can be separated into two groups, named hyperdiploid (HRD) and non-hyperdiploid (NHRD). The prevalence of NHRD and HRD tumors is approximately the same, but most hMCLs represent NHRD (Våtsveen et al., 2012). The two groups are distinguished by chromosome content (Borset, Waage, Brekke, & Helseth, 1994; Drexler & Matsuo, 2000). The HRD tumors contain 48-74 chromosomes, typically with extra copies of at least four of eight odd-numbered chromosomes (3, 5, 7, 9, 11, 15, 19 and 21). NHRD tumors contain <48 and/or >74 chromosomes (Våtsveen et al., 2009b).

Most hMCLs derive from extramedullary MM (EMM) tumors (~75 %) with a recurrent immunoglobulin heavy chain (*IGH)* translocation. 50 % of the MM tumors are HRD and ~90 % of these do not have one of the five recurrent *IGH* translocations. Likewise, the other 50 % of the MM tumors are NHRD and ~90 % of these do have one of five recurrent *IGH* translocations (Våtsveen et al., 2009b). Less than 10 % of EMM tumors are HRD, which suggests that intramedullary HRD tumors are less likely to develop into EMM tumors or hMCL (Bergsagel et al., 2005). The difficulties of establishing hMCL from HRD tumors are unclear. It has been suggested that cells with the characteristic genotype of HRD tumors make them more dependent on the microenvironment than cells with primary *IGH* translocations (Våtsveen et al., 2009b).

The hMCL OH-2 is a stroma-independent cell line derived from an EMM tumor (Borset et al., 1994). OH-2 retains the same HRD genotype as the EMM tumor cells and lacks an *IGH* translocation. This makes the OH-2 a unique hMCL. OH-2 was established at St. Olavs University Hospital, Trondheim, in 1991. These cells were harvested from pleural fluid from an MM patient in terminal stage of the disease (Borset et al., 1994). OH-2 is a demanding cell line that grows slowly, with a doubling time of four days. In addition, OH-2 is completely dependent on human serum and IL-6, in contrast to most hMCLs that grow in FCS. These properties of OH-2 might be explained by the fact that HRD tumors rarely develop to EMM tumors and hMCLs, and that tumors with a lack of one of five recurrent *IGH* translocations are more dependent on their microenvironment (Våtsveen et al., 2009b).

1.2.3 Multiple myeloma and its microenvironment

The bone marrow microenvironment plays an essential role for the growth and proliferation of MM, and consists of various different extracellular matrix proteins, e.g., proteoglycans, glycoaminoglycans and bone marrow stromal cells (BMSC) (Podar, Chauhan, & Anderson, 2009). Various factors are secreted in the bone marrow, including interleukin-6 (IL-6), insulin-like growth factor (IGF-1), and vascular endothelial growth factor (VEGF) (Hideshima, Mitsiades, Tonon, Richardson, & Anderson, 2007). Cellular and non-cellular components of the MM microenvironment are shown in Figure 1.

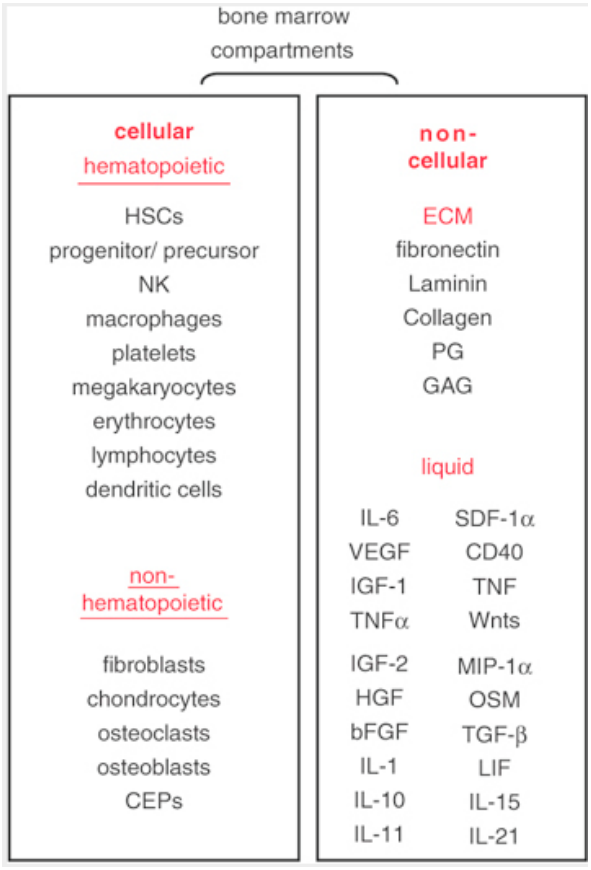

Figure 1. The multiple myeloma bone marrow microenvironment and its cellular and non-cellular components (Podar et al., 2009).

The cytokine IL-6 is produced by bone marrow stromal cells and by plasma cells (Ribatti, 2014). It is considered very important in cell growth of myeloma cells *in vivo* and *in vitro*. IL-6 induces proliferation of MM cells and serves as a growth factor for the cells *in vitro* (Akiyama et al., 2002). The cytokine is also known to promote angiogenesis by stimulating secretion of VEGF. Transforming growth factor-beta (TGF-β) increases secretion of IL-6 and VEGF by bone marrow stromal cells (Dong & Blobe, 2006). VEGF secretion is indirectly stimulated by IL-6, which further promote angiogenesis (Dankbar et al., 2000). Many hMCLs are dependent on externally added IL-6 for growth and survival (Hideshima et al., 2007).

IGF-1 is another important growth-promoting cytokine in myeloma cell lines. It is considered a weak mitogen that stimulates cell proliferation and cell cycle progression by increasing survival of MM cells (Georgii-Hemming, Wiklund, Ljunggren, & Nilsson, 1996; Jelinek, Witzig, & Arendt, 1997; Podar et al., 2009). IGF-1 contributes to an inhibition of apoptosis of MM cells by inducing *BCL3* expression in human myeloma cell lines. *BLC3* is an oncogene that encodes the protein Bcl-3 of the inhibitory κB-family (Brenne et al., 2009).

The proliferative effect of various cytokines on MM cell lines has been widely studied. Figure 2 and 3 show how IL-6 dependent MM cell lines respond to externally added cytokines. Both of the figures show that IL-6 and IGF-1 are important cytokines in supporting growth and survival of the MM cells. TGF-β was reported to have an inhibitory effect on the cells (Brenne et al., 2009; Drexler & Matsuo, 2000).

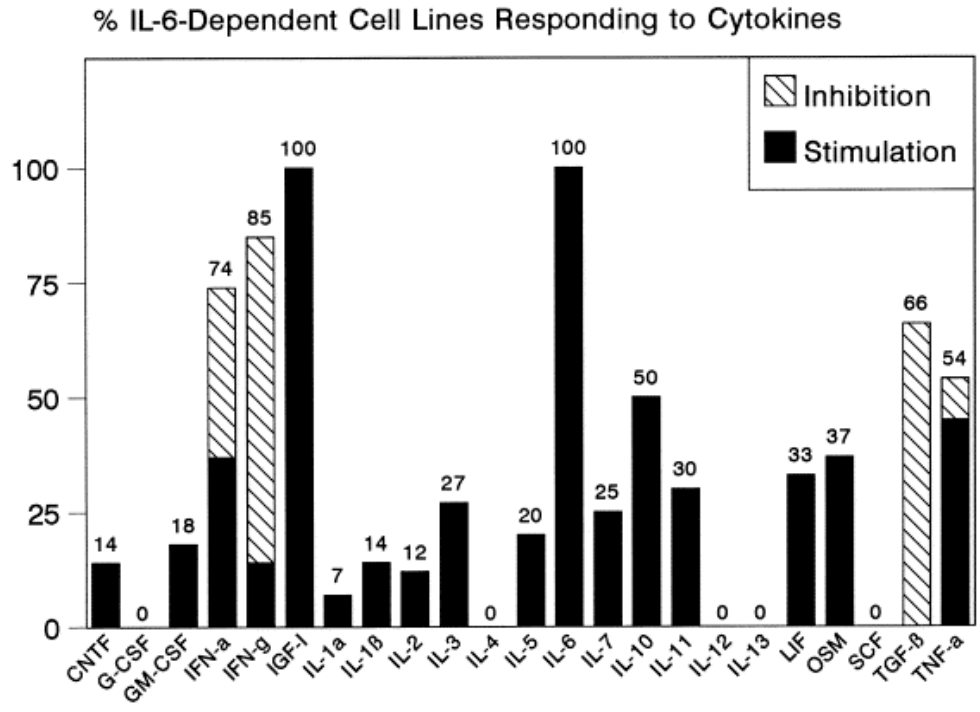

Figure 2. Various cytokines were added to different IL-6 dependent MM cell lines. The cytokines were added to three or more cell lines. Shown are the percentages of cell lines that responded to the externally added cytokines, and both growth stimulatory and growth inhibitory effects are shown. IL-6, IL-10 and IGF-1 were stimulatory in more than 50 % of the cell lines examined, whereas TGF-β had only inhibitory effect (Drexler & Matsuo, 2000).

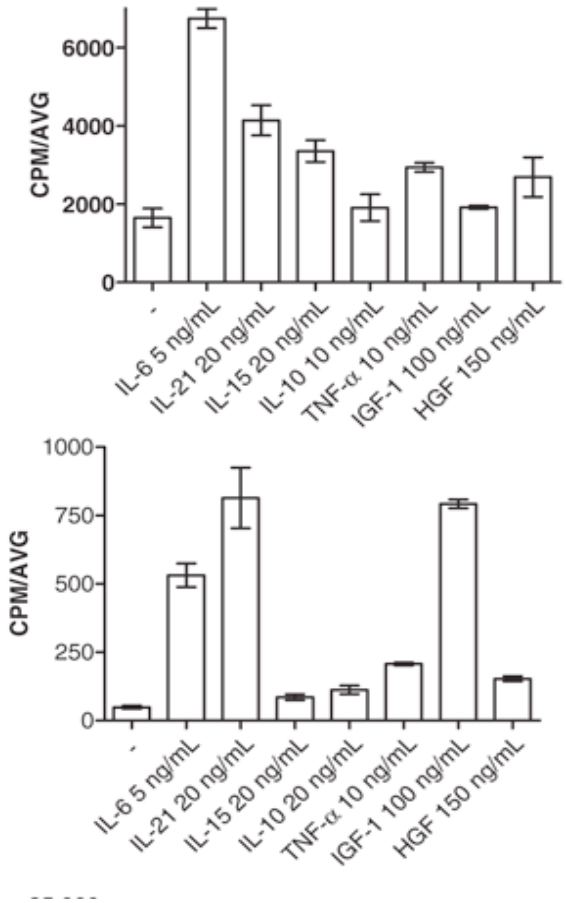

Figure 3. IH-1 and OH-2 cells were stimulated with various cytokines. The upper part represents IH-1 cells and the lower part OH-2 cells. The bars represent cell proliferation after cytokine stimulation. Mean +SD of triplicate wells is shown in the figure. Average counts per minute (CPM/AVG) are plotted along the y-axis (Brenne et al., 2009).

1.3 Growth medium in cell cultures

1.3.1 Plasma and serum

Blood can be divided into two parts: a cellular part that consists of red and white blood cells and platelets, and a liquid part called plasma. Plasma comprises approximately 50-55 % of the blood volume and the blood cells account for the remaining portion. The blood cells are suspended in the plasma. Plasma is obtained by adding anti-coagulants to a blood sample, which is further centrifuged to remove the non-cellular portion. Another liquid that is similar

to plasma is serum. Serum lacks fibrinogen and various clotting proteins, and is less viscous than plasma. The difference between processing plasma and serum is to avoid adding anti-coagulants to the blood sample. Plasma and serum contain about 95 % water and a variety of substances. Among them are proteins and peptides (e.g., enzymes, hormones, albumins and globulins), nutrients (e.g., amino acids, carbohydrates and lipids) including other organic molecules (Psychogios et al., 2011).

1.3.2 Serum in cell cultures

The culture medium is one of the most important single factors in cell culture. Basal culture together with animal serum of different origin has been essential for cell growth and proliferation (Gstraunthaler, 2003). Serum is important in cell cultures because it provides growth factors, binding- and transport proteins, amino acids, vitamins, fatty acids and lipids which all promote cell proliferation and survival (Freshney, 2010; Gstraunthaler, 2003). According to Gstraunthaler (2003), it is common knownledge that serum stimulates cell proliferation at a higher degree than plasma due to the release of polypeptides of platelets upon clotting processes.

Serum also has a number of disadvantages. Serum has physiological variability, meaning that serum contains minor components that have not been fully determined. Serum also varies from batch to batch. The availability of serum can be restricted because of political and economical reasons, or there may have been a spread of disease among the cattle (i.e., fetal bovine serum). In addition, serum can be contaminated with viruses that may have additional unknown effects on the cells being cultured (Freshney, 2010; Gstraunthaler, 2003).

Serum from FBS is commonly used as a culture medium constituent. FBS is rich in growth factors and comprises most of the factors required for cell proliferation and maintenance. HS is used for more demanding cell lines, sometimes with human cell lines. HS needs to be screened for HIV and Hepatitis B (Freshney, 2010). There are few reports that compare the use of FBS and HS. However, at the Department of Cancer Research and Molecular Medicine in Trondheim there are some well-established hMCLs that are dependent on HS as a supplement in the cell culture (Borset et al., 1994; Hjertner et al., 2001). The latter is true for the hMCLs IH-1, KJON and OH-2. As previously mentioned, OH-2 is a demanding cell line

that grows slowly with a doubling time of four days. Våtsveen et al. (2009b) performed an experiment in an attempt to study effective ways to culture OH-2 cells in terms of various types of medium. The experiment included FCS with various cytokines in RPMI 1640, as well as HS in various conditions. OH-2 cells did not survive in FCS. They were only able to survive in HS with externally added IL-6 for long-term culture (Figure 4).

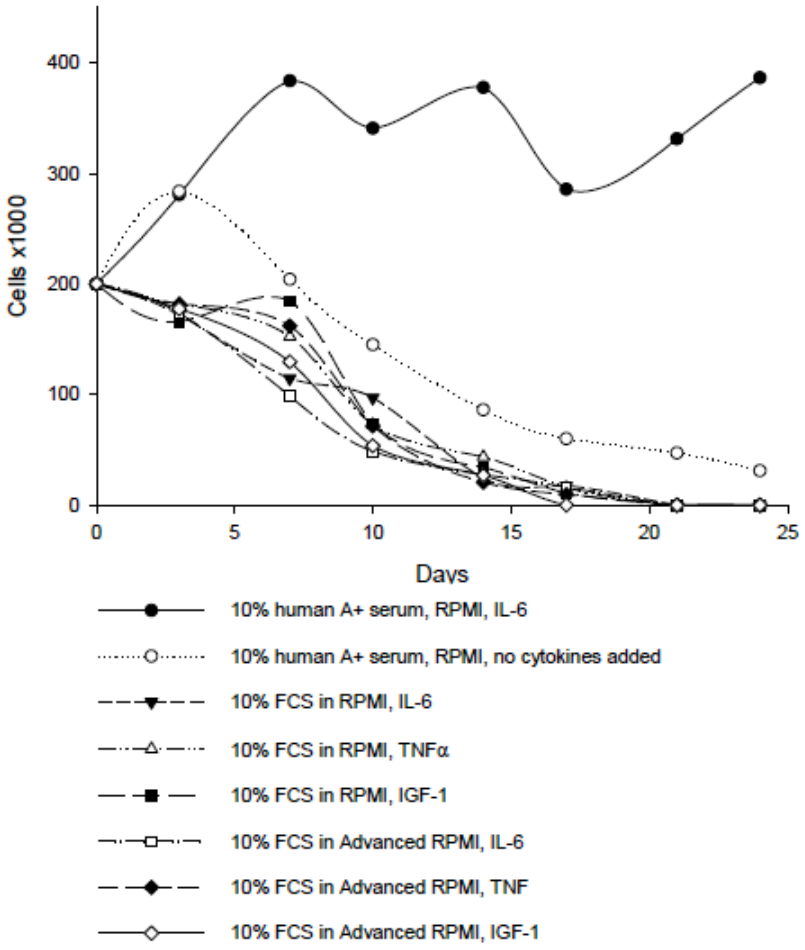

Figure 4. The hMCL OH-2 was grown in 10 % FCS with various conditions, and in 10 % HS with and without IL-6. Cells were only able to survive in 10 % HS with IL-6 over longer periods of time (Våtsveen et al., 2009a).

An alternative to animal serum is serum-free media. This may be advantageous in terms of less batch variations, ethical considerations and less microbial contaminations compared to animal serum. A disadvantage of serum-free media is that it often is cell specific, which

means that the media must be specified for each cell line. Serum-free media is therefore largely limited to well-established cell lines (Bjare, 1992; Gstraunthaler, 2003).

1.4 Platelet lysate

1.4.1 Platelet biology

1.4.1.1 Biological function and structure

Platelets, or thrombocytes, are fragments of cells and contain small amounts of cytoplasm surrounded by a cell membrane (Figure 5). They derive from megakaryocytes that are produced in the red bone marrow. Platelets are important in preventing blood loss (VanPutte, Regan, & Russo, 2010). Upon injury, the platelets adhere to the vascular endothelium and empty their granules, thereby releasing platelet-derived growth factor (PDGF) and other constituents. Adhesion to vascular wall activates the platelets, which further results in changes in shape; from smooth discs to pseudopod-like structures that facilitate platelet aggregation. When fibrinogen binds to an adhered platelet, a platelet plug is formed to reduce the blood loss from the injured vessel (VanPutte et al., 2010).

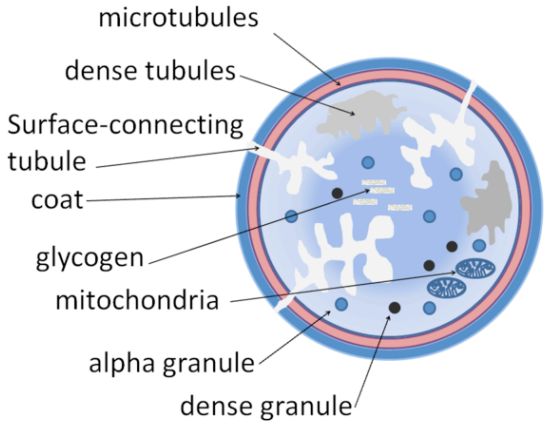

Figure 5. Platelet structure. The structure can be divided into three zones: the peripheral-, the sol-gel-, and the organelle zone. The peripheral consists of the outer surface of the platelet, while the sol-gel zone and the organelle zone consist of microtubules and α-granules, respectively (Ulrich, 2013).

Platelets are on average 2.0 to 5.0 μm in diameter and 0.5 μm in thickness, and circulate in the bloodstream for 7-10 days. The interior of a platelet can be divided into three different zones, the peripheral zone, the sol-gel zone and the organelle zone (White & Michelson, 2007). As shown in Figure 5, platelets consist of both α-granules and dense granules, which are located centrally in the organelle zone. α-granules are rich in soluble factors such as growth factors, coagulation factors, chemokines and other proteins (Reed, 2002).

1.4.1.2 Platelets are rich in growth factors

Platelets contain many different growth factors that include platelet-derived growth factor, IGF, TGF-β1 and TGF-β2, epidermal growth factor (EGF) epithelial growth factor (ECGF) and hepatocyte growth factor (HGF) (Doucet et al., 2005; Fekete et al., 2012; Kiuru, Viinikka, Myllylä, Pesonen, & Perheentupa, 1991). PDGF is a polypeptide consisting of two disulfide-bonded amino acids chains, A and B, which combine to hetero- and homodimers. PDGF binds to and activates a cell surface receptor, more specifically the tyrosine kinase receptor. Activation of the receptors stimulates cell growth, motility and shape. Hence, PDGF serves as a highly potent mitogen for cells (Heldin & Westermark, 1999).
Fekete et al. (2012) analyzed the amount of cytokines and chemokines of human PL (Figure 6). Three different batches were analyzed. The highest concentration of cytokines was found for PDGF-AB/BB (571,734 pg/ml) and PDGF-AA (239,412 pg/ml). The high content of PDGF in platelets has also been reported by Schallmoser et al. (2007), albeit the concentrations differ.

Chemokines are important in migration and trafficking of leukocytes. RANTES, GRO, IL-8, MIP-1β and MIP-α are examples of chemokines, and these were also detected in the previously mentioned analysis of PL, as shown in Figure 6. The highest level of chemokines was found for RANTES, which is a small protein that acts on a range of cells and mediate trafficking and homing of cells. RANTES is mainly produced by fibroblasts, epithelial cells and platelets (Appay & Rowland-Jones, 2001; Zlotnik & Yoshie, 2000).

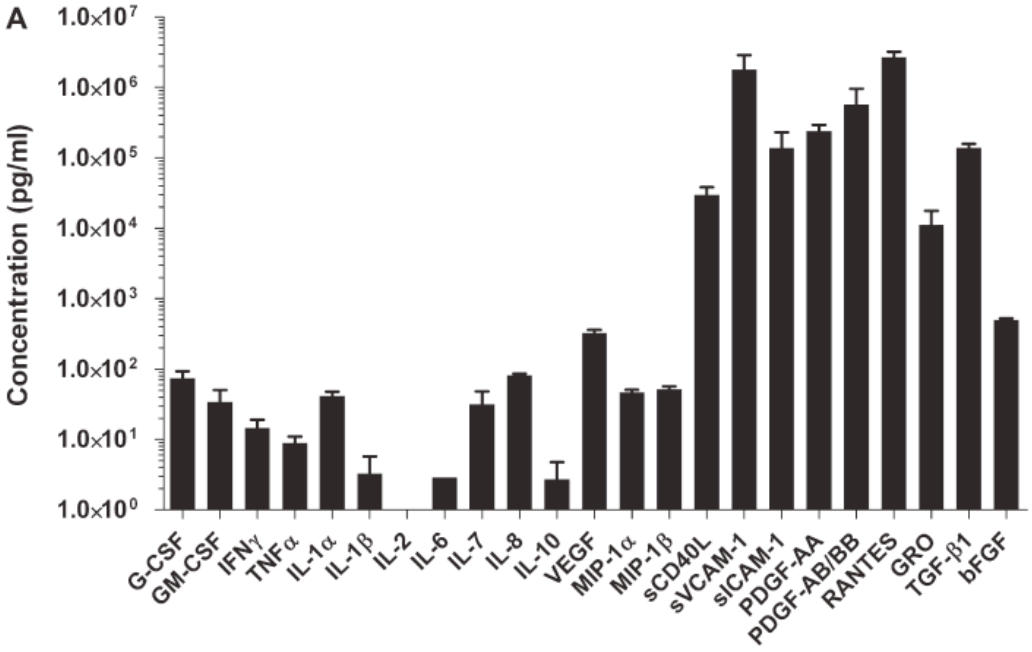

Figure 6. Analysis of human platelet lysate from pooled platelet concentrates (PL-PPC) by Multiplex assay. Three batches of PL were analyzed. The highest concentrations (pg/ml) of cytokines were found for PDGF-AA and PDGF-AB/BB, and the highest level of chemokines was found for RANTES (Fekete et al., 2012).

Rauch et al. (2011) performed an ELISA assay to determine the growth factor content of PL. The experiment included 11 batches of PL that were obtained from outdated human donor platelets. The lysates were prepared from platelet concentrates containing 1.5 x 10^{10} platelets/ml. Two HS samples were also quantified for comparison (Figure 7). PL was reported to contain higher levels of growth factors than the samples with serum. However, the level of IGH-1 and proteins was higher in the serum samples. Overall, PLs have a high content of PDGF, EFG, VEGF, bFGF and TGF-β1, but the differences in concentrations are likely due to individual variety and different preparation methods (Rauch et al., 2011).

Lysate Samples	Growth Factors [ng/ml]							Protein [mg/ml]
	EGF	PDGF-AB	TGF-β1	HGF	IGF-1	bFGF	VEGF	
# 56	11.63	57.28	1,179.7	1.17	12.81	0.96	8.46	7.93
# 69	15.34	76.48	533.2	1.38	16.70	1.04	3.47	5.88
# 70	20.55	53.60	1,462.6	1.46	8.90	1.05	10.63	9.03
# 71	13.45	58.26	503.7	2.48	16.13	0.90	19.65	7.10
# 72	16.69	46.34	1,272.5	0.68	9.32	0.81	5.17	7.16
# 73	14.27	51.88	1,084.3	1.22	16.66	0.98	19.99	6.94
# 108	13.44	89.26	518.1	1.65	25.89	1.33	1.27	9.34
# 134	16.40	96.08	669.2	1.39	n.d.	2.55	1.36	12.34
# 162	22.58	118.79	692.9	2.57	n.d.	2.97	1.12	10.55
# 173	37.11	162.75	350.1	1.78	n.d.	4.52	1.01	15.63
# 194	20.27	115.32	191.2	1.28	n.d.	4.41	5.32	14.15
Mean ±SD	18.34 ±6.76	84.19 ±34.86	768.9 ±395.0	1.55 ±0.53	15.20 ±5.34	1.96 ±1.36	7.04 ±6.72	9.64 ±3.04
Human Serum	1.52 0.02	4.64 5.20	46.87 33.85	0.80 0.62	75.96 83.98	0.0019 0.018	0.063 n.d.	70.00 70.00

Figure 7. Growth factor contents in platelet lysate from platelet concentrates containing 1.5 x 10^{10} platelets/ml. Concentrations of the growth factors bFGF, EGF, HGF, IGF-1, PDGF-AB, TGF-β1 and VEGF were quantified by ELISA assay in 11 batches of platelet lysates (Rauch et al., 2011). (n.d., not determined).

1.4.2 Processing and use of platelets in cell culture medium

1.4.2.1 How to obtain thrombocyte concentrates

Thrombocyte concentrates (also referred to as platelet concentrate) can be obtained by a blood bank. These concentrates can be produced from leukocyte concentrates, known as buffy coats, from blood or from platelet donors (Singh, Marwaha, Malhotra, & Dash, 2009). Whole blood collected from blood donors is centrifuged to separate erythrocytes from plasma (Figure 8). Buffy coat is a byproduct in the processing of pooled blood products and is located in the interlayer of plasma and erythrocytes (Figure 9) (pluriSelect, 2014; Singh et al., 2009). Buffy coat contains plasma, erythrocytes, thrombocytes and leukocytes. After the centrifugation of the whole blood, the plasma and erythrocytes are separated from each other and the erythrocytes are further filtrated (pluriSelect, 2014). Since buffy coats contain most of the platelets, they are used to obtain thrombocyte concentrates.

At the blood bank by St. Olavs University Hospital in Trondheim, thrombocyte concentrates are obtained by pooling 8 buffy coats that are centrifuged (Appendix 1). After centrifugation

the thrombocytes are isolated in a new bag by a mechanical press. Subsequently, the thrombocytes are pathogen-inactivated, a process called *Intercept treatment* (Appendix 2). This process inactivates viruses, bacteria, leukocytes and parasites. The technology involves UV-light and Amotosalen HCl that interacts with the DNA or RNA and thereby inactivates the pathogens. After having processed one bag with thrombocyte concentrate, certain specification requirements must be met. A bag with a total volume of 180-200 mL should comprise of >240 x 10^9 thrombocytes, <1.0 x 10^6 leukocytes, a pH > 6.4 and a bacteria value of negative (Appendix 3).

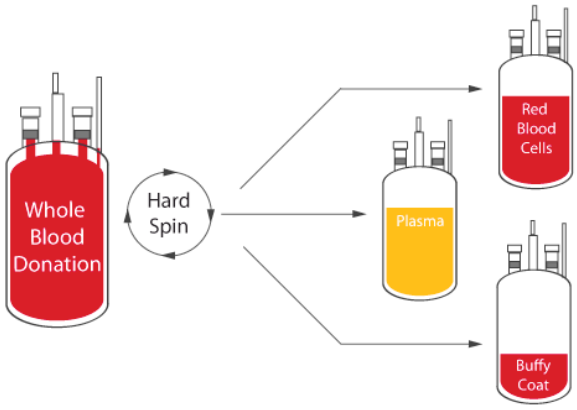

Figure 8. Whole blood from blood donors is centrifuged to separate erythrocytes (red blood cells), plasma and buffy coat (pluriSelect, 2014).

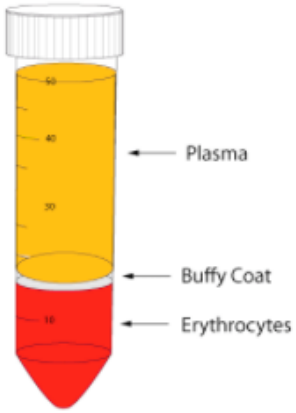

Figure 9. Buffy coat is a byproduct when whole blood from donors is centrifuged to separate plasma and erythrocytes. Buffy coat is located in the interlayer of the red blood cells and plasma (pluriSelect, 2014).

Platelet concentrates are rich in various growth factors, but platelet counts and growth factor content may depend on the type of method used to obtain the concentrate. A study by

Weibrich, Kleis, Hafner, and Hitzler (2002) was done to reveal whether the platelet counts and growth factor content are affected by the technique used to obtain the platelet concentration, or affected by biological conditions such as sex, age and thrombocyte concentration of the donor. It was reported that three types of growth factors were found in high concentrations (PDGF-AB, IGF-1 and TGF-β1), and that there were no significant differences between age and gender concerning growth factor concentration. In addition, there were found no significant correlations between the platelet count and growth factor levels. According to Weibrich et al. (2002), this can be explained by high individual variation in the production of cytokines.

1.4.2.2 Platelet lysate as a supplement in cell culture

Studies in 1980 defined human PL as a rich source of growth factors that could activate established cell lines (Eastment & Sirbasku, 1980). PL contains all the factors platelets consist of (Bieback, 2013). As mentioned earlier, platelets are rich in PDGF-AB, TGF-β1, bFGF and IGF-1, compared to FCS (Naaijkens et al., 2012). Freezing and thawing the platelets will release the factors. The mechanical lysing of the platelets is easy, less time-consuming and less cost-effective compared to chemical activation of the platelets (Bieback, 2013). The morphological changes of human platelets by freezing and thawing them are seen in Figure 10 (Rauch et al., 2011). After one freeze/thawing cycle, normal resting platelets with a round shape form long pseudopodia-like structure.

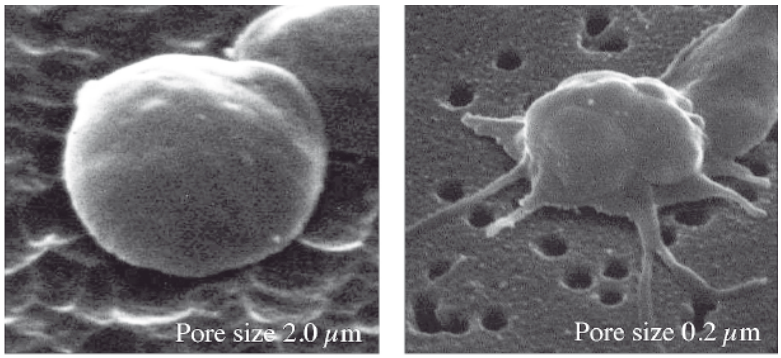

Figure 10. Human thrombocytes analyzed by scanning electron microscopy on Millipore filters (pore size 2.0 μm for control and 0.2 μm for activated platelets). Magnification is 10,000x. Shown are platelets in resting state (left) and after one cycle of freezing and thawing (right) (Rauch et al., 2011).

PL as a supplement in cell culture with MSC has been shown to induce proliferation at a higher rate than FBS (Jonsdottir-Buch et al., 2013; Prins et al., 2009). A study by Jonsdottir-Buch et al. (2013) that included variables such as fresh and expired platelet concentrates in cell cultures showed no differences in results between the cell cultures. According to Jonsdottir-Buch et al. (2013), PL from expired platelet concentrations can fully replace FBS in cell cultures with MSC. Additionally, use of several different batches of PL does not affect proliferation, differentiation or cloning efficiency of MSC, indicating a tendency of the presence of various growth factors and cytokines in PL (Prins et al., 2009).

Chiara Barsotti et al. (2013) evaluated the *in vitro* effect of PL concentrations ranging from 1% to 20% on proliferation and activity of monocytes, endothelial cells, fibroblasts and keratinocytes. 10 % and 20 % PL were shown to increase cell activity and proliferation, which may support the idea of using PL as a substitute for FBS and growth factors with a dose-related effect. Another evaluation of different thrombocyte concentrations as a 5 % supplement in basal medium with MCS, showed that a platelet concentration below 1.5 x 10^9/ml reduced the proliferative effect on MSC (Lange et al., 2007). This concentration is consistent with the previously mentioned requirements that must be met by the blood bank at St. Olavs University Hospital when manufacturing platelet concentrates.

The use of PL has both advantages and disadvantages compared to animal and human serum. Use of platelet lysate will likely decrease the risk of contamination, xenogenic infections and immunological reactions against proteins, compared to the use of FBS (Cholewa et al., 2011). However, PL is not fully defined. Although blood-derived products are tested for diseases and viruses, this does not exclude the risk of infections (Hemeda et al., 2014). PL is also rarely distributed commercially since it is easy to generate and therefore not comparable to the high profit of FBS/FCS. Further, use of expired platelets is considered free of ethical concerns, but it is important to remember that the use of any human-derived products can rise ethical constrains (Jonsdottir-Buch et al., 2013).

1.4.2.3 Stability of platelet lysate

Fekete et al. (2012) analyzed the stability of PL incubated at 4°C or 37°C for up to 28 days (Figure 11). A MILLIPLEX cytokine assay was performed to quantify human cytokines and

chemokines in PL. Six highly present soluble factors (>10 ng/ml) in PL was evaluated every week for 4 weeks. These six factors (sCD40L, PDGF-AA, PDGF-AB/BB, sVCAM-1, sICAM, CCL5/RANTES) are known to act as growth factors or adhesion factors. PDGF-BB, sICAM-1, sVCAM-1 and CCL5 maintained stable levels when incubated at 4°C. sCD40L levels declined from day 0. When PL was incubated in cell culture conditions (37°C, 5% CO_2), the cytokines showed progressively declining levels for all factors except RANTES. PDGF-BB levels were stable for up to at least 7 days.

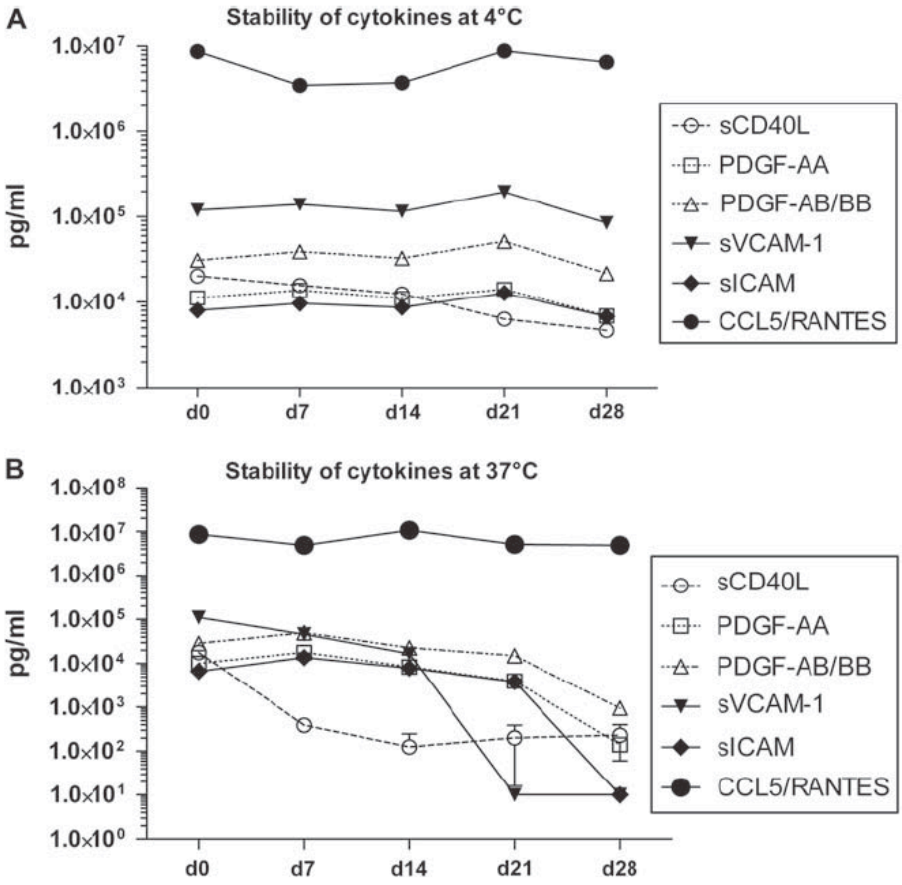

Figure 11. Stability of cytokines in human platelet lysate. Six soluble factors detected in human platelet lysate were evaluated by multiplex cytokine assay every week for 4 weeks. **A)** Analysis of cytokines from one batch of PL incubated at +4°C. **B)** Analysis of cytokines from one batch of PL incubated at 37°C (Fekete et al., 2012).

2. Materials and methods

2.1 Cell culture medium

2.1.1 Preparation of platelet lysate

Use of human materials in research needs to be approved by an ethical committee. The research conducted in this thesis was approved by the Regional Committees for Medical and Health Research Ethics (REC). It was approved as a sub-project of the REC number: 2009/2245.

Thrombocyte concentrates were made according to standard protocols at St. Olavs University Hospital in Trondheim (Appendix 1, 2 and 3). Briefly, eight pooled ABO-identical buffy coats were welded together with platelet-additive solution (PAS IIIM) and the solutions were mixed and stored for 30 min. Buffy coats were then centrifuged to obtain a supernatant rich in platelets. The supernatant was transferred to a new bag by a mechanical press (Optipress II) that stops pressing when red blood cells are detected. The result is a bag with a thrombocyte concentrate consisting of approximately 32 % plasma. The thrombocyte concentrates were subsequently incubated for one hour in 22 °C to stabilize the platelets before intercept-treatment to inactivate pathogens. The platelet rich concentrates have a shelf life for seven days before considered as expired, due to reduced platelet function and increased risk of bacterial contamination.

Two bags of 190 mL expired thrombocyte concentrates were kindly provided by the blood bank. The thrombocyte concentrates were aliquoted in 3 mL/tubes and frozen at -80 °C to lyse the cells and stored at -80 °C until use. Before use, PL was thawed and spun down at 953 g (~ 2,000 rpm) for 5 min and the supernatant was used as a medium supplement (Prins et al., 2009). PL was filtrated with 0.45 μm filter before applied to the medium. All experiments and passages of cells included fresh-thawed platelet lysate.

2.2 Cell lines and cell culture conditions

2.2.1 Human myeloma cell line, OH-2

The hMCL OH-2 was established at St. Olavs University Hospital in 1991 and was harvested from pleural fluid from an MM patient in terminal stage of the disease. The cells grow non-adherent mostly as single-cell suspension. OH-2 cells are cultured with RPMI 1640, L-glutamine (100 μg/ml), gentamicine (20 μg/ml), IL-6 (2 ng/ml), supplemented with 10 % HS and cultured in 5 % CO_2 atmosphere and 37 °C (Borset et al., 1994).

2.2.2 Human myeloma cell line, IH-1

The hMCL IH-1 was established at St. Olavs University Hospital in 1999. The IH-1 cells were isolated from pleural fluid from a stage II MM patient. The cells are grown in RPMI 1640, L-glutamine (2 mM), gentamicine (20 μg/ml) and supplemented with 10 % HS and IL-6 (2 ng/ml). The cells grow non-adherent with occasional clustering of cells (Hjertner et al., 2001).

2.2.3 Human myeloma cell line, KJON

The hMCL KJON was established at St. Olavs University Hospital in 2010. These cells were established from a MM patient at terminal stage. Cells derive from blood where 86.6 % of the cells were plasma cells. KJON cells are cultured in RPMI 1640, L-glutamine (2 mM) and gentamicine (20 μg/ml) and supplemented with 5 % HS and IL-6 (2ng/ml). They grow non-adherent as single-cells suspension in human serum (Våtsveen et al., 2012).

2.3 Experimental equipment

2.3.1 Cell counting

Cells were counted using a Beckman Coulter counter. This method uses a direct electronic resistance method for cell counting and sizing (Beckman Coulter, 2014). Briefly, the counter detects an electrical resistance that is caused by fluid with cells that drives through microchannels (Hughes & Mehmet, 2003). Particles that are suspended in electrolyte are counted by allowing them to pass through an aperture (Beckman Coulter, 2014). A voltage pulse is measured which is proportional to the volume of the particle. By using pulse height analyzer circuits, the number of particles in the sample can be determined. The concentration of particles in the sample can also be determined if the volume of the liquid passing through the aperture is controlled (Beckman Coulter, 2014).

2.3.2 Apoptosis and Flow Cytometry

Apoptosis was studied by analyzing Annexin V-FITC and PI uptake on flow cytometer. Flow cytometer is a device used to analyze a large number of cells. The method has been widely used to study apoptosis of liquid-suspended cells (Hughes & Mehmet, 2003). Flow cytometry involves fluorescent labeled antibodies to detect the cells. Parameters that can be measured in flow cytometry include forward and side light scatter, which is influenced by cell size and morphology, respectively. The side scatter reflects the complexity or the amount of granules in a particle (Wlodkowic, Telford, Skommer, & Darzynkiewicz, 2011).

Apoptotic cells express the negatively charged phospholipid *phosphatidylserine* (PS) on the outer membrane (Alberts et al., 2008). This occurs early in the apoptotic cell death during which the cell membrane remains intact (Olsen, 2012). Annexin V is a protein with anticoagulant properties that binds to phospholipids in a Ca^{2+}-dependent way (Lodish et al., 2008). Fluorescein isothiocyanate (FITC) labeled Annexin V is commonly used as a specific probe that will bind to PS. Annexin will not bind to viable cells. Emitted light from the cells that are bound to Annexin V-FITC is converted to electrical signals proportional to the amount of fluorochrome bound to cells (Wlodkowic et al., 2011).

Propidium iodide (PI) is used in flow cytometry to part dead cells from apoptotic cells (Van Engeland, Nieland, Ramaekers, Schutte, & Reutelingsperger, 1998). The dead and apoptotic cells can be presented in a diagram with four quadrants, Q1-Q4 (Figure 12). Q2 represents dead cells, Q3 represents viable cells and Q4 represent apoptotic cells (Olsen, 2012).

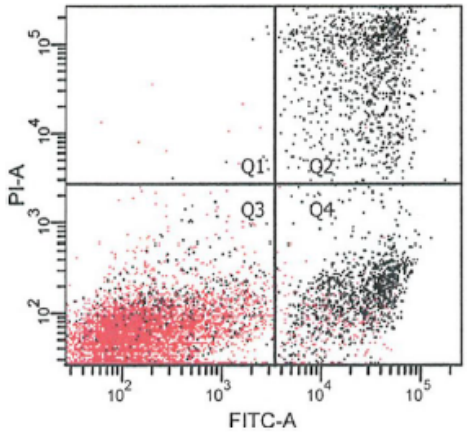

Figure 12. Apoptosis analysis of the hMCL IH-1 performed with flow cytometry. Viable cells are negative for both Annexin V-FITC and Propidium iodide (PI), represented in Q3. Early apoptotic cells that express phosphatidylserine (PS) bind only Annexin V-FITC, represented in Q4. Q2 represents dead cells (Olsen, 2012).

2.3.3 Proliferation analysis

The number of viable cells in a culture can be determined by analyzing the ATP content, which indicates the presence of metabolically active cells (Promega, 2012). The ATP content can be measured with CellTiter-Glo® Luminescent Cell Viability Assay, which is a fast and sensitive homogeneous method. The assay involves the addition of CellTiter-Glo® Reagent directly to cells cultured in serum-supplemented medium. By adding the reagent, the cells will become lysed and a luminescent signal proportional to the ATP content can be measured (Promega, 2012).

During the CellTiter-Glo® assay, a thermo-stable luciferase (Ultra-Glo™ Recombinant Luciferase) takes part in a reaction that generates a "glow-type" luminescent signal. The reaction involves mono-oxygenation of luciferine by luciferase in the presence of ATP, molecular oxygen and Mg^{2+}, shown in Figure 13. The luminescent signal reflects the ATP

content in the cell culture, and is proportional to the number of cells (Figure 14) (Promega, 2012).

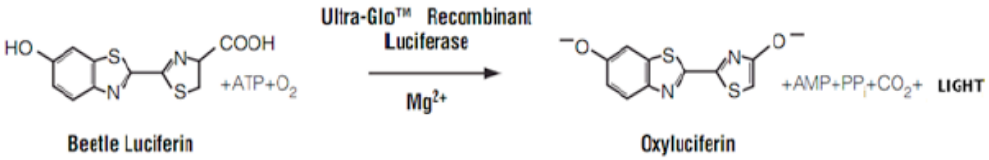

Figure 13. The luciferase reaction. Luciferase catalyzes the mono-oxygenation of luciferine in the presence of ATP, molecular oxygen and Mg^{2+} (Promega, 2012).

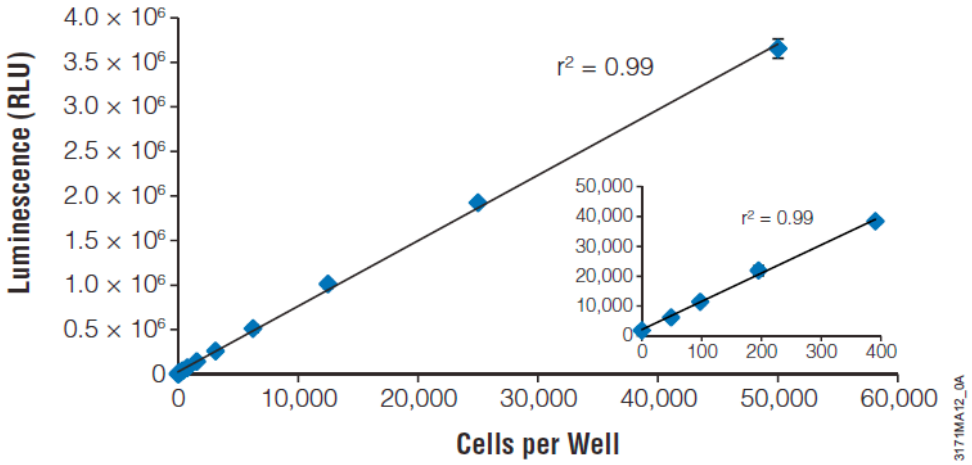

Figure 14. Luminescence (RLU) measured with CellTiter-Glo assay is directly related to the number of cells from 0 to 50,000 cells per well (Promega, 2012).

2.4 Experimental design

2.4.1 Proliferation assay

IH-1, KJON and OH-2 cells were seeded in 96-well culture plates with different concentrations of PL and/or HS (Table 1). IH-1 was seeded with a density of 10,000 cells/well. KJON and OH-2 cells were seeded with a density of 20,000 cells/well.

In short, RPMI-1640 (Sigma Aldrich) including gentamicin (20 μg/ml), L-glutamine (100 mg/ml) and IL-6 (2 ng/ml) (Biosource, CA, USA), was supplemented with PL or HS. Cells were spun down and suspended in RPMI 1640. The cells were counted using Coulter counter. 50 μL of each condition was seeded in each well in triplicates followed by 50 μL of cells. All the conditions were made twice as concentrated, as they would be diluted with 50 μL of cells. The culture plates were incubated for 3 days (5 % CO_2 atmosphere and 37 °C) before analyzed. Prior to the CellTiter-Glo assay, the cells were incubated in room temperature for 30 minutes and then stimulated with 100 μL CellTiter-Glo reagent for 10 minutes.

2.4.2 Apoptosis assay

Medium and cell preparations were identical to the proliferation assay (Table 1). Cells were seeded in 96-well culture plates (Corning Costar, Corning, New York, USA) with different concentrations of PL and HS. IH-1 was seeded with a density of 0.2 x 10^6 cells/ml and KJON and OH-2 was seeded with a density of 0.4 x 10^6 cells/ml. 100 μL of each condition and 100 μL of cells were added to each well in duplicates. The culture plates were incubated for 3 days (5 % CO_2 atmosphere and 37 °C) and then stained with Annexin V-FITC and PI according to protocol (APOPTEST-FITC kit, Nexins Research, Hoeven, Netherlands). In short, cells were washed and spun down and subsequently stimulated with Annexin V-FITC (0.25 μL/sample) in binding buffer. The cells were then incubated on ice for one hour and protected against light. PI (2 μl/sample) was added 5 minutes before running the samples on BD LSR II flow cytometry (BD, Biosciences, USA).

The results from the flow cytometry were analyzed using the software *FlowJo*. PI-negative and Annexin V-FITC positive cells are classified as apoptotic cells, and PI-positive cells are classified as dead cells. Cells that are both negative for PI and Annexin V are viable cells.

A statistical analysis was performed with a Student's t-test. A *p*-value of 0.05 or less was considered significant. The test was performed to reveal possible significant differences between the conditions *10 % HS* and *10 % PL,* and/or *10 % HS* and *10 % FCS.* The following formula was used:

$$t_{exp} = \frac{|(\overline{x_1} - \overline{x_2})|}{s_d/\sqrt{N}} = \frac{|(\overline{x_1} - \overline{x_2})|}{\sqrt{\frac{{s_1}^2}{N_1} + \frac{{s_2}^2}{N_2}}}$$

where:

$\overline{x_1}$ and $\overline{x_2}$ are the mean values of dataset 1 and 2

s_1 and s_2 are the standard deviations of dataset 1 and 2

N_1 and N_2 are the numbers of measurements of dataset 1 and 2

(Løvås, 2004).

Table 1. Various concentrations of PL and HS were supplemented in basal media for apoptosis- and proliferation assay. FCS was used as a control in some experiments.

Condition	Basal medium
20 % PL	RPMI 1640, gentamicin (20 μg/ml), glutamine (100 μg/ml), IL-6 (2ng/ml)
10 % PL	″
7.5 % PL	″
5 % PL	″
7.5 % PL + 2.5 % HS	″
5 % PL + 5 % HS	″
2.5 % PL + 7.5 % HS	″
10 % HS	″
10 % FCS	″

2.4.3 Long-term experiment

OH-2, KJON and IH-1 cell lines were cultured in flasks for long-term cultivation. RPMI 1640, including gentamicin (20 μg/ml) and L-glutamin (100 μg/ml), was supplemented with 10 % or 20 % fresh PL, or 10 % or 5 % HS. IL-6 (2 ng/ml) was added to the medium.

During the first long-term cultivation, IH-1 and OH-2 cells were cultured in 75 cm^2 flasks with 10 % PL and 10 % HS. OH-2 cells were seeded with a density of 0.2 x 10^6 cells/ml and IH-1 cells were seeded with a density of 0.1 x 10^6 cells/ml. During every passage the same initial number of cells was seeded in the flasks. Counting of cells and replenishment of fresh medium were performed two times a week.

A second long-term cultivation was performed with IH-1 and KJON cells. During this experiment, cells were cultured in 75 cm^2 flasks containing 20 % PL. IH-1 and KJON were also cultured with 10 % and 5 % HS respectively. The cells were counted and medium replenished two times a week. IH-1 and KJON were seeded with a density of 0.1 x 10^6 cells/ml and 0.15 x 10^6 cells/ml respectively. Both cell lines were cultured in 5 % CO_2 atmosphere and 37 °C.

3. Results

Proliferation and apoptosis assay of the cell lines IH-1, KJON and OH-2 were initially performed to compare proliferation and apoptosis of PL and HS as medium supplements. Long-term cultivation was then performed to further study the long-term effect of PL on cells.

3.1 Proliferation assay

Proliferation analysis was performed for the cell lines IH-1, OH-2 and KJON using CellTiter-Glo viability assay that measures metabolically active cells in term of ATP-levels. The results presented below represent one example out of two or three experiments. Shown in Figure 15, 16 and 17 are relative luciferase units (RLU) that reflect the number of metabolically active cells for each condition.

For OH-2, luminescence increased with an increasing level of supplemented HS in medium (Figure 15). Luminescence was highest for cells cultured in 10 % HS and lowest for cells cultured 5 % and 7.5 % PL, and higher in FCS than pure PL.

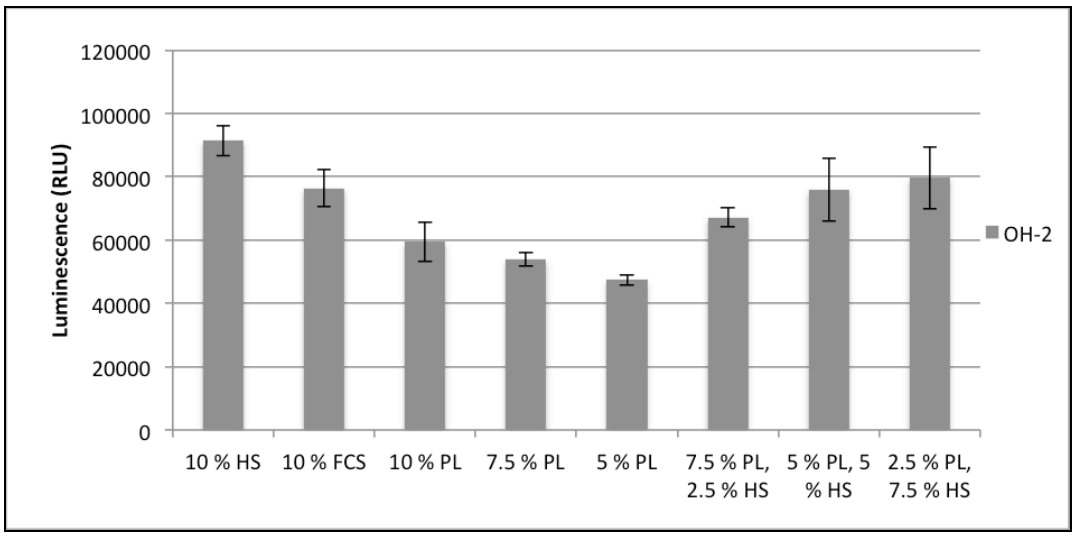

Figure 15. Proliferation assay of OH-2 cells. The cells were cultured in various concentrations of PL and HS and cultured for 3 days with 5 % CO_2 and 37 °C before analyzed by CellTiter-Glo assay. Bars represent mean ± SD of triplicate wells.

Proliferation assay of IH-1 showed a high luminescence for cells cultured in HS, and low luminescence with FCS (Figure 16). Similar to proliferation assay of OH-2 cells, luminescence declined in accordance with lower concentrations of PL. Luminescence seemed to increase with an increasing level of HS, except for the condition with both 2.5 % PL and 7.5 % HS.

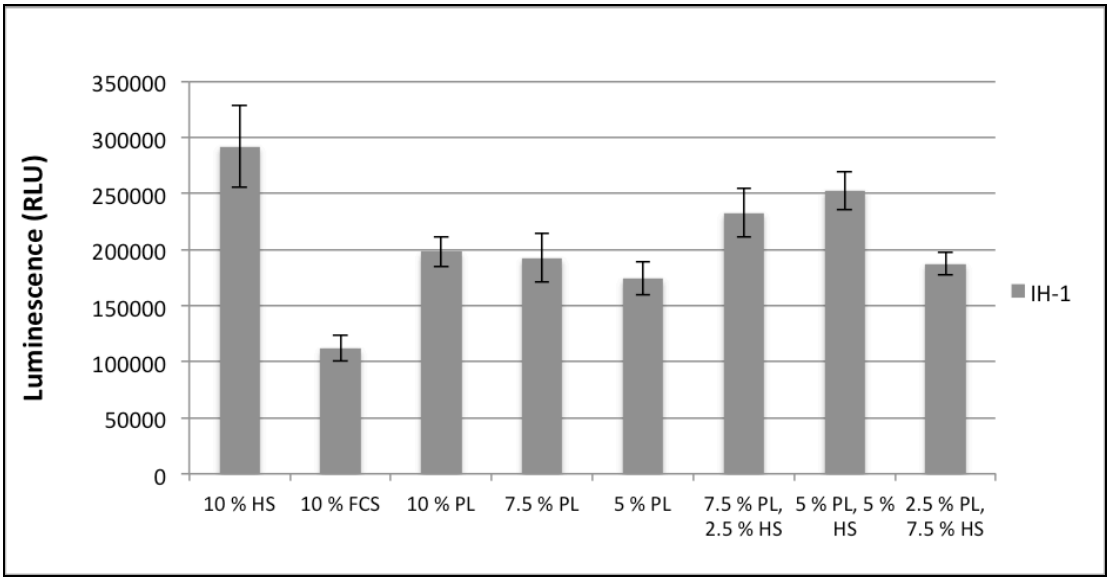

Figure 16. Proliferation assay of IH-1 cells. The cells were cultured in various concentrations of PL and HS and cultured for 3 days with 5 % CO_2 and 37 °C before analyzed by CellTiter-Glo assay. Bars represent mean ± SD of triplicate wells.

Proliferation analysis of KJON showed that HS outperformed all the other conditions, shown in Figure 17. FCS showed the lowest amount of metabolically active cells, indicating a higher amount of dead cells in the culture. Luminescence decreased with a decreasing concentration of PL, and increased in conditions with higher concentrations of HS, a similar trend to the proliferation assay of OH-2.

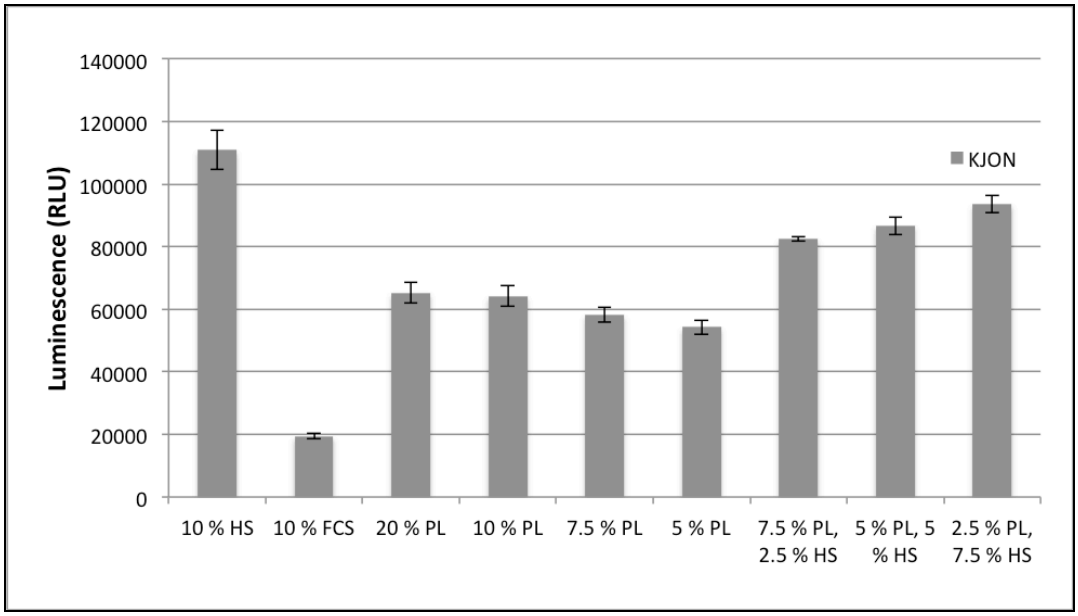

Figure 17. Proliferation assay of KJON cells. The cells were cultured in various concentrations of PL and HS and cultured for 3 days with 5 % CO_2 and 37 °C before analyzed by CellTiter-Glo assay. Bars represent mean ± SD of triplicate wells.

3.2 Apoptosis studies

Apoptosis analysis of OH-2, KJON and IH-1 cells was performed using Annexin V-FITC/PI staining and flow cytometry. The results are shown in a bar chart, which shows the percentage of viable cells in each condition (Figure 18, 19 and 20). Viable cells are negative for both Annexin V-FITC and PI.

Apoptosis analysis of IH-1 cells showed a high percentage of viability in all conditions, ranging from an average of 74.45 % to 91.3 %, shown in Figure 18. IH-1 cells had a lower percentage of viability in 10 % FCS than the other conditions, which is consistent with the results from the proliferation assay of IH-1.

OH-2 cells were mainly detected as viable or dead. OH-2 cells showed a higher degree of dead cells than IH-1 cells, with an overall viability less than 60 %, except for the condition with 10 % HS (~ 66.55 %). The viability of OH-2 cells ranged from an average of 38.5 % to 66.55 %, shown in Figure 19. A statistical analysis showed a significant difference between the conditions *10 % HS* and *10 % PL* ($p < 0.05$).

Apoptosis assay of KJON showed that FCS gave a low viability of 6.83 %, and a high amount of dead cells. Conditions with KJON in both HS and PL were somewhat higher than pure HS and pure PL. However, these differences were not significant different. Except for the condition with FCS, the overall viability for KJON cells ranged from 63 % to 74.8 %, as shown in Figure 20. A statistical analysis showed a significant difference between the conditions *10 % HS and 10 % FCS*, and *10 % FCS and 20 % PL* ($p < 0.05$).

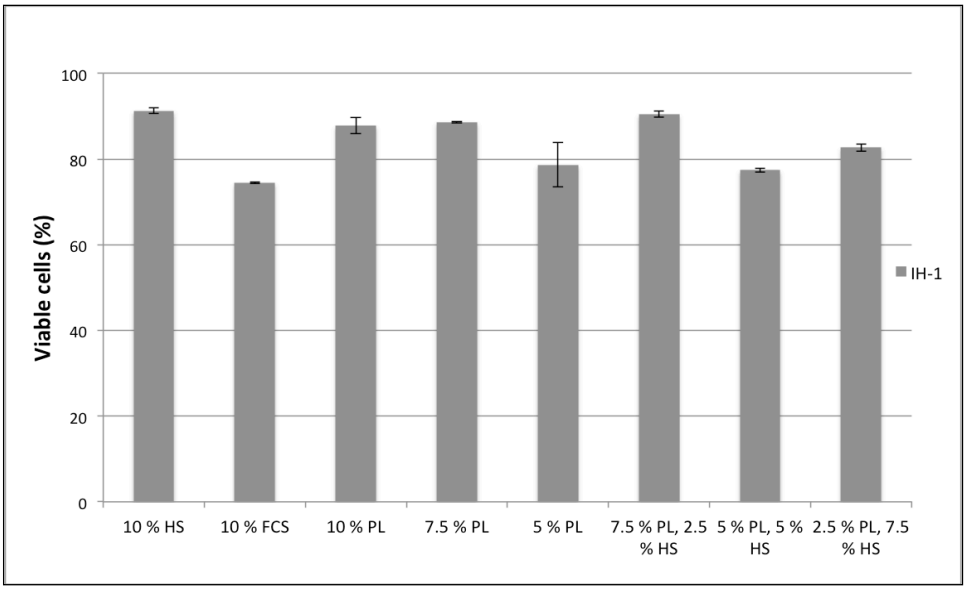

Figure 18. Apoptosis of IH-1 in various conditions was studied by Annexin- V-FITC/PI and flow cytometry. Viable cells are negative for both Annexin and PI. Bars represent mean ± SD of duplicate wells.

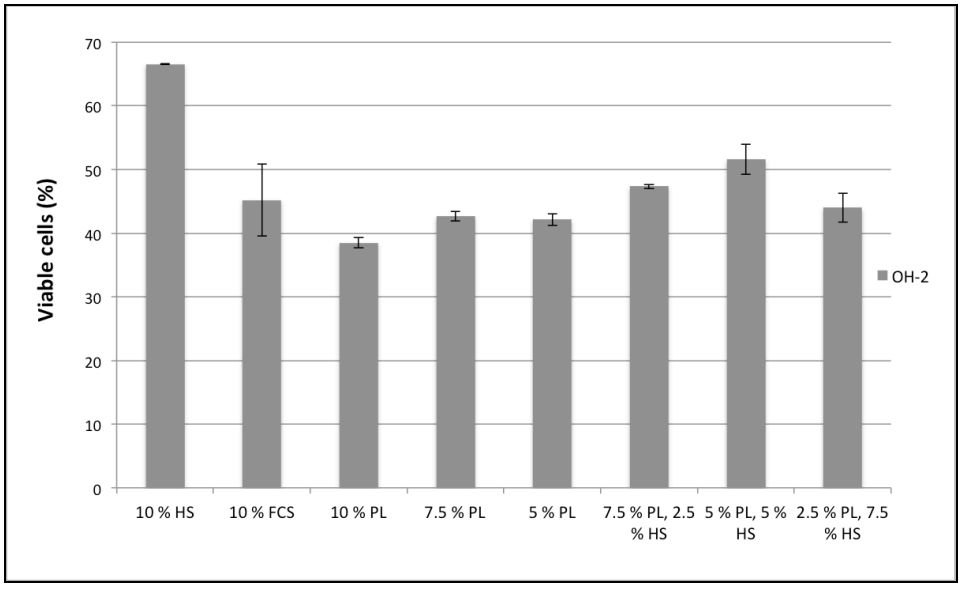

Figure 19. Apoptosis of OH-2 in various conditions was studied by Annexin V-FITC/PI and flow cytometry. Viable cells are negative for both Annexin and PI. Bars represent mean ± SD of duplicate wells. A statistical analysis of the results showed a significant difference between *10 % HS* and *10 % PL* ($p < 0.05$).

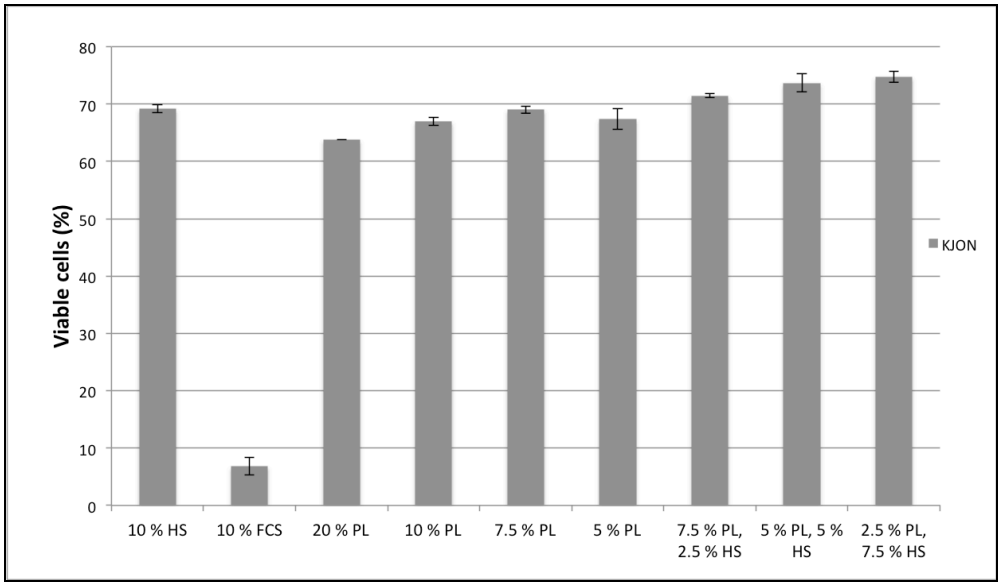

Figure 20. Apoptosis of KJON in various conditions was studied by Annexin V-FITC/PI and flow cytometry. Viable cells are negative for both Annexin and PI. Bars represent mean ± SD of duplicate wells. A statistical analysis of the results showed a significant difference between *10 % HS* and *10 % FCS*, and between *10 % FCS* and *20 % PL* ($p < 0.05$).

3.3 Cells behave differently in PL and HS

When cells were cultured in 96-well plates prior to proliferation and apoptosis assay, it was observed that the cells had different behavior in the various conditions. The same observation was true for the three cell lines IH-1, KJON and OH-2. Cells cultured in HS formed a crescent in the periphery of the well, while those cultured in conditions with PL were spread throughout the well. As the concentration of HS increased in the conditions, the cells seemed to gather more. Figure 21 shows a representation of KJON cells cultured in 10 % HS and 10 % PL.

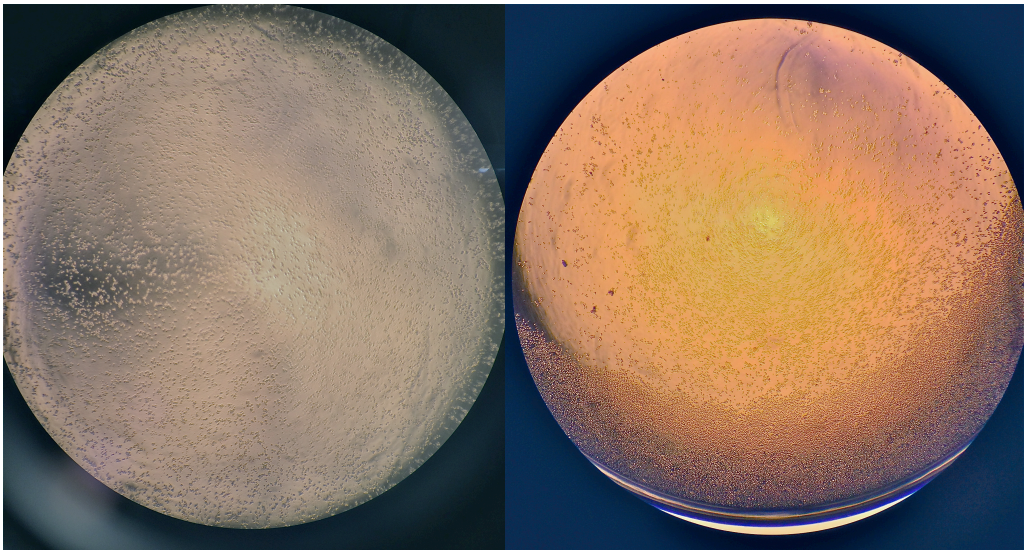

Figure 21. A representation of KJON cells cultured in 96-well plates in 10 % HS and 10 % PL. Cells cultured in PL are shown to the left. These cells were spread throughout the well. The condition with HS is shown to the right and these cells formed a crescent in the periphery of the well.

3.4 Long-term cultivation of cells

Long-term cultivation was performed for IH-1, OH-2 and KJON. First, IH-1 cells were cultured in medium supplemented with 10 % PL or 10 % HS. Cells were cultured for 10 days. The results are presented in Figure 22. After day 3, the IH-1 cells cultured in HS had a higher cell count at each subsequent measurement compared to the cells cultured in PL. On day 10 the theoretical total number of cells was 116.3 x 10^6 in PL and 806.7 x 10^6 in HS. The cells in HS had approximately a five-fold increase in cell number between each passage, while only a

two to three-fold increase in PL. There were no observed differences in cell morphology in microscopy in HS and PL.

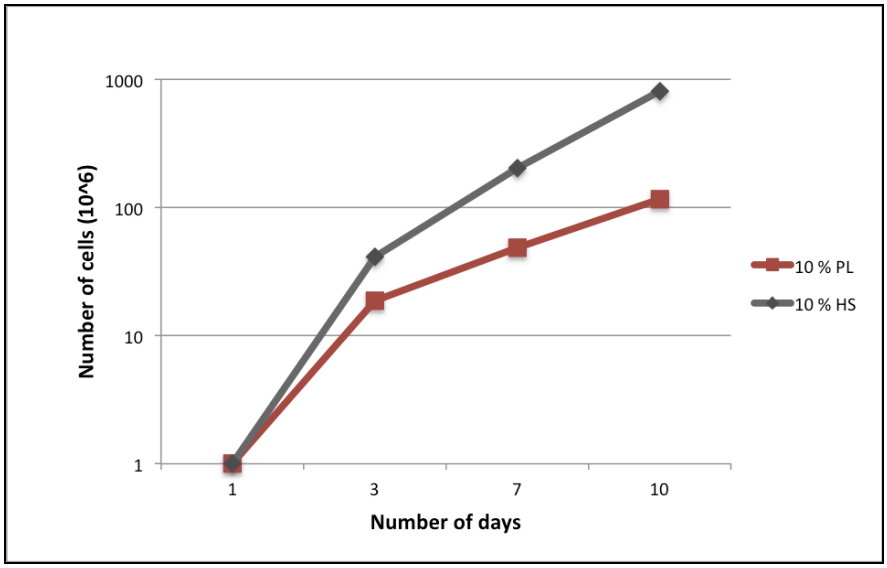

Figure 22. IH-1 cells were cultured in media supplemented with 10 % PL or 10 % HS for 10 days. Cells were counted before each passage and the same amount of cells was seeded every time. Cells were seeded with a density of 0.1 x 10^6 cells/ml and 0.075 x 10^6 cells/ml, each third and fourth day respectively.

OH-2 cells were cultured in 10 % PL or 10 % HS. Cells were seeded with a density of 0.2 x 10^6 cells/ml during each passage. The cells were demanding considering proliferation rate. During the first 7 days of cultivation, the total amount of cells increased in both culture flasks with HS and PL. However, the amount of cells in PL decreased from day 7, in which proliferation declined. The experiment was terminated after 10 days, as the cells in PL did not show an increase in the number of cells between day 7 and 10. As shown in Figure 23, it is a distinct difference between the cells cultured in PL and HS. The cells that were cultured in HS had a slow proliferation rate as well, but did not stop growing. By the end of the experiment the total theoretical number of cells in PL was 4.07 x 10^6 and 13.9 x 10^6 in HS. No differences in cell morphology between the respective culture flasks were observed in microscopy.

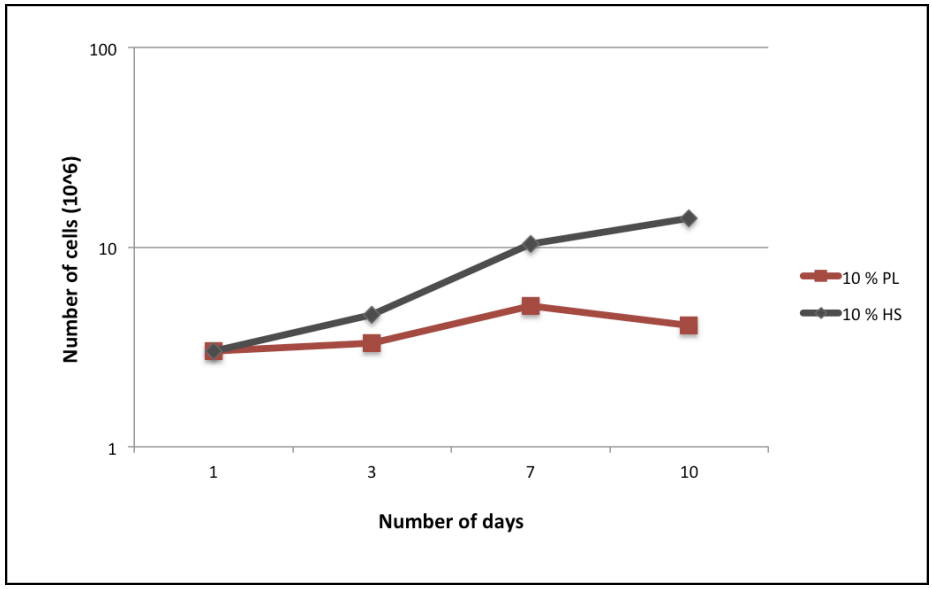

Figure 23. OH-2 cells were cultured in media supplemented with 10 % PL or 10 % HS for 10 days. Cells were counted before each passage and the same amount of cells was seeded every time. Cells were split twice a week and seeded with a density of 0.2 x 10^6 cells/ml.

A second long-term cultivation was performed with IH-1 and KJON. IH-1 was cultured in 20 % PL or 10 % HS, and KJON was cultured in 20 % PL or 5 % HS. The results are shown in Figure 24 and 25. The cell count for IH-1 in PL on day 3 was higher than in HS, but proliferation then subsequently declined in PL while maintained a stable rate in HS. By day 14, the IH-1 cells in PL had reached a theoretical cell number of 557.7 x 10^6 cells, while a number of 13,624.7 x 10^6 cells in HS.

KJON cells have in general a slower proliferation rate than IH-1 cells, but are far from as demanding as OH-2 cells. As shown in Figure 25, proliferation was seemingly slower in PL than HS. The cells grew similarly from day 1 to 3, but proliferation then declined in the condition with PL. By day 10 the total theoretical number of cells was 20 x 10^6 in PL and 135.8 x 10^6 in HS. There were no observed differences in cell morphology or amount of dead cells in microscopy.

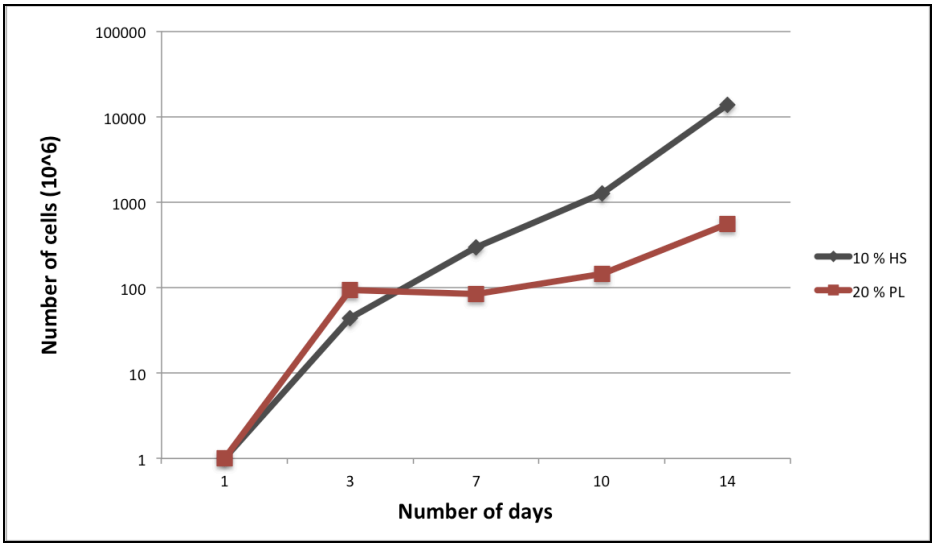

Figure 24. IH-1 cells were cultured in media supplemented with 20 % PL and 10 % HS for 14 days. Cells were counted before each passage and the same amount of cells was seeded every time. IH-1 cells were seeded with a density of 0.1 x 10^6 cells/ml and 0.075 x 10^6 cells/ml, each third and fourth day respectively.

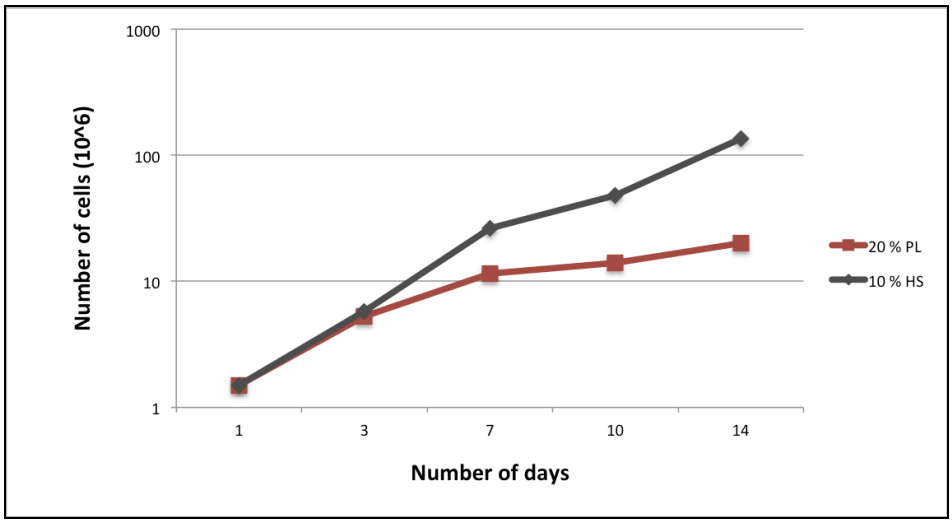

Figure 25. KJON cells were cultured in media supplemented with 20 % PL and 5 % HS for 14 days. Cells were counted before each passage and the same amount of cells (0.15 x 10^6 cells/ml) was seeded every third and fourth day.

4. Discussion

Lot of research on MM cell lines has identified various properties of the cancer cells with respect to proliferation, growth and responses on cytokines (Borset et al., 1994; Hjertner et al., 2001; Våtsveen et al., 2009b). The hMCLs IH-1, OH-2 and KJON are all cultured in HS, which is considered as the "gold standard" when it comes to medium supplement for these cell lines. Why these cell lines prefer HS rather than FCS still remains to be fully answered. PL and its proliferative effect as a medium supplement in cultures with the respective cell lines was tested in this thesis because a possible replacement to HS would be less ethical concerned, more economically favorable and give a more stable access to medium supplements.

The process to produce PL from platelet concentrates will be discussed below followed by the results from the experiments. The various experimental methods used will also be assessed.

4.1 Processing and use of PL in cell culture medium

Platelet concentrates can be obtained from pooled buffy coats collected from whole blood or apheresis from blood or from platelet donors. Some papers discuss the possible impacts of various methods used to obtain platelet concentrates and whether properties such as age and gender of the blood donors can influence the growth factor content in PL (Weibrich et al., 2002). PL is a biological product and differences in performance due to batch variations are thus important to consider (Johansson, Klinth, Holmqvist, & Ohlson, 2003). Nevertheless, PL derived from pooled buffy coats should minimize these possible factors due to less variation (Hemeda et al., 2014). Only one batch of platelet concentrate derived from eight pooled buffy coats was used in this thesis. Hence, it is not appropriate to discuss possible batch differences related to experimental results in this thesis.

The manufacturing of platelet concentrates is carefully executed at St. Olavs University Hospital and the respective blood bank follows strict protocols (Appendix 1, 2 and 3). Intercept treatment of thrombocyte concentrates further reduces the infectious risk from virus

and bacteria. The latter supports the idea that the use of platelet lysate decreases the risk of contamination and infection compared to animal serum (Cholewa et al., 2011).

Various methods are also used to process PL from platelet concentrates. In this thesis it was chosen to follow a similar protocol by Naaijkens et al. (2012). Most of the reports found related to PL include research on MSC. The methods to obtain PL are usually based on the same principle with shock freezing to lyse the cells and centrifugation of the respective lysate to obtain supernatants (Chiara Barsotti et al., 2013; Doucet et al., 2005; Hemeda, Kalz, Walenda, Lohmann, & Wagner, 2013; Jonsdottir-Buch et al., 2013; Mirabet et al., 2008; Schallmoser et al., 2007). However, many of these preparations of PL differ in terms of number of freezing/thawing cycles and gravity during centrifugation. Despite the various preparation methods, the use of PL as a medium supplement has approached the same results (i.e., PL induces proliferation of MSC). From this view it is less likely that the differences in preparations influence the quality of PL and its function. But, it is still not possible to predict how important the choice of preparation method is regarding the use of PL in cultures with hMCLs.

The process that was used to prepare PL as a medium supplement in this thesis was somewhat time-consuming. Platelet concentrates were aliquoted in 3 ml tubes, frozen at -80 degrees and then thawed prior to use. PL was then subsequently centrifuged and filtrated and the supernatants were used as a medium supplement. If PL were to replace HS it would be advantageous to find another preparation method. Preparations would have been less time-consuming if the PL had been centrifuged and filtrated after lysing and then restored at -80 degrees. It would also be more convenient if PL could be stored in a refrigerator rather than in a freezer after thawing and filtration. This would save time during laboratory work. However, the stability of PL over time is not defined. Fekete et al. (2012) analyzed the stability of various cytokines in PL in both 4°C and 37°C, but the results can not be used to predict the stability of the biological activity among the cytokines.

Three different methods were used in this thesis to study the effect of PL. Flow cytometry and CellTiter-Glo assay were performed initially to study apoptosis and proliferation respectively. Long-term cultivation of the cells was performed to study the long-term effect of PL. This was the most important method, because this type of experiment reveals how the various cell

types behave and proliferate in cultures over time. The results and methods will be discussed below.

4.2 Apoptosis analysis

The rapid events that happen during apoptosis can make apoptosis difficult to study. Annexin V is a phospholipid binding protein and has a high affinity for the surface exposed PS, which flips to the outer membrane leaflet during apoptosis. This exposure to the outer membrane happens early in apoptosis. Annexin V cannot bind to viable cells because it cannot penetrate the membrane. However, the integrity of the membrane is lost in dead cells, and Annexin V can then bind to the inner leaflet of the membrane. PI is therefore additionally used in flow cytometry to part dead cells from apoptotic cells. Flow cytometry is thus a simple and fast method to study apoptosis and the results are highly reliable (Van Engeland et al., 1998).

Flow cytometry was performed for the cell lines IH-1, OH-2 and KJON. The cells were cultured in both HS and PL for 3 days before running them on a flow cytometer. As shown in Figure 18, apoptosis analysis of IH-1 showed little differences in the percentage of viable cells between the different conditions. There were no distinct differences between PL and HS as a medium supplement in the apoptosis analysis of IH-1, and the variations in viability might be due to sources of errors, such as uneven seeding of cells and inaccuracy during the preparation process, rather than due to actual biological differences. Though there was no significant difference between the conditions *10 % HS* and *10 % FCS*, it is likely that the difference is biologically relevant, based on the results in Figure 18. FCS had the highest amount of dead and apoptotic cells compared to the other conditions. This is consistent with research that states that IH-1 thrives better in HS than FCS (Brenne et al., 2002).

Apoptosis analysis of OH-2, shown in Figure 19, showed a higher degree of viable cells in 10 % HS compared to any other condition, supporting the theory that the cells are demanding regarding growth (Våtsveen et al., 2009a). It was a significant difference between *10 % HS* and *10 % PL,* which reinforces HS as the optimal medium supplement. It was noteworthy that OH-2 cells had a higher amount of viable cells in FCS than PL. Despite a none-significant difference between FCS and the other conditions, this trend was also observed in the proliferation analysis of OH-2. Other research, however, shows that OH-2 cells die in FCS

over time (Våtsveen et al., 2009b). This strongly indicates that PL cannot be used in cultures with OH-2.

Apoptosis analysis of KJON cells showed no distinct differences between viability of cells in PL and HS (Figure 20). It was a significant difference between *10 % FCS* and *10 % HS*, and *10 % FCS* and *20 % PL.* The variations in viability between the other conditions might be due to sources of errors or coincidences, rather than due to biological differences. However, the amount of dead cells in FCS was high compared to apoptosis analysis of the other two cell lines. It is possible that KJON cells are more sensitive to FCS than the other respective cell lines and that the apoptotic events when KJON is cultured in FCS are initiated already after 3 days of cultivation.

Flow cytometry showed in general small differences between PL and HS in viability among the respective cell lines, especially for IH-1 and KJON. There were no observable trends, and the small differences in viability might be due to sources of errors such as cultivation conditions and inaccuracy in the preparation process, rather being actual biological differences. It is important to bear in mind that the percentage of viability not necessarily reflects the cells' ability to grow and proliferate in PL. It is possible that the cells that were grown in PL would undergo apoptotic events beyond three days of cultivation. Flow cytometry is thus not considered a suitable method alone to determine PL's ability to function as a culture medium supplement. On the other hand, apoptosis analysis of OH-2 cells had more distinct differences in 10 % HS and 10 % PL, and the method seems to be more suitable for these cells.

4.3 Proliferation analysis

Proliferation assay was performed in parallel to flow cytometry to get an overview of cell growth. CellTiter-Glo measures the ATP content in culture. The amount of ATP is proportional to the amount of viable and metabolically active cells. CellTiter-Glo offers high throughput screening. It is quick, sensitive and less hazardous than for example use of thymidine to study proliferation. Another advantage is that only a single reagent (CellTiter-Glo® Reagent) is to be added directly to the cell culture. Neither is removal of medium or cell washing required. The luminescent signal from the luciferase reaction has a half-life of at

least five hours, which makes the method very flexible in terms of multiple plates. A disadvantage with the method is that different treatments can affect the ATP content in the cell. As the number of cells is directly proportional to the ATP content, an uneven seeding of cells may affect the luminescent signal (Promega, 2012).

Proliferation analysis was performed for the cell lines IH-1, OH-2 and KJON. Proliferation analysis of IH-1 cells in various concentrations of HS and PL revealed that the cells were more metabolically active in 10 % HS and in conditions with both HS and PL, compared to pure PL (Figure 16). The increase in luminescence was likely due to the supportive growth effect of HS. FCS did not support proliferation of IH-1 cells in particular, which is consistent with the result from the apoptosis analysis. Proliferation assay of both KJON and OH-2 cells showed that the cells thrive better in HS than any other medium supplement tested (Figure 15 and 17). KJON cells grown in FCS contributed to the lowest amount of ATP measured, which indicates that FCS rather kills the cells than stimulates growth. For both KJON and OH-2, luminescence increased with an increasing concentration of HS, indicating that HS is important to stimulate growth of the respective cells.

Both proliferation and apoptosis assays were performed after three days of cultivation. Despite high precision and sensitivity, the methods are not very suitable to determine the long-term effect of PL. The assays do give an overview of how the cells respond to PL, but three days of cultivation seem to be too short to determine whether the cells will die in PL or not. However, long-term cultivation of the cells revealed a slowing proliferation rate in PL over time. CellTiter-Glo assays revealed more distinct differences between HS and PL as a culture medium for all cell lines, compared to the apoptosis assays. Porliferation assays confirmed that the cells from the respective cell lines thrive better in HS. The results from the proliferation assays are thus considered reliable because all experiments showed the same trend in which the cells thrive better in HS than PL and FCS.

4.4 Cells behave differently in PL and HS

An interesting observation was made when the various cell lines were cultured in 96-well plates in both apoptosis and proliferation assay. In all experiments and after 3 days of cultivation, cells seemed to behave differently in HS and PL. As shown in Figure 21, cells

cultured in HS formed a crescent in the periphery of the well. Cells that were cultured in PL spread throughout the wells. Many factors can contribute to this. One hypothesis is that PL contains different levels of chemokines compared to HS. Fekete et al. (2012) analyzed various chemokines in PL and found high levels of RANTES, GRO, IL-8, MIP-1β and MIP-α, which are all cell migration factors. RANTES are among the chemokines that are mainly produced by platelets.

4.5 Long-term cultivation

Long-term cultivation of the cells was the most important method in this thesis. This type of experiment revealed how the cells responded to PL over time. IH-1, OH-2 and KJON were cultured in HS or PL.

During the first long-term cultivation of IH-1 it was a distinct difference in proliferation of cells in the flasks with 10 % PL and 10 % HS. Cell counting every third and fourth day revealed that the number of IH-1 cells in PL increased two to three times in three and four days, in contrast to the cells in HS that had a five-fold increase during the same period. The experiment was terminated after ten days due to declining proliferation rate of IH-1 cells in 10 % PL. Early termination of the experiment makes it difficult to decide whether culturing the cells in PL would eventually kill the cells or simply just contribute to a slower proliferation rate. It was not possible to observe a distinct difference in the amount of dead cells in PL or HS in microscopy. IH-1 cells in PL had a small declining level in cell number at the end of the experiment and it is possible that the cells would eventually stop proliferating due to a lack of nutrients. IH-1 cells were also cultured in 20 % PL. A doubling of PL concentrate in culture did not seem to increase proliferation of the cells. Cells cultured in 10 % HS had a higher theoretical number of cells compared to those grown in 20 % PL. This might indicate that PL lacks certain substances needed for optimal growth rather than having too low concentrations of them. Though it is clear that IH-1 cells proliferate and thrive better in HS, it seems that the cells can utilize PL to some extent since they were able to survive and proliferate for ten days. It is therefore reasonable to assume that PL contains certain substances that can support growth of IH-1 cells to some extent.

Long-term cultivation of OH-2 cells was terminated after 10 days because the cells stopped proliferating in 10 % PL. The cells seemed to proliferate and thrive much better in HS, which is consistent with the results from the apoptosis and proliferation assays. OH-2 cells are known to be slow growing and demanding, and they have been impossible to wean from their strict dependence on HS. It is assumed that cell lines (e.g., OH-2) derived from HRD tumors are more dependent on the microenvironment and mitogenic cytokines than other MM cell lines. The importance of the microenvironment can explain why for instance OH-2 cells benefit from HS rather than FCS. Why OH-2 cells cannot utilize PL cannot be explained in this thesis, but it is reasonable to question whether PL is deficient in certain necessary growth and survival factors. The results, however, reinforce the evidence that OH-2 cells are difficult to wean from their dependence on HS (Våtsveen et al., 2009b).

KJON cells were cultured in 20 % PL and 5 % HS. Despite a four-doubling of the amount of medium added to the culture, 5 % HS seemed to outperform PL in terms of proliferation. Culturing the cells in PL did not seem to kill the cells within 14 days of cultivation, but proliferation had a small decline at the end of the experiment. The decline in proliferation can be due to various factors. Both KJON and IH-1 cells seemed to only have a slower proliferation rate in PL than in HS. The cells in PL were split as often as those grown in HS (twice a week) regardless of the increase in cell number. It is therefore difficult to predict whether the decrease in the proliferation of in PL indicates an eventual cell death or simply that the cells need more time to proliferate. As the amount of medium (i.e., PL) to be refilled also declined during each passage of cells, a consequence could be a possible lack of nutrients. A longer cultivation time of the cells cultured in PL between the passages could be an option, but then it would be difficult to compare the cell proliferation in PL to HS. Another alternative way to perform the long-term experiments could be to keep the cells in each respective culture flask and only add fresh medium to the flasks twice a weak without splitting them. This method would probably prove faster whether growing the cells in PL kills them or that PL has a lower stimulatory effect on proliferation. This would thus lead to large amounts of medium in the flask, which could affect the access to CO_2, lead to an increased contamination risk and otherwise be disadvantageous to handle.

hMCLs are in general considered difficult to establish. In addition, the cells have often extended doubling times and limited availability compared to other hematopoietic cell lines (Drexler & Matsuo, 2000). Many hMCLs are reported to be dependent on IL-6, and some cell

lines become IL-6 independent after cultivation for extended periods (Våtsveen et al., 2009b). This is not the case for OH-2, KJON and IH-1. IL-6 is externally added to the medium to promote growth and survival (Brenne et al., 2009; Våtsveen et al., 2012). An analysis of human PL from pooled platelet concentrates shown in Figure 6 reveals that PL contains low concentrations of IL-6 compared to other cytokines (e.g., PDGF, TGF-β and bFGF). The concentration of IL-6 in both HS and PL used in this thesis is unknown, and it is therefore no basis to discuss whether the concentration of IL-6 in PL is too low to support growth and survival of the cells. A possible difference in the IL-6 concentration in these two mediums would still most likely be negligible because of the externally added IL-6 (2 ng/ml) in the cell cultures. On the other hand, IGF-1 concentrations have been reported to be in average one-fifth in PL compared to HS, shown in Figure 7 (Rauch et al., 2011). Similar to IL-6, IGF-1 is considered an important growth-promoting cytokine for hMCLs. It stimulates cell proliferation and cell cycle progression by increasing survival of MM cells (Georgii-Hemming et al., 1996; Jelinek et al., 1997). It is interesting that both of these growth-promoting factors are found in low concentrations in PL compared to other cytokines, considering the fact that they are important for growth and survival of MM cells.

Another interesting observation is from an overview of IL-6 dependent hMCLs' response to various externally added cytokines (Drexler & Matsuo, 2000). As Figure 2 shows, IL-6 and IGF-1 were both growth stimulating, while TGF-β1 had only an inhibitory effect on growth. TGF-β is one of the major components in PL (Figure 6 and 7). Since it is reported that TGF-β has an inhibitory proliferative effect for a selection of IL-6 dependent cell lines, it is reasonable to question whether the low concentrations of IL-6 and IGF-1 and high concentration of TGF-β1 in PL can be among the factors that resulted in poor proliferation of the cells cultured in PL. This hypothesis can be interesting for further research.

4.6 Conclusion and further perspectives

Platelet lysate is a promising medium supplement in cell cultures with MSC. Many ethical questions have been raised regarding the use of human and animal serum in cultivation. HS is an important product that could be used in treatments of patients in terms of plasma, which is a scarce internationally. This raises questions concerning the use of HS in research. PL is thus a good alternative choice considering ethical questions.

For further research it might be interesting to investigate whether PL can substitute FCS/FBS in cultures with other MM cell lines, as most of the MM cell lines are grown in FBS. Such a replacement would be advantageous in terms of both ethical questions that are raised against animal serum and the infectious risk for research groups that consume large amounts of animal serum. It would also be interesting to analyze the content of HS and compare it to PL. This could probably give a closer perspective of what types of substances and growth factors that must be present to stimulate proliferation and growth of the respective hMCLs.

Two important factors had to be present if PL was to replace HS. First, PL should provide a good stimulatory effect on proliferation, and second, the preparation method should be as easy and effective as for HS. None of these factors were present. In summary, HS outclassed PL as a medium supplement. HS must provide a cocktail of growth and survival factors that IH-1, OH-2 and KJON need for optimal growth and survival. This also corresponds to previous research that shows that HS is preferred as a medium supplement in cultures with these cell lines (Våtsveen et al., 2009b). To answer the basis of this thesis it must be concluded that PL is not a good alternative medium supplement to HS, in terms of both practical reasons and stimulatory effect on proliferation of IH-1, KJON and OH-2. HS really is the gold standard as a medium supplement for the respective cell lines studied in this thesis.

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APPENDIX

Appendix 1

Trombocyttkonsentrat -fremstilling fra buffycoat

Versjon:	4.0	ID:	6991
Gyldig fra:	29.08.2012	Forfatter:	Margrete Skjelstad (Bioingeniør) Ingvild Telgum (Fagansvarlig Bioingeniør)
Revisjonsfrist:	25.07.2014	Godkjent av:	Ellen Berg (Kvalitetskoordinator)

Hensikt

Å produsere trombocyttkonsentrat fra buffycoat (restprodukt ved fremstilling av erytrocyttkonsentrat og plasma) som tilfredsstiller gjeldende kvalitetskrav.

Omfang

Prosedyren omfatter utvelgelse av buffycoater, registrering, pooling av buffycoater, pakking for sentrifugering, sentrifugering og framstilling av trombocyttkonsentrat.

Tidspunkt for utførelse av prosedyren / retningslinjen

Trombocyttkonsentrat fra buffycoat produseres normalt tirsdag til lørdag.

Grunnlagsinformasjon

Veileder for transfusjonstjenesten i Norge, siste utgave.
Blodforskriften, siste utgave.
Guide to the preparation, use and quality assurance of blood components, siste utgave.
Pakningsvedlegg for poolingsett og PASIIM (SSP+).

Trombocyttenes oppgave er å hindre, eventuelt stoppe blødninger.
Trombocyttene er klebrige og strømmer til og "tetter" igjen sårflater.
Trombocyttene har ABO-antigener, HLA-klasse-I-antigener og spesifikke HPA-antigener, men ikke Rh-antigener.

Trombocyttkonsentrat fra buffycoat blodtype O kan gis til alle pasienter uansett blodtype.
Trombocyttkonsentrat fra buffycoat blodtype A2 kan i praksis brukes som blodtype O.
Trombocyttkonsentrat fra buffycoat blodtype A kan gis til pasienter med blodtype A og AB, men kan også gis til pasienter med blodtype O ved akutt behov for trombocytter ved pågående blødning.

Buffycoat (BC) får vi ved fremstilling av blodkomponenter ved hjelp av OPTI-systemet.
Blodkomponenter, framstilling .
Buffycoat inneholder plasma, erytrocytter, trombocytter og leukocytter.
Trombocyttene har en egenvekt (tetthet) som gjør at de sentrifugeres ut i samme skikt som storparten av leukocyttene (dvs. i buffycoat) i fullblod.

Vi lager trombocyttkonsentrat ved å poole 8 buffycoater.

Etter framstilling skal alle trombocyttkonsentrat gjennomgå patogen inaktivering.

Arbeidsbeskrivelse

Ansvar

Bioingeniør
Lege, ved akutte situasjoner

Fremgangsmåte

Råvare

Buffycoat, se punkt "Utvelgelse av buffycoat til trombocyttkonsentrat".

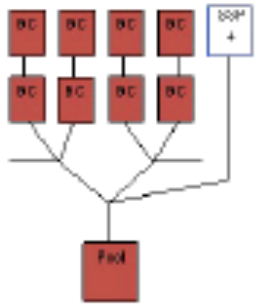
Utstyr

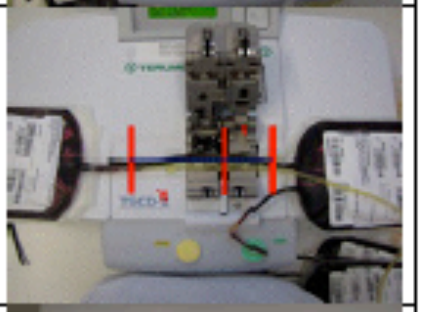
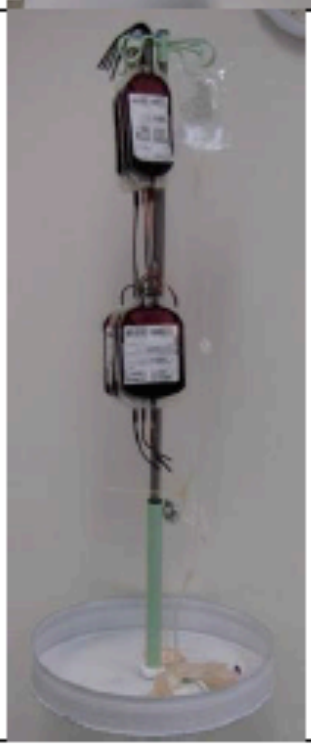
- SSP+ 280 ml suspensjonsmedium for trombocytter (PAS IIM)
- Teruflex BP-kit med IMUGARD® II-S PL filter (poolingsett)
- Sterilsveis
- Peang (blå slangeklemme)
- Håndsveis
- Blodbanksentrifuge
- Optipress II
- Trombocyttagitator i klimaskap

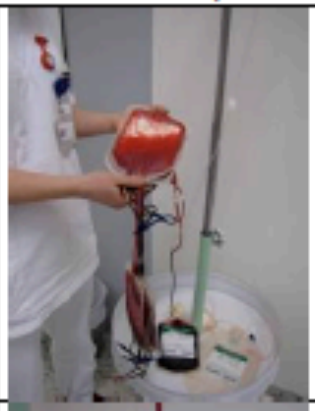
Utvelgelse av buffycoat til trombocyttkonsentrat:

- BC fra tappinger uten anmerkninger (skrevet på blodposen).
- BC som er lagret minst 2 timer i ro, og deretter minst 2 timer (vanligvis natten over) på agitator i klimaskap, ved 22°C±2°C.
- BC er holdbare til kl 24 dagen etter tapping.
- Kontroller at BC ikke inneholder koagler.
- Velg 8 enheter ABO-identiske BC pr. batch trombocyttkonsentrat.
  For å utnytte BC kan inntil 2 A1-negative buffycoater blandes med blodtype O (gir trombocyttkonsentrat av blodtype O)
  Det kan også lages rene A1-negative batcher.
  For Rh (D) negative trombocyttkonsentrat, benytt kun Rh (D) negative BC.
- Velg BC der givernes trombocytt-tall gir et gjennomsnitt i poolen på ca $250x10^9$/L (hvis mulig).
- BC som blir "til overs" ved morgenens produksjon legges tilbake på agtitaor og kan evt brukes ved ny produksjon senere samme dag.

Pooling av buffycoater:

Skjematisk tegning av sveising av 8BC, SSP+ og poolingsett med 6+1 armer	
Lukk alle fargede klemmer SSP+ sveises sammen med poolingsettet (til den lange slangen med filter på enden). Pass på at klemma ikke sveises av.	
Legg de 8 BC i to bunker med 4 i hver, og sveis sammen med poolingsettet til to "tog":	

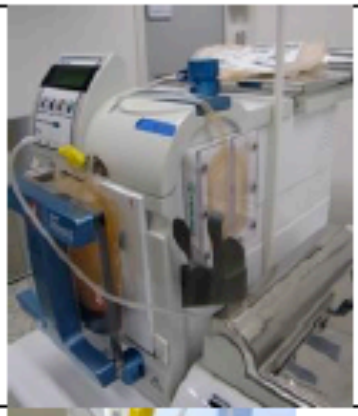
Plasmaslangen (10cm, til venstre) på 4 BC sveises mot poolingsettet (5cm). Se merking på TSCDII.	
Plasmaslangen (10cm, til venstre) i 4 nye BC sveises mot RBCslangen (5cm) i de 4 første BC	
Lag buffycoat-poolen i Prosang, Rutine P345; Pooling av produkter. Produktkode E7555 Scan først den buffycoaten som bestemmer blodtype og Rh(D) på poolen. Kell anmerkes for hånd.	
Heng opp "buffycoat-toget" og SSP+-posen. Åpne sveisene mellom buffycoatposene og pool-posen og la BC renne ned i pool-posen. Lukk klemma mellom buffycoatposene og pool-posen.	

Åpne sveisen til SSP+ og la all væske renne vha tyngdekraften ned i BC-posene. Lukk klemma til SSP+ Sett på klemme mellom øvre og nedre BC, og bland forsiktig frem og tilbake 3 ganger. Åpne klemma og overfør SSP+ til de øvre BC. Bland forsiktig frem og tilbake 3 ganger. Heng BC-toget opp igjen, slik at pool-posen henger, og slipp all væske ned i pool-posen.	
Press lufta fra pool-posen opp til de nederste BC slik at slangene er fylt, og slipp all væske ned Lukk klemma til pool-posen og sveis av med kort slange over pool-posen.	
Bland pool-posen forsiktig, men godt slik at innholdet blir homogent. La poolen hvile på benk ved 22°C±2°C i minst 30 min (maks en time). Bland pool-posen forsiktig, men godt slik at innholdet blir homogent før sentrifugering.	

Pakking og sentrifugering:

Sett pool-posen i en sentrifugekopp. Etiketten på poolposen skal vende inn mot midten av sentrifugekoppen. Pakk filteret og prøvetakingsposen inn i oppbevaringsposen og legg "pakken" i motsatt sentrifugekopp sammen med 500ml (for eksempel ACD-poser) motvekt. Vei to og to kopper for likevekt. Balansevekt for sentrifuge	
Koppene settes i sentrifugen med pool-posen i ytre ring. Sentrifuger umiddelbart i blodbanksentrifuge, program 10 Blodposesentrifuger, - bruk og vedlikehold	

Fremstilling av trombocyttkonsentrat:

Avpresses umiddelbart (innen 15 min) etter sentrifugering, i Optipress II, program 2-2C med flat bakplate. Optipresse II, bruk og vedlikehold . Filteret plasseres i Terumo-holderen med den ruglete sida ut og slangen fra poolposen i bunnen, Slangen fra filteret til produktposen føres gjennom Hb detektoren og øvre slangeklemme. Dersom avpressing ikke kan skje umiddelbart, må enheten blandes og sentrifugeres på nytt. (Dette stresser trombocyttene, og må unngås så langt det er mulig).	
Vurder skillet og fargen på trombocyttkonsentratet før avpressing starter. Fargen skal være GUL, og skillet klart og tydelig. Dersom det er dårlig skille og /eller rødt konsentrat kan enheten blandes og sentrifugeres på nytt. (Maks 2 ganger sentrifugering totalt). Poolen skal da blandes godt, men forsiktig opp, og hvile minst en time på benk, blandes opp og sentrifugeres umiddelbart.	
La trombocyttkonsentratene hvile på benken ved 22°C±2°C i 1 time, deretter på agitator i klimaskap i 10 min. Etter hviletid skal trombocyttkonsentratene gjennomgå patogen inaktivering (Intercept behandling) Trombocyttkonsentrat -behandling og oppbevaring	

I akutte situasjoner, i samråd med vakthavende lege:

- Dersom det er akutt behov for å lage trombocyttkonsentrat kan det lages fra BC som er lagret minst 2 timer ved 22°C±2°C. Holdbarhet på slike trombocyttkonsentrat reduseres til 1 døgn.
- Hviletid før interceptbehandling kan reduseres.

Relaterte dokumenter

Tapperegistrering og merking av blodposer og prøveglass.

Appendix 2

Trombocyttkonsentrat -behandling og oppbevaring

Versjon: 4.2 ID: 7842
Gyldig fra: 29.07.2013 Forfatter: Ingvild Telgum (Fagansvarlig Bioingeniør)
Revisjonsfrist: 03.07.2015 Godkjent av: Ellen Berg (Avdelingssjef)

Hensikt

Sikre at patogen inaktivering og oppbevaring av trombocyttkonsentrat utføres riktig.

Omfang

Patogen inaktivering av trombocyttkonsentrat ved hjelp av Intercept-behandling.
Oppbevaring av ferdig behandlede trombocyttkonsentrat.

Grunnlagsinformasjon

Intercept-prosessen består av Helinx teknologi der en benytter seg av Amotosalen HCl og UVA- lys for å inaktivere både virus, bakterier, leukocytter og parasitter i trombocyttkonsentratene. Interaksjonen mellom amotosalen og DNA eller RNA er høyt spesifikk og danner irreversible kryssbindinger med minimal proteininteraksjon.
Amotosalen har vist seg å inaktivere både virus som HIV, HBV, HCV, HTLV 1+2, CMV, Parvovirus- B19, gram negative og gram positive bakterier, parasitter (Plasmodium, Babesia) og nye patogener som SARS og West Nile Virus.

For å kunne gjennomføre Intercept-prosessen må følgende kriterier til trombocyttkonsentratene oppfylles før behandling kan gjennomføres.

	Dual Storage (DS)			Small Volume (SV)
Brukes til	Buffycoat			Aferese
Amotosalen	17,5mL			15mL
Trombocyttinnhold x10^9/enhet	250-700	710-800	Validert, AIT: 550-800	250-600
Volum	300-420mL	375-420mL	380-420mL	255-325mL
RBC	<4x10^{6}mL			
Plasma	32-47%			
Illuminasjon avsluttes	Innen kl. 24 dagen etter tapping			
CAD-tid	6-16t			4-16t

Kvalitetskrav og kvalitetskontroller til ferdigbehandlede trombocyttkonsentrat er omhandlet i prosedyren Blodkomponenter, kvalitetskontroll .

Pakningsvedlegg for Interceptsett DS og SV.
Blodforskriften, siste utgave.
Veileder for transfusjonstjenesten i Norge, IS-1184, utgitt av Sosial- og helsedirektoratet, siste utgave.
Guide to the preparation, use and quality assurance of blood components.

Arbeidsbeskrivelse

Ansvar
Bioingeniør, lege

Utstyr

Trombocyttkonsentrat fra poolede buffycoat tilsatt SSP+, eller aferese tilsatt Intersol
Intercept fremstillingssett for poolede buffycoat-trombocytter (Dual Storage DS)
Intercept fremstillingssett for aferese-trombocytter (Small Volume, SV)
Sterilsveis
Håndsveis
Peang (blå slangeklemme)
Intercept Illuminator
Manuell blodpresse
Trombocyttagitator i klimaskap

Fremgangsmåte

Forbehandling og registrering av trombocyttkonsentrat fra aferese:

- Aferesene produseres i Prosang:
  P700 Produksjon av komponenter, produktkode E4976 (1. enhet) og E4978 (2. enhet).

- Aferesene skal hvile på benk i 2 timer, deretter på agitator i klimaskap i 2 timer før behandling. Fyll ut skjema for tidsplan (vedlegg).

- Doble afereser deles i to like deler ved å henge dem opp og åpne klemmene.
  Bruk vekt for å kontrollere at enhetene er tilnærmet like store.

Patogen inaktivering av trombocyttkonsentrat fra buffycoat og aferese:

- Velg produktene som skal behandles.
  Kontroller at produktet har erytrocytter < 4.0×10^6. Dette gjøres visuelt med sammenligning av fargekart. Dersom produktet blir vurdert til å ha for høyt innhold av erytrocytter skal det kasseres.

- Kontroller om produktet har fnokker(afereser). Produkt med fnokker kan behandles dersom fnokkene har løst seg opp innen behandlingsfristen. De kan også behandles dersom det fortsatt er litt fnokker igjen, men da må det anmerkes på det ferdige konsentratet slik at det blir tatt kvalitetskontroller før frigiving.

- Kontroller bruttovekt på produktet (pr enhet).

Trc-konsentrat fra	Bruttovekt	Kommentar
Buffycoat	439-484g (375-420mL)	Dobbel enhet, Inkludert prøvetakingspose.
	Ved vekt >484g fjernes det overskytende til prøvetakingsposen Ved vekt 361-438g skal den ikke deles til to enheter. Sveis av den ene oppbevaringsposen før behandling.	
Amicus aferese	297-369g (255-325mL)	Etter deling
Trima aferese	303-375g (255-325mL)	Etter deling Sveis av prøvetakingssettet med kort slange før veiing

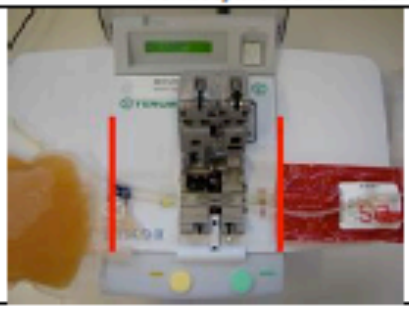
- Velg riktig Intercept-sett (for Buffycoat; DS, eller aferese; SV).

IDMS = Intercept Data Management System. (grønne punkter).

Sveising:

Sveis sammen trombocyttkonsentrat og interceptsett vha sterilsveis. Sterilsveis TSCD, bruk og vedlikehold .

Bruk kantene på TSCD som mal for slangene.



Endre produktkode i Prosang, rutine P346:

Buffycoat-trombocytter fra E7555 til E6642.
Del etiketten og merk begge oppbevaringsposene i interceptsettet (den nedre "flippen" settes på den ytterste posen slik at det blir lett å scanne under behandlingen.)

Aferesetrombocytter fra E4976 til E4977 og fra E4978 til E4979.
Merk oppbevaringsposen i interceptsettet med etiketten.

Dersom aferesetrombocyttene er forbeholdt pasient med trombocyttantistoff/HLA-antistoff (merket med Forlikelige trombocyttkonsentrat, etiketter , se prosedyre Forlikelige trombocyttkonsentrater, rutiner ved AIT).

Legg til tilleggstjeneste i rutine P655:
- Situasjon: Inför reservasjon
- Legg til: T-tillegg
- Kode: 130 (HLA-forlikelighet)

Registrer produktet, interceptsettet og sveisingen i IDMS (3 trinn):

Scan tappenummer og produktkode for hvert trinn.
Følg instruksjonen på skjermen.

Tilsett amotosalen:

Heng opp trombocyttkonsentratet, stativet heves til merket.

Åpne nedre brekkstift på den røde posen for å se om amotosalenet renner igjennom.

Åpne øvre brekkstift, sveisen og evt klemme til trombocyttkonsentratet slik at alt renner ned i belysningsposen.

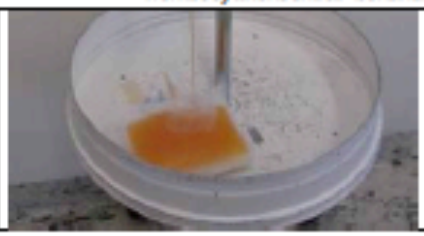
Mens trombocyttkonsentratet renner gjennom amotosalenet, fjern luft fra prøvetakingsposen med en klemme

Bland trombocyttkonsentratet og amotosalenet forsiktig men godt.

Klem lufta og noen ml trombocyttkonsentrat gjennom amotosalenposen og opp i den øverste posen (for å sikre at alt amotosalenet blir blandet med trombocyttkonsentratet).

La alt trombocyttkonsentrat renne ned igjen til belysningsposen (litt luft er ok).

Sveis av til en kort slange på belysningsposen, pass på at slangestussen er fylt med trombocyttkonsentrat.



Belysning: Interceptbehandling, bruk av utstyr

Legg trombocyttkonsentratet i Illuminatoren.
Følg instruksjonen på skjermen og start belysningen ved å trykke på start. Tar ca. 5 min.
Ta trombocyttkonsentratet ut av Illuminatoren.

Registrer belysningen i IDMS.

Scan tappenummer og produktkode, og godkjenn belysningen

Overfør trombocyttkonsentratet til CAD-posen:

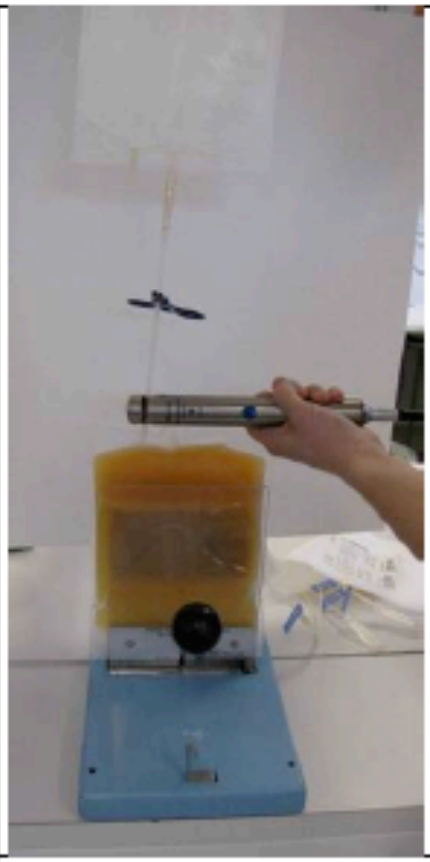
INNEN 30 MIN. ETTER BELYSNING

Fjern strikken og plastbeskyttelsen, og sjekk at CAD-puten er hel.

Heng opp trombocyttkonsentratet og overfør innholdet fra belysningsposen til CAD-posen

Fjern luft ved hjelp av manuell presse. CADen må ikke bøyes eller brettes. Pass på å få med så mye trombocyttkonsentrat som mulig, slipp opp litt på presset for å få med alt. Litt luft er ok.

Sveis av belysningsposen med kort slange.



Registrer start for CAD behandling i IDMS:

Scan tappenummer og produktkode.

Velg skuffe på agitatoren i klimaskapet der produktet legges og scan utstyrs-ID (skuff).

Sjekk agitasjonsliste i IDMS:
(Ikon som viser to piler som går frem og tilbake ⇤⇥).

Sjekk at alle behandlede trombocyttkonsentrat er registrert.

CAD-behandling:

Trombocyttkonsentrat behandlet med DS-sett (fra buffycoat) skal ligge til CAD-behandling på agitator i klimaskapet i 6-16 timer.

Trombocyttkonsentrat behandlet med SV-sett (fra aferese) skal ligge til CAD-behandling på agitator i klimaskapet 4-16 timer

Avslutning i IDMS:

Se på agitasjonslisten på skjermen. Der vises alle produktene som ligger til CAD- behandling. Finn posene som er ferdig CAD- behandlet:

Grå skrift: Ikke ferdig.
Svart skrift: Ferdig
Rød skrift: Produktene har ligget mer enn 16 timer. Produktet kan ikke brukes.

Registrer overføring til lagringspose i IDMS.

Scan tappenummer og produktkode (i Intercept startbildet). Klikk pil oppe til venstre i bildet på agitasjonslista(←) eller ikon som det står Intercept på.)

Bekreft at du har overført produktet fra CAD-posen til lagringsposen ved å hake ut.

Avslutning DS:

Overfør trombocyttkonsentratet til oppbevaringsposene:
Heng opp trombocyttkonsentratet.

Åpne brekkstiften og la produktet renne ned i oppbevaringsposene

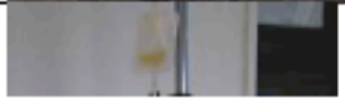
Steng klemma under filteret (bruk evt peang).

Fjern luft:
Overfør all luft fra den posen som har klemme på slangen til den andre posen.
Hele slangen opp til y-koblingen skal være fylt med konsentrat.

Steng poseslangen med klemma.

Åpne klemma under filteret og press all luft fra den andre oppbevaringsposen opp i CAD-posen.

La alt konsentrat renne ned, hold utløpet på filteret slik at det tømmes helt.

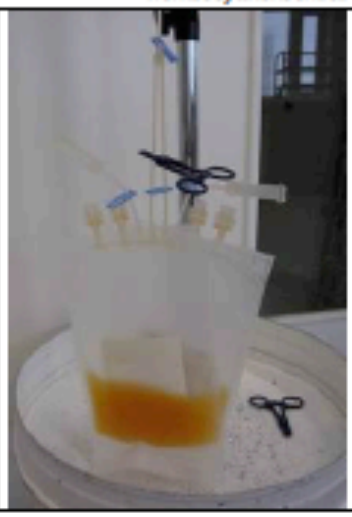


Press forsiktig den siste lufta opp i filteret.

Hele y-koblingen skal være fylt med konsentrat.

Steng klemma under filteret og sveis av rett over y-koblingen.

Det skal være så lite luft og så mye trombocyttkonsentrat som mulig i oppbevaringsposene.



Skriv ut behandlingsrapport i IDMS:

Trykk på ikonet "Rapporter", trykk på firkant nede til høyre. (Ser ut som et foldet papir).

Scan inn tappenummer og produktkode, hak ut rute til høyre. Bekreft med grønn pil. Trykk på skriver-ikonet nede til venstre, og så "skriv ut". Dette må gjøres for hvert produkt. En får ut en rapport per produkt.
For å komme tilbake til bildet der man scanner tappenummer, trykk på pilen øverst til venstre.

Rapportene legges i arkiv-eske.

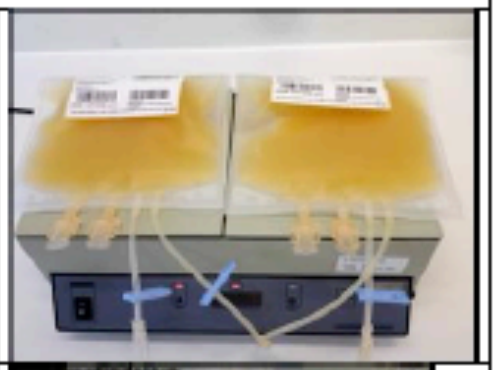
Lag to splitter i Prosang, rutine P344, og merk oppbevaringsposene.

Utjevning av volum:

Legg de to enhetene på hver sin side på balansevekta Balansevekt for sentrifuge , og fordel slik at det blir likevekt.

Bruttovekt skal være ca 240g på hvert konsentrat.

Sveis av y-koblingen med lang slange til konsentratene.

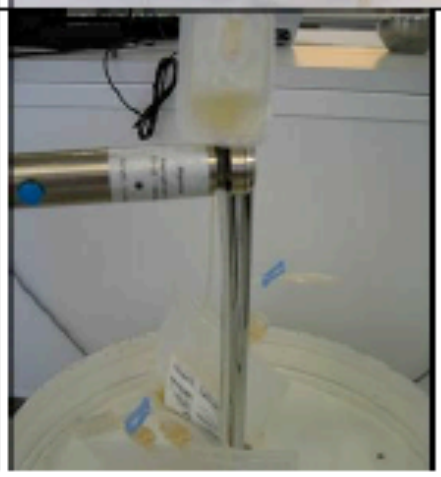


Avslutning SV:

Overfør trombocyttkonsentratet til oppbevaringsposen:
Heng opp trombocyttkonsentratet.
Åpne brekkstiften og la produktet renne ned i oppbevaringsposen.

Fjern luft fra oppbevaringsposen SV:
Press lufta forsiktig opp i filteret og videre opp i CAD-posen.
La alt trombocyttkonsentratet renne tilbake og fjern siste rest av lufta opp i filteret..

Sveis av CAD- posen med lang slange (rett under



filteret). Det skal være så lite luft og så mye trombocyttkonsentrat som mulig i oppbevaringsposene	
Skriv ut behandlingsrapport i IDMS: Se punkt under avslutning DS	

Manuell stopp og overstyring, IDMS:

Ved behov (for eks dersom et trombocyttkonsentrat må kasseres pga virussvar eller syk blodgiver) kan prosessen stoppes ved å klikke på ikon for manuell stopp (oppe til venstre). Det kan også være behov for å overstyre, (f eks ved registreringsfeil eller ved avvik som godkjennes av vakthavende lege). Klikk på ikon for overstyring (oppe til venstre).
Følg instruksjonen på skjermen, og legg inn passende kommentar. Skriv ut rapport og før på eventuell tilleggskommentar. Skriv evt avvik /fravikstillatelse.

Oppbevaring og holdbarhet av trombocyttkonsentrat

Intercept-behandlede trombocyttkonsentrat er holdbare i 168 timer (7 dager) fra tappedato kl 16:00.

Trombocyttkonsentrat oppbevares på trombocyttagitator ved + 22°C ± 2°C.

Trombocyttkonsentrat i karantene oppbevares på trombocyttagitator i Klimaskap ved Enhet for Komponentproduksjon
Frigitte trombocyttkonsentrat oppbevares på trombocyttagitator i Klimaskap i slusa ved Enhet for Transfusjonsmedisin.

Blodkomponenter, frigjøring, AIT
Helmer klimaskap og agitator

Ved datastans:

IDMS (Intercept Data Management System):

Interceptbehandling kan utføres uten bruk av IDMS:

- Utfør alle trinn som vanlig, men uten dataregistrering.
- Scan som normalt i lyskassene.
- Skriv ut belysningsrapport fra lyskassene. (Velg "skriv ut registreringer" og følg instruksjonen på skjermen).
- Fyll ut skjema for manuell registrering av CAD-behandling (vedlegg).

Prosang:

Dersom Prosang-etiketter mangler, kan tappenummer og produktkoder legges inn manuelt i lyskassene:

- Merk settene manuelt med tappenr, produktkode og blodtype, og sørg for korrekt Prosang-merking så snart det er mulig.
- I lyskassene legges tappenummer og produktkoder inn vha tastaturet (trykk flere ganger for å få fram bokstaver).
  Eks tappenr: J00401100123441 (41 er suffix, og må være med).
  Eks produktkode: E6642000

Dokumentasjon ved avviksmelding med fravikstillatelse.

Ved akutt behov for trombocyttkonsentrat:

Ved lavt lager og akutt behov for trombocyttkonsentrat der vakthavende lege vurderer at det ikke er tid til å

utføre Interceptbehandling, kan vakthavende lege vurdere å gi fravikstillatelse for å bruke trombocyttkonsentrat som ikke er Interceptbehandlet.
Trombocyttkonsentratet må da bestråles.

Produktkoder:

- Trombocyttkonsentrat fra buffycoat; Lag 2 splitter i P344 og del produktet i to. Legg til tilleggstjeneste i rutine P655:
  - Situasjon: Infør reservasjon
  - Legg til: D-komponent-tillegg
  - Kode: EC0 (bestrålt)

- Trombocyttkonsentrat fra aferese; E5715 og E5716 (PAS III (Intersol), filtrert og bestrålt)

Holdbarhet:
120 timer (5dager) fra tappedato kl 16:00 på trombocyttagitator ved + 22°C ±2°C

Dokumentasjon ved avviksmelding med fravikstillatelse.

Se fanen relatert for vedlegg.

Relaterte dokumenter
Trombocytkonsentrat -fremstilling fra buffycoat
Trombocytkonsentrat, -utvelgelse og utlevering
Trombocytkonsentrat, vasket
Vedlegg:
Interceptbehandling_aferese_v4.2.xls: Interceptbehandling av aferese, tidsplan
Intercept_manuell_CAD_v4.2.xls: Manuell registrering, CAD

Appendix 3

Blodkomponenter, kvalitetskontroll

Versjon:	3.3	ID:	12307
Gyldig fra:	10.04.2013	Forfatter:	Ingvild Telgum (Fagansvarlig Bioingeniør)
Revisjonsfrist:	19.03.2015	Godkjent av:	Ellen Berg (Avdelingssjef)

Hensikt

Å sikre gjennomføring av kvalitetskontroller på blodkomponenter og iverksettelse av tiltak hvis resultatene ikke tilfredsstiller gjeldende spesifikasjonskrav.

Omfang

Prosedyren beskriver de kvalitetskontroller som skal gjennomføres, fremgangsmåte og beskrivelse av tiltak ved avvik fra spesifikasjonskravene.

Grunnlagsinformasjon

Spesifikasjonskravene fastsettes av:

- Veileder for transfusjonstjenesten i Norge, Helse- og sosialdirektoratet, siste utgave.
- Blodforskriften, siste utgave.
- Guide to the preparation, use and quality assurance of blood components, siste utgave.
- Minimumskrav for humant plasma til fraksjonering, Baxter, siste utgave.

Se forøvrig Blodkomponenter, kvalitetskontroll: Bakgrunnsinformasjon

Erytrocyttkonsentrat og plasma fremstilles fra fullblod vha. Fenwals OPTI-system eller ved aferese.
Trombocyttkonsentrat fremstilles fra buffycoat fra 8 givere, eller ved aferese.

For å sikre optimal kvalitet på blodkomponentene må kravene til produksjonsbetingelser overholdes. Følgende parametre skal kontrolleres:

Romtemperatur:
Spesifikasjonskrav: 22±2°C
Kontinuerlig overvåking ved pakkebenken og Optipressene vEMS Temperaturloggesystem, EMS. AMB og AIT. .

Innfrysningskurve for plasma: Frysekurve for plasma

Hygienekontroll: Hygienekontroll, Clean Trace

Kvaliteten på blodkomponentene fra hver fremstillingsprosess overvåkes vha statistisk prosesskontroll, se prosedyrene *Blodkomponenter, statistisk prosesskontroll* ***og*** *NWA Quality Analyst statistikkprogram* ***. Følgende komponenter og parametere skal kontrolleres:***

Buffycoat, vekt:
Spesifikasjonskrav: 40,5mL ± 2mL (bruttovekt 76-81g)

Plasma, spesifikasjonskrav

Parameter	Plasma fra fullblod
Erytrocytter	≤ 6000 x 10^6 / liter
Leukocytter	≤ 100 x 10^6 / liter
Trombocytter	≤ 50 x 10^9 / liter
Faktor VIII	> 0,7 IU/mL (gjelder også aferese)
Totalprotein	> 50 g/L (gjelder også aferese)

Bakterie-undersøkelse	Negativ (gjelder også aferese)

Erytrocyttkonsentrat, spesifikasjonskrav

Parameter	Erytrocyttkonsentrat fra fullblod	Erytrocyttkonsentrat fra aferese
Volum	200 – 320 mL	200 – 320 mL
Leukocytter	< 1,0 x 10^6 /enhet	< 1,0 x 10^6 /enhet
Hemoblobin	> 40 g/enhet	> 40 g/enhet
EVF	0,50 – 0,70 %	0,50 – 0,70 %
Hemolyse i utdaterte	< 0,8 %	< 0,8 %
Bakterie-us i utdaterte	Negativ	Negativ
Totalprotein i vaskede	<0,5g/enhet	<0,5g/enhet

Trombocyttkonsentrat, interceptbehandlede, spesifikasjonskrav

Parameter	Trombocyttkonsentrat fra 8 BC	Trombocyttkonsentrat fra aferese
Volum	180-200 mL	>215 mL
Trombocytter	>240 x 10^9 /enhet	>240 x 10^9 /enhet
Leukocytter	< 1,0 x 10^6 /enhet	< 1,0 x 10^6 /enhet
pH	>6,4	>6,4
Bakterie us	Negativ	Negativ

Arbeidsbeskrivelse

Ansvar

Bioingeniører med opplæring i kvalitetsarbeid.
Lege.

Fremgangsmåte

Registrering av kontroller og resultater gjøres i respektive kvalitetskontrollskjema i Excel:
\\Blodbank\Felles\Seksjon Blodbank\Enhet for komponentproduksjon\Kvalitetskontroller

Prøvemateriale

- Buffycoat
- Erytrocyttkonsentrat
- Trombocyttkonsentrat
- Plasma

Utvelgelse av enheter som skal kontrolleres

Frekvens av prøvetaking dersom det ikke er nevnt under, rekvirering, type glass og hvem som utfører analysene går fram av dokumentene:
Blodkomponenter, kvalitetskontroll: Ukentlige kontroller
Blodkomponenter, kvalitetskontroll: kortversjon rekvirering og fremgangsmåte
Blodkomponenter, kvalitetskontroll: Periodiske kontroller

- Buffycoat vektkontroll: En buffycoat fra hver Optipresse hver dag.
- Celletall i plasma, erytrocyttkonsentrat og trombocyttkonsentrat: Den bioingeniør som har arbeidsoppgaven "Plasma/spesialprodukt/QC" (nr.3) har ansvar for å velge ut tilfeldige enheter som skal kontrolleres. Enhetene (plasma og erytrocyttkonsentrat) merkes med farget lapp slik at de sveises av med lang slange. Denne bioingeniøren skal ikke selv produsere disse enhetene (gjelder plasma).
- Ved skifte av lotnummer på blodposene skal 12 poser av det nye lotnummeret kontrolleres på celletall i plasma og erytrocyttkonsentrat. Disse blodposene er merket "QC nytt lotnr". Blodposer - mottak/lagring . Dersom kontrollene er innenfor kravene, godkjennes lotnummeret for bruk ved å gi beskjed til Enhet for blodgivning. Dersom en eller flere kontroller er utenfor kravene vurderes tiltak i

samråd med relevant personale/leverandør.

- Til kontroll av hemolyse og bakteriologisk kontroll av utdaterte erytrocyttkonsentrat velges ut 2 enheter som testes på utløpsdato hver 5. uke. Det må sikres at det taes kontroll av enheter produsert både fra fullblod og aferese.
- Til kontroll av pH og bakteriologisk kontroll av utdaterte trombocyttkonsentrat, velges enheter når det finnes tilgjengelige utdaterte enheter. Det må sikres at det taes kontroll av enheter produsert både fra fullblod og aferese.

Utstyr

Fargede etiketter
Sveiseapparat
Rulletang, arteriepinsett og saks
Glass uten tilsetning (hvit topp), glass med EDTA (lilla topp), gelglass (rød/gul topp) og polypropylenglass
Utstyr for prøvetaking
Vekt
Sentrifuge
pH-meter med tilhørende utstyr

Buffycoat vektkontroll

- Buffycoatene merkes med presse-nummer.
- Slangene sveises av til standard lengde: Plasmaslange 20cm, rbc-slange 15cm, tappeslange 5cm.
- Vei og registrer bruttovekta.

Volum og celleinnhold i erytrocyttkonsentrat, trombocyttkonsentrat og plasma:
Hb og EVF i erytrocyttkonsentrat

Prøvetaking:

- Erytrocyttkonsentrat:
  Sveises av med ca 40cm lang slange.
  Slangen strippes med rulletang og innholdet i posen blandes.
  Gjenta 3 ganger.
  Sveis av slangen og overfør innholdet til 2 merkede glass uten tilsetning (hvit topp)
  Veies, bruttovekt føres på glasset.
  Erytrocyttkonsentratet merkes med etikett stemplet "Leucocyttinnhold<1,0*10^6/enhet"
- Plasma:
  Sveises av med ca 40cm lang slange.
  Slangen strippes med rulletang og innholdet i posen blandes.
  Gjenta 3 ganger.
  Sveis av slangen og overfør innholdet til et merket EDTA-glass (lilla topp).
  Veies, bruttovekt føres på glasset.
- Trombocyttkonsentrat:
  Veies, bruttovekt føres på 2 merkede glass, et uten tilsetning (hvit topp) til leukocytt-telling og et med EDTA (lilla topp) til trombocytt-telling.
  Blandes forsiktig og ca 3ml konsentrat overføres til prøvetakingsposen (la det renne forsiktig ned i prøvetakingsposen, press evt ut luft fra prøvetakingsposen en gang for å få den full). Sveis av prøvetakingsposen og overfør innholdet til de merkede glassene.

Erytrocyttkonsentrat og plasma oppbevares i karantene i egne merkede kurver inntil resultatene foreligger.

Registrering:

- Prøvene registreres som kontroller i NSL, og ønskede analyser rekvireres.

- Prøvene merkes med NSL-etikett, og glasset som skal til leucocount-us påføres nettovekt (beregnes i de respektive kvalitetskontrollskjema)
- Dagens dato, tappenummer, NSL-nummer og evt. andre opplysninger registreres i de respektive kvalitetskontrollskjema.

Analysering:
- Prøvene leveres til analyse så snart som mulig (samme dag). Se prosedyrene Leukocyttkontroll av blodprodukt-Leucocount og Kvalitetskontroll av blodbankprodukter, ADVIA 120, Sysmex XE-2100, AMB/AIT for holdbarhet og oppbevaring.
- Leukocytter i trombocyttkonsentrat og erytrocyttkonsentrat analyseres ved Enhet for cytometri, øvrige parametre analyseres ved Enhet for hematologi.

Faktor VIII-måling og totalproteinmåling i plasma (i pool av 10 enheter fra fullblod og aferese)

Prøvetaking:
- Sveises av med ca 40cm lang slange.
  Slangen strippes med rulletang og innholdet i posen blandes.
  Gjenta 3 ganger.
  Sveis av slangen og overfør plasma til et merket polypropylenglass.
  Dersom en ikke har 10 enheter til å lage en pool, må prøven fryses ned ved -40ºC inntil en har nok prøver.
- Når en har samlet 10 prøver, tines disse ved 37 ºC.
  Lag en pool av 500µl fra hver prøve.
  Bland godt.
  Del prøven i 3, en del til totalprotein-måling og 2 til Faktor VIII-måling.

Registrering:
- Prøvene registreres som kontroller i NSL, og ønskede analyser rekvireres.
- Prøvene merkes med NSL-etikett,
- Dagens dato, tappenummer og NSL-nummer registreres i de respektive kvalitetskontrollskjema

Analysering:
- Prøvene leveres til AMB, Felles prøvemottak.så snart som mulig,
- NB! Prøven til Faktor-VIII-måling er holdbar 1 time, evt. kan prøven fryses ved -80ºC inntil den kan leveres.

Bakterie-undersøkelse i plasma

- Se prosedyre Bakteriologisk kontroll av blodkomponenter, - prøvetaking, AIT

Hemolyse og bakterie-undersøkelse i utdatert erytrocyttkonsentrat

Prøvetaking:
- Vei erytrocyttkonsentratet og registrer bruttovekt.
- Bland erytrocyttkonsentratet forsiktig.
- Ta prøve til bakterie-undersøkelse. Bakteriologisk kontroll av blodkomponenter, - prøvetaking, AIT
- Ta prøve til hemolysemåling fra et poseuttak med blodprøvetakingsutstyr:
  - et 3 ml glass med hvit topp.
  - et 5 ml glass med rød/gul topp som sentrifugeres ved 3500RPM i 6 min (til Hb i supernatant). Supernatanten overføres til et glass med hvit topp, den skal være fri for røde blodlegemer.

Registrering:
- Registrer erytrocyttkonsentratet i Prosang P303 "til labbruk", "kvalitetskontroll".
- Prøvene registreres som kontroller i NSL, og P-Hb og B-Hb rekvireres.
- Prøvene merkes med NSL-etikett.

- Alle opplysninger registreres i kvalitetskontrollskjema for erytrocyttkonsentrat.

Analysering:
- Prøvene leveres til Enhet for hematologi.

Beregning:
- Alle resultater registreres i kvalitetskontrollskjemaet, og % hemolyse beregnes automatisk.
- For manuell beregning, se formler i *vedlegg*.

Hemolyse og Totalprotein i vasket erytrocyttkonsentrat

Prøvetaking:
- Vei enheten og registrer bruttovekta.
- Sveis på en transferpose (150mL) og bland frem og tilbake 3 ganger. La det være igjen ca 10mL blod i transferposen som fordeles på
  - et 3 ml glass med hvit topp for Hb-måling
  - et 5 ml glass med rød/gul topp som sentrifugeres ved 3500RPM i 6 min.
    Supernatanten fordeles på to 3 ml glass med hvit topp, et til Hb-måling og et til totalprotein.

Registrering:
- Rekvirer totalprotein på rekvisisjon til AMB (Rekvirentkode BB-RIT, spesifiser at det gjelder SAG-væske (annet materiale)).
- Prøvene til Hb registreres som kontroller i NSL, og P-Hb og B-Hb rekvireres og glassene merkes med NSL-etikett. Se Blodkomponenter, kvalitetskontroll: kortversjon rekvirering og fremgangsmåte
- Alle opplysninger registreres i kvalitetskontrollskjemaet for erytrocyttkonsentrat.

Analysering
- Totalprotein: Leveres AMB, Felles prøvemottak sammen med rekvisisjon.
- Hemolyse: Leveres enhet for hematologi.

pH og bakterie-undersøkelse i utdatert trombocyttkonsentrat

- Utføres dagen etter utdatering.
- Vei konsentratet og registrer bruttovekta.
- Ta evt. prøve til bakterie-undersøkelse Bakteriologisk kontroll av blodkomponenter, - prøvetaking, AIT .
- Ta ut ca. 3 ml prøvemateriale på EDTA-glass for trombocytt-telling.
- Ta ut prøvemateriale som overføres til målebeger for pH-måling.
- Mål pH i konsentratet. Jenway pH-meter, bruk og vedlikehold

Resultatbehandling

- Resultater fra Enhet for hematologi, Enhet for cytometri og AMB hentes opp i NSL.
- Resultater fra AMM foreligger på svarrapport.
- Alle resultater registreres i de respektive kvalitetskontrollskjema i Excel:
  I\Blodbank\Felles\Seksjon Blodbank\Enhet for komponentproduksjon\Kvalitetskontroller\
- Resultater som er utenfor spesifikasjonskravene blir rødmerket i regnearket.
- Dersom resultatene for celleinnhold er innenfor kravene:
  - Plasma: Enhetene flyttes til kurv for pakking til plasmaforsendelse i karantenefryser.
  - Erytrocyttkonsentrat: Etikett for leukocyttinnhold signeres, og enheten frigis for bruk.
  - Trombocyttkonsentrat: Vil oftest være frigitt for bruk før resultatene foreligger.

Tiltak når resultatene ikke tilfredsstiller spesifikasjonskravene

Se prosedyren Blodkomponenter, statistisk prosesskontroll for tillatt antall avvik, vurdering av

kvalitetskontrollene og eventuelle tiltak i forhold til prosessene.

Generelle retningslinjer er angitt nedenfor. Ved tvil kan vakthavende lege vurdere om den aktuelle komponent kan brukes til pasient, evt. skal kasseres eller kan utleveres til teknisk bruk.
Blodkomponenter til teknisk bruk, oppbevaring og utlevering
Frigitte komponenter må tilbaketrekkes dersom de ikke kan brukes til pasient.
Se prosedyre Blodkomponenter og plasmaprodukter, tilbaketrekking og kassasjon .

Romtemperatur:
Juster romtemperaturen.

Buffycoat, vektkontroll
- Avklar om avviket skyldes tilfeldige eller systematiske feil ved å veie 3 buffycoater til for den aktuelle Optipressa.
  - Tilfeldige feil kan skyldes feil lengde på slangene.
  - Systematiske feil kan skyldes feil ved innstillingen til den aktuelle Optipressa.
- Dersom det er en systematisk feil, beregnes og justeres parametrene for den aktuelle Optipressa (utføres av bioingeniør med spesiell opplæring).
- Den/de aktuelle buffycoat benyttes ikke i produksjon av trombocyttkonsentrat.

Leukocytter i erytrocyttkonsentrat og trombocyttkonsentrat:
- Komponentene kasseres.

Vekt, celleinnhold og hematologiske parametere:
- Erytrocyttkonsentrat og plasma benyttes til teknisk bruk eller kasseres.
- Trombocyttkonsentrat kan benyttes.

Hemolyse under produksjon (observert).
- Alle produkt fra samme tapping utleveres til teknisk bruk eller kasseres.
- Enhet for blodgiving kontaktes ang. tappingen.
- Dersom hemolyse inntreffer flere ganger under produksjon, og annen årsak til hemolysen ikke kan fastslås, kontaktes leverandør av blodposer umiddelbart om mulig feil i konsentrasjon av SAGMAN-væske. (Leverandøren vil eventuelt ha tilsendt det hemolyserte produktet for videre undersøkelser.)

Hemolyse i utdaterte erytrocytter.
- Kontroller temperaturen i kjølerommet.
- Det taes umiddelbart 4-6 nye kontroller for å se om avviket er tilfeldig.
  - Bruk de eldste enhetene vi har, bland forsiktig og sveis på en transfer-pose for prøvetaking.
- Dersom det er forhøyet hemolyse i flere enheter, vurderes videre tiltak i samråd med kvalitetskoordinator/vakthavende lege.

Bakterieundersøkelse
- Vakthavende lege varsles umiddelbart.
- Dersom det er oppvekst i erytrocyttkonsentrat eller plasma, skal også andre blodkomponenter med samme tappenummer kasseres.
- Dersom plasma er sendt fraksjoneringspartner må denne varsles.
- Dersom det er produsert trombocyttkonsentrat av samme tappenummer., og dette er transfundert til pasient, må vakthavende lege ved AIT varsle behandlende lege.
  Det bør vurderes om det skal tas blodprøve av pasienten til dyrkning.
- Rutinene for fremstilling av erytrocyttkonsentrat gjennomgåes.
- Rutinene for prøvetaking til bakteriekontroll gjennomgåes.
- Enhet for blodgivning kontaktes for kontroll av rutinene i forbindelse med tapping.
- Vakthavende lege kontakter evt. blodgiveren som ga den aktuelle enheten for å utelukke sykdom, evt. ta prøver til dyrkning.

Vedlegg:
QC_manuell_hemolyseberegning_v0.3.doc: Manuell beregning av hemolyse

NTNU HMS/KS	Risikovurdering	utarbeidet av HMS-avd.	Nummer HMSRV2603	Dato 04.02.2011
		godkjent av Rektor	side 1 av 2	Erstatter 9.2.2010

Enhet: Institutt for biologi
Dato: 09.09.2013
Linjeleder: Lisbeth Aune
Deltakere ved risikovurderingen (m/ funksjon): Magne Børset, Ingrid E. Bruvoll

ID nr	Aktivitet fra kartleggings-skjemaet	Mulig uønsket hendelse/ belastning	Vurdering av sannsyn-lighet	Vurdering av konsekvens:				Risiko-verdi	Kommentarer/status Forslag til tiltak
			(1-5)	Menneske (A-E)	Ytre miljø (A-E)	Øk/ materiell (A-E)	Om-dømme (A-E)		
	Tilberedning av næringsmiddelløsning								
	Dyrking av myelomaceller	Virusinfeksjon i det biologiske materialet	1	A	A	A	A	1A	Bruk av verneutstyr og sikkerhetsbenk for å redusere risiko

Sannsynlighet
1. Svært liten
2. Liten
3. Middels
4. Stor
5. Svært stor

Konsekvens
A. Svært liten
B. Liten
C. Moderat
D. Alvorlig
E. Svært alvorlig

Risikoverdi (beregnes hver for seg):
Menneske = Sannsynlighet x Konsekvens Menneske
Ytre miljø = Sannsynlighet x Konsekvens Ytre miljø
Økonomi/materiell = Sannsynlighet x Konsekvens Øk/materiell
Omdømme = Sannsynlighet x Konsekvens Omdømme

Sannsynlighet vurderes etter følgende kriterier:

Svært liten 1	Liten 2	Middels 3	Stor 4	Svært stor 5
1 gang pr 50 år eller sjeldnere	1 gang pr 10 år eller sjeldnere	1 gang pr år eller sjeldnere	1 gang pr måned eller sjeldnere	Skjer ukentlig

Konsekvens vurderes etter følgende kriterier:

Gradering	Menneske	Ytre miljø Vann, jord og luft	Øk/materiell	Omdømme
E Svært Alvorlig	Død	Svært langvarig og ikke reversibel skade	Drifts- eller aktivitetsstans >1 år.	Troverdighet og respekt betydelig og varig svekket
D Alvorlig	Alvorlig personskade. Mulig uførhet.	Langvarig skade. Lang restitusjonstid	Driftsstans > ½ år Aktivitetsstans i opp til 1 år	Troverdighet og respekt betydelig svekket
C Moderat	Alvorlig personskade.	Mindre skade og lang restitusjonstid	Drifts- eller aktivitetsstans < 1 mnd	Troverdighet og respekt svekket
B Liten	Skade som krever medisinsk behandling	Mindre skade og kort restitusjonstid	Drifts- eller aktivitetsstans < 1uke	Negativ påvirkning på troverdighet og respekt
A Svært liten	Skade som krever førstehjelp	Ubetydelig skade og kort restitusjonstid	Drifts- eller aktivitetsstans < 1dag	Liten påvirkning på troverdighet og respekt

Risikoverdi = Sannsynlighet x Konsekvens
Beregn risikoverdi for Menneske. Enheten vurderer selv om de i tillegg vil beregne risikoverdi for Ytre miljø, Økonomi/materiell og Omdømme. I så fall beregnes disse hver for seg.

Til kolonnen "Kommentarer/status, forslag til forebyggende og korrigerende tiltak":
Tiltak kan påvirke både sannsynlighet og konsekvens. Prioriter tiltak som kan forhindre at hendelsen inntreffer, dvs. sannsynlighetsreduserende tiltak foran skjerpet beredskap, dvs. konsekvensreduserende tiltak.