T.C.
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ASSESSMENT OF THE ROLE AND ANTIOXIDATIVE AND ANTIAPOPTOTIC EFFECTS OF DELTA-OPIOID PEPTIDE D-ALA2-LEU5-ENKEPHALIN ON THE VIABILITY OF HEMATOPOIETIC STEM CELLS

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MASTER THESIS

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Aynura Mammadova

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ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with supervisor Assist. Prof. Dr. Fatima S.F. AERTS KAYA and written according to the rules of thesis writing of Hacettepe University Graduate School of Health Sciences.

29 / 07 / 2019

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ÖZET

Mammadova A., Delta-opioid Peptide D-Ala2-Leu5-Enkephalin'in Hematopoetik Kök Hücrelerin Sağ Kalımındaki Rolü ve Antioksidatif ve Antiapoptotik Etkilerinin Araştırılması, Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Kök Hücre Programı Yüksek Lisans Tezi, Ankara, 2019. Hematopoetik kök hücreler (HKH), kendini yenileyebilme ve bütün kan hücrelerine farklılaşabilme özelliklerine sahip erişkin kök hücrelerdir. HKH'ler; nakil ve/veya gen tedavi amacıyla kullanıldığında kısa dönemde hücre kültürüne ve/veya dondurma koşullarına maruz bırakılmaktadır. Ancak, bu süreçte oluşabilen oksidatif ve endoplasmik retikulum (ER) stresi HKH'lerin canlılığı ve fonksiyonunu negatif etkileyebilmektedir. HKH'lerin yüksek verimli kullanımı için toksik olmayan, hücre canlılığını koruyan, özgün ve kısa süreli hücre ekspansiyon protokollerine ihtiyaç vardır. Bu nedenle, Delta Opioid Reseptör (DOR) aktivasyonu sağlayan sentetik opioid peptidi D-Ala2-Leu5-Enkephalin (DADLE)'in *in vitro* çoğaltma, oksidatif stres, ER stresi ve hipotermik koşullara maruz bırakılan HKH'lerin üzerindeki etkilerinin araştırılması amaçlanmıştır. Göbek Kordon Kanı CD34+ HKH'ler; H_2O_2 ile indüklenmiş oksidatif strese veya Tünikamisin (TM) ve Tapsigargin (TG) ile endoplasmik retikulum stresini (ER stres) indükleyen koşullara maruz bırakılmıştır. Hücre proliferasyonu WST-1 ile; hücre döngüsü BrdU ile; apoptoz Annexin-V/Propidium İyodür ile, ve Reaktif Oksijen Türlerin oluşumu H_2DCFDA ile değerlendirilmiştir. RT-qPCR ile HKH'lerde stres koşullarında oluşan gen ifade değişimlerine bakılmıştır. DADLE; HKH'leri *in vitro* oksidatif stres ve ER stresi indükleyen moleküllerin zararlı etkilerinden korumaktadır, ancak hipotermiye maruz bırakılan HKH'leri apoptozdan koruyamamaktadır. Bu çalışmada ilk defa DADLE'nin HKH'ler üzerindeki etkileri değerlendirilmiştir. Başka anti-oksidan veya ER stresinden koruyan maddeler ile birlikte veya tek başına DADLE; *ex vivo* hücre kültürü ortamında HKH'lerin sağ kalımı ve canlılığının koruyarak, HKH'lerin ömürünü artırarak ve/veya HKH'lerin köklülüğü koruyarak klinikte kullanılabilir ekspansiyon protokollarının geliştirilmesine önemli bir katkı sağlayabilecektir.

Anahtar Kelimeler: DADLE, HKH, Oksidatif Stres, ER Stres, Hipotermi.

ABSTRACT

Mammadova A., Assessment of the Role and Antioxidative and Antiapoptotic Effects of Delta-opioid Peptide D-Ala2-Leu5-Enkephalin on the viability of Hematopoietic Stem Cells, Hacettepe University Graduate School of Health Sciences Stem Cell Program Master Thesis, Ankara, 2019. Hematopoietic Stem Cells (HSCs) are adult stem cells with the capacity for self-renewal and differentiation into all blood cell lineages. When HSCs are used for transplantation or gene therapy purposes, they can be exposed to short term cell culture and/or freezing procedures. However, the viability and function of HSCs may be negatively affected by oxidative and endoplasmic reticulum stress induced by these processes. There is a need for the development of novel short term HSC expansion protocols that are highly effective, non-toxic and protect cell viability. Therefore, in the framework of this thesis, the role of Delta Opioid Receptor (DOR) activating synthetic peptid D-Ala2-Leu5-Enkephalin (DADLE) on *in vitro* expansion, oxidative stress, ER stress and hypothermic storage of HSCs was assessed. Umbilical Cord Blood CD34+ HSCs were exposed to oxidative stress induced by H_2O_2 and ER stress induced by Tunicamycin (TM) and Thapsigargin (TG). Cell proliferation was assessed using WST-1; cell cycle analysis were done using BrdU; apoptosis was measured with Annexin-V/Propidium Iodide and the formation of Reactive Oxygen Species were assessed using H_2DCFDA. RT-qPCR was used to investigate the effect of these stress conditions on gene expression of HSCs. Although DADLE was able to protect HSCs *in vitro* from oxidative stress and ER stress inducing molecules, DADLE was not able to protect HSCs from hypothermia-induced apoptosis. In this study, the effects of DADLE on HSCs were determined for the first time. Alone or in combination with other anti-oxidants or ER stress protective agents, DADLE might be helpful in the development of novel protocols for the *ex vivo* expansion of HSCs for clinical use, by promoting their survival through enhanced viability and by supporting their long-term maintenance and/or stemness.

Key Words: DADLE, HSCs, Oxidative Stress, ER Stress, Hypothermia.

Table of Contents

ABBREVIATIONS

AGM	Aorta-Gonad-Mesonephros
Ang-1	Angiopoietin-1
Ann-V	Annexin-V
BFU-E	Burst Forming Unit-Erythroid
BM	Bone Marrow
BMP	Bone Morphogenetic Protein
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CD26 (DPPIV)	Dipeptidylpeptidase IV
CFU-E	Colony Forming Unit-Erythroid
CFU-G	Colony Forming Unit-Granulocyte
CFU-GEMM	Colony Forming Unit-Granulocyte/Erythrocyte/ Monocyte/Megakaryocyte
CFU-GM	Colony Forming Unit-Granulocyte/Monocyte
CFU-M	Colony Forming Unit-Monocyte
CFU-Meg	Colony Forming Unit-Megakaryocyte
c-kit	Receptor of Stem Cell Factor
CKs	Cytokines
CNS	Central Nervous System
CXCR4	Receptor of SDF-1
DADLE	D-Ala2, D-Leu5-Enkephalin
DCF	2',7'-Dichlorofluorescein
DMSO	Dimethyl Sulfoxide
DOR	Delta Opioid Receptor
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
FGF	Fibroblast Growth Factor
FITC	Fluorescein İsothiocyanate
FL	Flt3-ligand
G-CSF	Granulocyte-Colony Stimulating Factor

GPCRs	G-coupled Receptors
H_2DCFDA	2',7'-Dichlorodihydrofluorescein Diacetate
HGFs	Hematopoietic Growth Factors
HRP	Horse Radish Peroxidase
HSCs	Hematopoietic Stem Cells
HSPCs	Hematopoietic Stem and Progenitor Cells
IGF	Insulin Growth Factor
IMDM	Iscove's Modified Dulbecco's Medium
KOR	Kappa Opioid Receptor
Lin-	Lineage negative
LSK	$Lin^-/Sca\text{-}1^+/c\text{-}kit^+$ murine hematopoietic stem cells
MOR	Mu Opioid Receptor
MSCs	Multipotent Stromal Stem Cells (Mesenchymal Stem Cells)
OBs	Osteoblasts
Opr	Opioid receptors
PB	Peripheral Blood
PBN	PBS, BSA, NaN3 solution
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PE	Phycoerythrin
PFA	Paraformaldehyde
PI	Propidium Iodide
PNS	Peripheral Nervous System
PVDF	Polyvinylidene
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
Sca-1	Stem Cell Antigen-1
SCF	Stem Cell Factor
SDF-1 (CXCL12)	Stromal Derived Factor-1
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis
TG	Thapsigargin

TGF-α	Transforming Growth fFctor-alpha
TM	Tunicamycin
TNCs	Total Nucleated Cells
TPO	Thrombopoietin
VEGF	Vascular Endothelial Growth Factor
WB	Western Blot
Wnt	Wingless-related integration site

FIGURES

TABLES

1. INTRODUCTION

Hematopoietic stem cells (HSCs) are self-renewing cells with the potential to develop all types of specialized blood cells. HSCs are widely used in clinical transplantation for treatment of both hematological and non-hematological diseases. When HSCs are used for transplantation, they exposed to short-term cell culture and/or freezing procedures. These procedures are used for expansion, storage or transportation of HSC products until the patient is ready. However, HSCs can get damaged during these processes and survival/cell viability may be affected due to the use of cryoprotective chemicals required for freezing, such as DMSO, cell culture medium and physical conditions. Particularly reactive oxygen species (ROS), which may result from oxidative stress and endoplasmic reticulum (ER) stress during freezing/thawing, may occur during cell culture and can negatively affect the viability and function of HSCs. In addition, the use of DMSO has been shown be related to a range of minor to severe side effects, when directly infused together with the stem cell product. D-Ala2, D-Leu5 Enkephalin (DADLE) is a synthetic delta opioid peptide that serves as a ligand for the delta opioid receptor (DOR). It has been shown to stimulate mechanisms of cell repair and helps in the prevention of cell death, especially during ischemia (1-3). In addition, DADLE has been found to prolong the survival of organs, such as lungs, heart, liver and kidneys, when stored under temporary hypothermic conditions for transplant purposes (4). Addition of DADLE could also prevent cell death of serum-deprived PC12 neuronal cells in an *in vitro* experimental model of ischemia (5). Therefore, we hypothesized that DADLE might be able to confer a similar protection of HSCs and we aimed within the scope of this master thesis project to investigate the protective effects of the synthetic opioid peptide DADLE on HSCs during *in vitro* culture on induced oxidative stress, ER stress and hypothermic conditions. Finding new agents that can be used in stead of DMSO, and protect HSCs during storage or transport may have direct clinical implications for storage and expansion procedures of HSCs and may help prevent HSC infusion-related side effects.

2. BACKGROUND

2.1. Hematopoiesis and Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are unique and unspecialized self-renewing cells with the potential to develop into all types of specialized blood cells, including erythrocytes, monocytes, lymphocytes, thrombocytes and granulocytes (6). HSCs are widely used in clinical transplantations for the treatment of hematologic and non-hematologic diseases, which may be either inherited, such as Thalassemia, Fanconi Anemia or Immune Deficiences and/or acquired (e.g. aplastic anemias, leukemia or non-hematologic malignancies) (7). Currently these diseases can be treated with HSC transplantation, but these cells also hold a huge promise for their use in (future) gene therapy applications (8-10). The formation of blood cellular components, which is called hematopoiesis, is initiated early during embryonic development and is maintained throughout adulthood to produce and replenish the blood system (11). Hematopoiesis during the early stages of embryogenesis occurs in the yolk sac, the aorta-gonad-mesonephros (AGM) region and in the fetal liver and around 5 month of gestation it shifts to the marrow cavity, where it remains for the remainder of the life span of the individual (12). After birth, HSCs are primarily found in the bone marrow (BM) and are responsible for the production of all blood cells, as well as protection of the stem cell reserves. Through tight regulation of the balance between self-renewal, proliferation and quiescence, HSCs manage to maintain both the HSC pool and sufficient numbers of differentiated mature cells throughout life. The hematopoietic stem and progenitor cells (HSPCs) not only generate the complete blood forming system, they also obviously play a significant role in the regulation of the immune response. Furthermore, a small fraction of the HSPCs is able to leave the BM and circulate throughout the body in the peripheral blood (PB), where they assist in the normal repair and defence of the body (13).

The discovery and characterization of HSCs in the 1960-70's led to the definition of the inherent features of all stem cells, that is capability of self-renewal and the ability to generate differentiated daughter cells. Today, these HSCs are among

the best-characterized adult stem cells and their discovery has revolutionized the treatment of many diseases. Approximately 20 years ago, murine HSCs were conclusively distinguished based on their characteristic cellular phenotype, i.e. Lin-/Sca-1+/c-kit+ (LSK), meaning the absence of expression of specific lineage markers found on specialized cell types (Lin-) and the positive surface expression of Stem Cell Antigen-1 (Sca-1) and the receptor for Stem Cell Factor (c-Kit) (14). Based on this phenotype, murine HSCs can be easily isolated from the BM and this system has been used extensively to characterize and further investigate the properties and potentials of HSCs. In humans, a small group of CD34+ cells that adhere to the ventral side of the aortic endothelium within the embryonic compartment was shown to display the distinct cell-surface and molecular characteristics of the earliest primitive hematopoietic precursor cells (CD45+, CD34+, CD31+, CD38−) (15), with absence of lineage-specific marker expression and expression of GATA-2+, GATA-3+, c-myb+, SCL/TAL1+, c-kit+, flk-1/KDR+) (16). After birth, human HSCs are determined to be CD34+, CD59+, CD90/Thy1+, CD38low/-, c-Kit-/low and Lin-.

2.2. The Hematopoietic Niche

In the BM, HSCs are located in an organized microenvironment, also known as the BM niche, which plays a key role in the regulation of stem cell behavior, self-renewal and differentiation (17). The BM niche directs its effects on HSCs through distinctive signals emanating from and activated by different components, such as direct cell-cell interactions, interactions between cells and the extracellular matrix (ECM), the presence of soluble and surface-bound hematopoietic growth factors (HGFs) and cytokines (CKs), mechanical forces, physicochemical clues, such as calcium and oxygen gradients and pH level (10). Each niche may be specific for the type of stem cell it harbors: the activation of molecular pathways is likely to depend on the niche, and may be essential in some and redundant in other niches and thus may have different roles depending on the specific niche. The most important molecular pathways shown to be involved in the regulation of the hematopoietic niche are activated through interactions with Wnt/beta-catenin, bone morphogenetic protein (BMP), Notch, Angiopoietin-1 (Ang-1)/Tie2, fibroblast growth factor (FGF), insulin

like growth factor (IGF), vascular endothelial growth factor (VEGF), transforming growth factor-alpha (TGF-ß) and platelet derived growth factor (PDGF). From these, the BMP and Wnt signaling pathways have been shown to be highly conserved mechanisms controlling self-renewal and lineage commitment of stem cells in both invertebrates and mammals (18).

During homeostasis two types of stromal cells in the BM called multipotent stromal stem cells or mesenchymal stem cells (MSCs) and osteoblasts (OBs) produce a chemotactic protein called SDF-1 (CXCL12), that both attracts HSCs over a gradient and anchors them firmly into the endosteal niche (19). Also endothelial cells (ECs) lining the BM blood vessels are a source of SDF-1 and release it into the BM. As SDF1 is released or secreted in the BM a chemotactic gradient is formed with higher concentrations of SDF-1 located closer to the MSCs and OBs. SDF-1 interacts with the chemokine reseptor called CXCR4, which is expressed on the surface of BM resident HSCs, ECs and other stromal cells (10). The chemotactic signal prevents HSCs from leaving the BM to the peripheral blood (PB). In order to collect adult HSPCs, the cells first need to be stimulated to enter the PB circulation (20). In response to exogenous administration of Granulocyte-Colony Stimulating Factor (G-CSF) the HSPCs are stimulated to expand and differentiate into neutrophilic granulocytes. These cells produce enzymes including Neutrophil Elastase, Pepsin G and MMP9, which are activated inside the BM and cleave the SDF-1 protein, and inhibiting its ability to interact with HSPCs (21). This results then in the rapid release of HSPCs from the BM. In addition the CD26 antigen (DPPIV/ dipeptidylpeptidase IV) plays an important role in mobilization of HSCPs. CD26 is a membrane-bound peptidase that is expressed on the surface of a subpopulation of stem and mononuclear cells and degrades several chemokines, including SDF-1 (10). When the interaction between SDF-1 and CXCR4 is broken, the HSPCs are no longer held in place inside the BM and are released into the PB (22).

2.3. Opioid Receptors and D-Ala2,D-Leu5 Enkephalin

Endogenous opioid signaling plays a critical role in the management of analgesia, euphoria, homeostasis, anorexia/obesity, immune responses and the cardiovascular system (15). Exogenously administered opiates (morphines) have been shown to have an analgetic and sedative effect through interation with specific opioid receptors (Opr) that can be found both in the central nervous system (CNS) and in the peripheral nervous system (PNS) (16). The endogenous opioids β-endorphins, enkephalins, and dynorphins are together known as the endorphins (or endogenous morphines) (23). Although the endorphins interact with different Oprs, they have the tetra-peptide Tyrosine-Glycine-Glycine-Phenylalanine motif in common. Whereas the effects of opioids on pain perception are largely regulated through signaling from pain receptors (nociceptors), opioids have also been shown to work peripherally and decrease the activation of primary neurons, inhibiting immune and inflammatory responses (24). Opioid reseptors are present on both presynaptic and postsynaptic neurons. Binding of an opioid to a receptor on the presynaptic neuron results in a decreased calcium ion influx, decreasing the amount of stimulating neurotransmitters released into the synapse. Binding of opioids receptors present on the postsynaptic neuron has been shown to decrease the response to neurotransmitters released from the presynaptic neuron (25).

The opioid receptors MOR, KOR and DOR are respectively encoded by the μ (Oprm1), κ (Oprk1) and δ (Oprd1) genes and belong to the superfamily of the seven-transmembrane G-coupled receptors (GPCRs) (26-28). Kappa receptor stimulation has been typically linked to hallucinations and dysphoria and an overwhelming sense of dissatisfaction, anxiety and or restlessness (29). Activation of the delta or mu receptors can cause respiratory depression due to the fact that opioid stimulation of the midbrain results in loss of the ability to sense CO_2 levels (30). DOR has further been shown to be involved in the protection of especially neuronal cells (31), cardiomyocytes (1) and MSCs (32, 33). In 1992, DOR was detected in mice, followed by identification in rodents, amphibians and humans (27). D-Ala2, D-Leu5 Enkephalin (DADLE), a synthetic delta opioid peptide serves as a ligand for DOR. It has been shown to stimulate mechanisms of cell repair and helps in the prevention of cell death, especially

during ischemic processes (1-3). In addition, DADLE has been found to prolong the survival of organs, such as lungs, heart, liver and kidneys, when stored under temporary hypothermic conditions for transplant purposes (4). DADLE has been shown to maintain the viability of cultured primary dopaminergic neuronal cells in a dose-dependent manner and transplantation of DADLE-treated dopaminergic cells into the brain of Parkinson rats resulted in a doubling of the surviving cells (34). Thus, DADLE as a single agent provides protection against deterioration of dopaminergic cells in this rodent Parkinson model. Similarly, DADLE was shown to be able to reverse methamphetamine (meth)-induced dopaminergic terminal damage (35). In addition to the neuroprotective effects of DADLE against meth-induced neurotoxicity, DADLE has been shown to attenuate gene expression of Tumor Necrosis Factor, p53 and c-phosphine. Parallel to these beneficial effects on the dopaminergic system, DADLE was also observed to improve neuronal damage following ischemia-reperfusion-induced brain damage. Addition of DADLE could also prevent cell death of serum-deprived PC12 neuronal cells in an *in vitro* experimental model of ischemia (5). Evenmore, the neuroprotective effects of DADLE were further underlined by showing that these cells retained their ability to replicate. Therefore, DADLE has been proposed as a therapeutic preservative for neuroprotection during ischemic and hypothermic conditions. Furthermore, DADLE has been shown to protect cortical neurons in dose-dependent fashion from oxygen/glucose deprivation or damage (OGD) in a similar manner to hypoxia (36). DADLE was shown to protect neurons from hypoxia-induced neuronal damage through activation of DOR and prevent OGD-induced damage by increasing cellular ERK phosphorylation and stimulating p38 phosphorylation (37). This neuroprotective effect of DADLE can be blocked by Naltrindole, a DOR antagonist (31, 32, 38).

2.4. Oxidative Stress

The reactive oxygen species (ROS) superoxide ($\cdot O2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$) can arise from microenvironmental stress or can be the result of the cell's own oxidative metabolism (39). Either way, when ROS production exceeds the cell's capacity to scavenge the ROS, the cells enter a situation

known as oxidative stress (40). The DNA damage resulting from the oxidative stress has been known to play a critical role in the development of many degenerative diseases, including aging, cancer, neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, immune damage and cardiovascular diseases (41). In culture, oxidative stress is more likely to occur since non-physiologic, high oxygen culture tension may lead to more ROS generation, whereas the cellular antioxidant defenses may be simultaneously relatively impaired. For example, specialized serum-free and protein-free defined media, used for the expansion of HSCs may lack many of the anti-oxidative properties of serum. Thus, during ex vivo culture expansion of HSCs, the cells may inadvertently be exposed to fluctuating (usually elevated) levels of O_2, which may affect the stemness properties of these cells. Culture induced-oxidative stress can further lead to induction of apoptosis, certain protein modifications, cross-linking, as well as precipitation (42). Therefore, as a protective measure, cell culture media usually contain some level of antioxidants although these themselves may affect the experimental results, due to degradation products or interference with certain reactions (43).

2.5. Endoplasmic Reticulum Stress

Constituting more than half of the total membranes in the cell, the endoplasmic reticulum (ER) is a large cellular organelle extending throughout the cytoplasm. The ER has a key function in the biosynthesis of secreted proteins, but is also important for the control of proper folding of many proteins (44). When increased levels of unfolded or misfolded proteins surpass the functional abilities of the ER, cells enter a condition known as ER stress (45). In response to ER stress the unfolded protein response (UPR) is initiated, resulting in the activation of three different intracellular signal transduction pathways with the aim to reestablish ER homeostasis (46). Whereas the cell initially tries to resolve ER stress through ER expansion, an increase in ER chaperones, increased ER-associated protein degradation (ERAD) and decreased production of proteins, the inability to restore ER functions will eventually induce cell death due to apoptosis or autophagy (47). Similar to oxidative stress, prolonged ER stress may contribute to the etiology of many human diseases, including diabetes, aging,

neurodegenerative diseases and cancer (48). Molecularly, the UPR is characterized by the activation of one or more of the three ER transmembrane sensors, known as the pancreatic ER kinase (PKR)-like ER kinase (PERK) (49), the inositol-requiring enzyme 1 (IRE1), and the activating transcription factor 6 (ATF6) (50). These sensors are under physiological circumstances inactive and bound to a small ER resident chaperone molecule called BiP/Grp78 (Immunoglobulin-binding protein 78/Glucose-regulated protein 78). During ER stress, BiP/Grp78 is released from the sensors and its dissociation initiates the activation of the ER sensors through autophosphorylation. Downstream of p-PERK initiation factor 2α (eIF2α) is phosphorylated, resulting in a reduced rate of protein translation and upregulation of the activated transcription factor 4 (ATF4) (51), which further upregulates expression of *CHOP*, *XBP1* and *GADD34* genes (49). IRE1 autophosphorylation results in the alternate splicing of XBP1 causing the production of the slightly longer protein XBP1s, which functions as a transcription factor activating genes involved in ER expansion (52). Dissociation of BiP/Grp78 from ATF6 results in its transport to the Golgi complex and recognition by proteases (53). The truncated ATF6N is then transported to the nucleus where it causes upregulation of ER chaperone production and increased *XBP1* gene expression (54). However, since different ER stress inducing factors may activate any or all of the three sensor pathways, responses towards ER stress may vary depending on the situation.

2.6. Hypothermic Stress

In addition to irradiation and high dose chemotherapy, patients with specific types of cancer, including acute leukemia, lymphoma and certain solid tumors, may be eligible for treatment with hematopoietic stem cell therapy (55). Clinically, hemopoietic stem cell products are derived from BM, mobilized peripheral blood or UCB. If storage is required to sustain cellular viability, these blood products are frozen in the presence of dimethyl sulfoxide (DMSO), which is a chemical solvent widely used as a cryopreservative agent (56). DMSO is a highly polar organic liquid that has one polar sulfinyl domain and two apolar methyl groups that help reduce freezing damage induced by storage in vapor or liquid phase nitrogen, thus preserving the cells' physiological structure and functions and hypothermal storage of stem cells. Because

of its ability to modulate membrane permeability and to penetrate membranes of viable cells, it prevents cellular dehydration and shrinkage of cells during the freezing process. During cryopreservation, cells are slowly frozen using a rate-controlled freezing during which the hygroscopic cryoprotectant binds the water molecules in solution, thus reducing the formation of intracellular ice crystals. Despite its concentration-related toxicity and observed side effects following intravenous infusion DMSO it remains as the gold-standard cryoprotective agent for clinical use (57, 58).

DMSO toxicity has been reported as a result of a dose-dependent vasoconstriction in animal studies. Clinically both minor and more severe adverse reactions, including nausea, vomiting and abdominal cramps, more rarely cardiovascular and respiratory problems, such as hypotension and bradycardia, have been shown to be associated with the infusion of thawed cell products containing DMSO (59).

DMSO has also been reported to exert neurological toxicity causing leukoencephalopathy, epileptic seizures and even stroke. In addition, DMSO toxicity has been associated with a negative impact on stemness, as well as induction of epigenetic changes. Furthermore, DMSO used in current cryopreservation protocols, may cause decreased cell viability during thawing of the product. However, for short-term storage, eg for transport only, of blood cell products, recent literature suggests that cryopreservation may not even be necessary. Therefore, the development of novel, storage protocols for cellular products that do not include DMSO, are non-toxic, are easy-to-use, protect cell viability and do not require special transport systems should be assessed.

More than 40.000 transplants using HSPCs obtained from BM, umbilical cord blood (UCB) or mobilized peripheral blood (PB) are used in Europe annually and numbers of transplants in Turkey have been increasing steadily for the last 10 years (60). To allow sufficient time for product quality control, patient conditioning or transport of the cells, the cellular products are generally frozen and stored. Current cryopreservation protocols make use of DMSO, a chemical that protects cells during freezing, but may result in low cell viability during thawing of the product and may induce toxic side effects when infused into patients. In fact, cryopreservation may not

even be necessary for cell products that require only short term storage before being used for cellular treatments in patients or for transport only. Therefore, the development of novel, short-term storage protocols for cellular products that do not include DMSO, are non-toxic, are easy-to-use, protect cell viability and do not require special transport systems should be assessed. DADLE has been tested for efficacy during hypothermic conditions in several cell lines and whole organs and may be able to induce a similar reversible protection of HSCs (61).

2.7. Quiescent Stem Cell Gene Signature

Forkhead box (FOXO) proteins are a family of transcription factors that control the expression of genes effective in apoptosis, cell growth, development, proliferation and survival. The main defining feature of FOXO proteins is the forkhead box region, which can bind to the DNAs of target genes, also known as the winged helix of 80-100 amino acids. FOXO proteins may either activate or inhibit target genes. FOXO3 expression is upregulated in quiescent stem cells and is involved in transcriptional regulation and stem cell fate decisions and controls HSC quiescence by regulating ROS levels (5,9).

SMARCA2/BRM or alternatively the BAF complex (containing BRG/BRM associated factors) and is part of the large ATP-dependent chromatin remodeling complex SNF/SWI, which plays a key role in aortic smooth muscle cell differentiation, negative regulation of cell growth, cell proliferation, nervous system development and has been implicated in HSC maintenance and the regulation of erythroid, lymphoid and myeloid lineage decisions (10,11).

3-Phosphoinositide-dependent protein kinase 1 (PDK1) is a pivotal regulator of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway and has been shown to play an important role in defining the functions and development of hematopoietic cells, including T cells and B cells (7,8). Deletion of the PDK1 gene caused HSCs to enter the cell cycle and to produce a higher number of phenotypic HSCs with loss of progenitor cells. Furthermore, lack of PDK1 expression in T cells results in a significantly decreased T cell number in the thymus and causes a T cell differentiation

block (6). PDK1-deficient HSCs were also shown to be unable to reconstitute the hematopoietic system as a result of the inability to control cellular ROS levels.

Proliferating cell nuclear antigen (PCNA) plays an essential role in DNA replication and chromosome segregation. As a component of the replication complex PCNA is important for lineage determination and differentiation (12).

As a conserved member of the RNase III family of endoribonucleases, the miRNA-processing enzyme, DICER1 gene provides instructions for the production of a protein that plays a role in the regulation of the activity (expression) of other genes which aids in the production of miRNA. Through this role in regulating the expression of genes, Dicer1 has revealed the involvement of miRNA in many processes including cell growth, survival, division, maturation of cells and homeostasis of peripheral T cells (13,14).

2.8. Aim of the Thesis

When HSCs are used for transplantation or gene therapy purposes, they maybe exposed to short-term cell culture and/or freezing procedures. These procedures are used for expansion, storage or transportation of HSC products until the patient is ready. However, HSCs can get damaged during these processes and survival/cell viability may be affected due to the use of cryoprotective chemicals required for freezing, such as DMSO, cell culture medium and physical conditions. Particularly reactive oxygen species (ROS), which may result from oxidative stress and endoplasmic reticulum (ER) stress during freezing/thawing, may occur during cell culture and can negatively affect the viability and function of HSCs. In addition, various bone marrow syndromes and niche disorders may be caused by oxidative stress and/or endoplasmic reticulum stress. DADLE has been tested for its efficacy during hypothermic conditions in several cell lines and whole organs and may be able to induce a similar reversible protection of HSCs. Therefore, we aimed within the scope of this master thesis project to investigate the protective effects of the synthetic opioid peptide DADLE on HSCs during *in vitro* culture on induced oxidative stress, ER stress and hypothermic conditions.

3. MATERIALS AND METHODS

3.1. Umbilical Cord Blood Collection

Umbilical cord blood (UCB) was collected in the Hacettepe University Department of Gynecology by Assoc. Prof. Dr. Özgür Özyüncu in sterile glass bottles containing 20 mL PBS + 200 µL Heparin (final concentration 50 IU/mL) as an anticoagulant. The Medical Ethical Committee of the Hacettepe University approved collection of UCB (GO18/680) and written informed consent was obtained from healthy full-term pregnant women prior to donation and indicated for Cesarion Section. UCB samples were transported to the Hacettepe University Center for Stem Research and Development laboratories and stored at room temperature and processed within 4-48 hours after collection.

3.2. Isolation of Mononuclear Cells from Human UCB

Collected UCB samples (maximally 60 mL) were counted manually with Turks solution and resuspended in an equal volume of Phosphate Buffered Saline (PBS, pH 7.4) and 30 mL UCB/PBS mixture was carefully layered onto 15 mL Biocoll (1.077 g/mL, Biochrom, Merck, Germany, cat no L 6113) in 50 mL conical tubes. Tubes were subjected to 15 min centrifugation without brake at 2000 rpm in a table top centrifuge (Eppendorf 5810R, Germany) followed by careful removal of the buffy coat containing the mononuclear cell (MNC) fraction using a Pasteur pipette. The cells were washed twice with PBS and centrifuged at 2000 rpm for 10 min to remove remaining Biocoll. The final cell pellet containing mononuclear cells (MNCs) was either resuspended in 5 mL of PBS and cell number and recovery were determined using Turk's Solution.

3.3. Selection of CD34+ Hematopoietic Stem Cells Using Anti-CD34 Microbeads

CD34 positive (CD34+) cells were isolated from the MNC fraction using anti-CD34 magnetic microbeads (Miltenyi, cat no 130-046-703) and the MidiMACS

Separator (Miltenyi, Germany) system according to manufacturer's protocol. Briefly, CD34+ cells are magnetically labeled with anti-CD34+ MicroBeads using 100 µL anti-CD34+ MicroBeads, 10^8 cells, 100 µL FcR blocking reagent and 300 uL MACS Buffer (PBS, pH 7.2, 0.5% BSA and 2 mM EDTA) per 10^8 MNCs. Cells were incubated on the MACS Rotator (Miltenyi, Germany) for 30 min at 4ºC. Cells were washed twice with MACS Buffer and resuspended in 6 mL MACS Buffer on ice. Before separation, magnetically labelled cells were run through a 30 µm pre-separation filter (Miltenyi, cat no 130-041-407). The LS Column (Miltenyi, cat no130-042-401) were place into the MidiMACS magnetic field and the cell suspension was run through the prewetted column. The column was rinsed 3-5 times with 3 mL MACS Buffer each. The first fraction collected contained the lineage negative cells, whereas the CD34+ cells were retained CD34 in the colum. After removal of the column from the separator, CD34+ cells were flushed out using up to 12 mL MACS Buffer. To ensure purity of the isolated CD34+ cells, if necessary, the cells were run through a second column. Purity and CD34 expression of the cells was confirmed using FACS analysis.

3.4. Hematopoietic Colony Forming Assays

The colony forming capacity of HSPCs can be evaluated using colony assays. Depending on the type of HGF used and the capacity of the HSPCs, many different types of colonies can be identified: colony forming unit-granulocyte/erythrocyte/ monocyte/megakaryocyte (CFU-GEMM), which define the earliest HSPCs with the capacity to form both myeloid and erytrhoid colonies; burst forming unit-erythroid (BFU-E), which consist of early erythroid progenitors and are bright red in color; colony forming unit-erythroblast (CFU-E), which are small colonies consisting of late erythroid progenitors; colony forming unit granulocyte/monocyte (CFU-GM), colony forming unit granulocyte (CFU-G) and colony forming unit monocyte (CFU-M), which are white colonies that contain respectively both granulocyte and monocyte/macrophage precursors, granulocyte only or monocyte/macrophage precursors only, and colony forming unit-megakaryocyte (CFU-Meg), which consists of megakaryocytes and in humans are difficult to assess. Here, CFU-GEMM, CFU-GM and BFU-E numbers were counted after 10-14 days of culture in semisolid

medium. Briefly, 10.000 CD34 enriched cells were diluted in 2 mL Iscove's Modified Dulbecco's Medium (IMDM) and mixed. 300 µL of this cell suspension was then taken and resuspended in 2,7 mL HGFs containing semisolid methylcellulose medium (Stem Cell Technologies, Methocult Classic H4434) and seeded in duplicate at 1 mL/35 mm dish. Dishes were incubated in a humidified tray at 37°C and 5.0% CO_2. After 10-14 days, colonies were examined and counted using phase contrast microscopy.

3.5. Immunophenotyping of Cells

Flow cytometric analysis was used to characterise the cells isolated from human UCB samples. UCB total nucleated cells (TNCs), MNCs, CD34 negative and CD34 positive cell fractions were assessed for surface expression of CD34, CD38 and DOR. TNCs and where necessary MNCs samples were subjected to lysing in 10 mL 1X NH_4Cl Lysing Buffer for 10 minutes at 4°C to remove excess red blood cells. Cells were washed twice with PBN (PBS, 0.5% BSA and 0.05% NaN_3) before staining. Cells were then stained with 5 uL of phycoerythrin (PE)-conjugated anti-human CD38 (BD Biosciences, cat no 555459) and anti-human CD34-APC (BioLegend, Cat no BioLegend, 343510) in PBN with 2% human AB serum for 15 min at room temperature in the dark. Cells were then washed twice in 2 mL PBN and spun down at 1500 rpm, 5 min. For FACS analysis, cells were resuspended in 200 µL PBN Buffer. Flow cytometric analysis was performed using a FACSAccuri (Becton Dickinson, USA). 10.000 list mode events were collected and data was analyzed using the BD CSampler software (Becton Dickinson, USA). An unstained control sample was used to set the voltage for each channel, gates were set on forward (FSC) and sideward light scatter (SSC), to exclude erythrocytes and cellular debris.

3.6. Immunofluorescent Staining

Cells were cytospun onto slides and fixed with 2 mL of %4 Paraformaldehyde (PFA) solution for 15 minutes. If appropriate, cells were permeabilized by incubating

with 2 mL 0.1% Triton X-100 in icecold PBS for 15 minutes and then washed three times with PBS. Cells were incubated with blocking buffer, consisting of 3% human AB Serum, 10% Bovine Serum Albumin (BSA, Sigma-Aldrich, USA), 0.1% Tween-20 (Merck, Germany) for 1 hour. They were stained with primary donkey anti-human DOR antibody (Abcam, cat no epr5029), diluted in blocking buffer and incubated at 4ºC overnight in a dark humidity chamber. After each incubation, they were washed three times with 0.1% Tween-20 in PBS (PBS/Tween-20). Samples were then incubated in Fluorescein Isothiocyanate (FITC)-conjugated secondary antibody donkey anti-rabbit IgG (Thermofisher Scientific, cat no A16024) in the dark at 4ºC for 1 hour. After three washes in PBS/Tween-20, mouse-anti-CD34 primary antibody was added, and slides were incubated for 30 min at 4ºC. The samples were washed three more times in PBS/Tween-20 and stained with rat-anti-mouse IgG-FITC (1/1000, BD Biosciences, cat no 553443) secondary antibody. Cell nuclei were stained with DAPI (1/4000 ratio) at room temperature for 1 min. Slides were washed three more times with PBS/Tween-20. Slides were assessed using a Leica fluorescent microscope and photographs were taken using Leica Application Suit 3.1 software.

3.7. CD34+ HSC in vitro Culture

For cell proliferation analysis using WST-1 (see below), CD34+ cells were cultured in 96-well plates at a concentration of 10.000 cells/well in 200 µL medium. All culture conditions were tested in triplicate. For induction of different stress conditions, cells were cultured in 24-well plates at a concentration of 100.000 cells/well in 2 mL medium. For cell culture serum-free StemMACS™ HSC expansion medium XF (Miltenyi, cat no 130-100-463) was used and supplemented with different cocktails of hematopoietic growth factors (HGFs), including Thrombopoietin (TPO), Stem Cell Factor (SCF) and/or Flt3-ligand (FL) or a combination of the three HGFs (STF, StemMACS™ HSC expansion cocktail, 100x, Miltenyi, cat no 130-100-843).

3.8. WST Cell Proliferation Assay

Cell proliferation was assessed using the WST-1 cell proliferation reagent (Roche, cat no 11 644 807 001). The WST assay quantitatively demonstrates cell proliferation based on the cleavage by mitochondrial dehydrogenases of tetrazolium salt WST-1 to formazan. Formazan production correlates with with the number of metabolically active cells in the culture and therefore non-viable cells do not produce a signal. CD34+ UCB cells were plated into the wells of 96-well plates and cultured in serum-free HSC expansion medium in presence of TPO, SCF and/or FL and cells were subjected to different stress conditions (described below). After a set period of cell culture, 10-20 µL of WST1 reagent was added to each well of the 96-well plate, leading to a 1:10 ratio WST-1 to cell and incubated for 4 hours at 37ºC, 5% CO_2 in a humified incubator. The absorbance was measured in a microplate (96-well plate) reader (Tecan, Switzerland) at 420-480 nm, with a reference value of >600 nm, and cell viability was calculated. A blank sample (HSC Expansion Medium without cells) was used to calibrate the spectrophotometer to zero absorbance. Doses of DOR agonist DADLE, DOR antagonist Naltrindole, H_2O_2, Tunicamycin (TM) and Thapsigargin (TG) were determined using WST-1.

3.9. Induction of Oxidative Stress

Hydrogen Peroxide (H_2O_2) has been previously used as an in vitro model of oxidative stress. The oxidative stress mimicking agent was exogenously used in this study to induce production of intracellular ROS by CD34+ UCB cells (38). The optimal dose of H_2O_2 was determined based on ~50% cell death in HSCs after 48h of treatment through dose-finding experiments using WST-1 (100-1000 µM) and used in subsequent tests. For DOR activation, the effects of 100-300 nM DOR agonist DADLE and /or 10 µM DOR antagonist Naltrindole were evaluated. To assess the effects of oxidative stress on HSCs, cells were cultured in serum-free HSC expansion medium with TPO in 96-well plates (WST-1 assay) or 24-well plates (cell cycle, ROS, apoptosis assays) and first pre-treated with Naltrindole and then 45 min later with

DADLE. 24 hours later 100-1000 μM H_2O_2 was added and effects on HSCs were assessed 48 hours later.

3.10. Induction of Endoplasmic Reticulum Stress

To assess the effects of DADLE on Endoplasmic Reticulum (ER) stress in HSCs, 10^4 or 10^5 CD34+ HSCs were cultured in 96-well (WST-1 assay) and 24-well culture plates (apoptosis, BrdU, Western Blot, RT-qPCR) respectively for 4 or 7 days in serum-free HSC expansion medium with 1x STF. Endoplasmic reticulum (ER) stress was induced by treating cultures with the ER stress-inducing chemicals Thapsigargin (TG, Sigma) at a dose of 1-100 nM or Tunicamycin (TM, Tocris) at a dose of 0.1-0.5 μg/mL.

3.11. Induction of Hypothermic Stress

CD34+ HSCs were cultured for 7 days in presence of serum-free medium HSC expansion medium supplemented with 1x STF. After 7 days of culture without any medium changes, different concentrations of DADLE were directly added to the wells and the plates were sealed airtight using parafilm and stored for 3, 7, 10 or 14 days at +4°C. After the hypothermic incubation period finished, the parafilm was removed and the cell culture plates were placed in a humidified incubator at 37°C and incubated for 4 hours before further analysis.

3.12. Analysis of Apoptosis Using Annexin-V and Propidium Iodide

Cell apoptosis was measured using double-labeling with Annexin-V-FITC (Ann-V, BioLegend, cat no 640906) and Propidum Iodide (PI, Sigma-Aldrich, cat no P4170) on the freshly isolated CD34+ cells from UCB and after culture under different stress conditions in the absence or presence of different concentrations of DADLE. Briefly, 100.000 cells were plated in 24-well plates and subjected to different stress conditions, as described below. Following treatment, the cells were carefully collected and tranferred into the 5 mL polystyrene FACS tubes for centrifugation at 1500 rpm

for 5 min and supernatant was discarded. Cells were resuspended in 100 µL Annexin Binding Buffer, consisting of 100 mM HEPES, 140 mM NaCl, 25 mM $CaCl_2$ at pH 7.4 and mixed gently with 5 µL of Ann-V and 5 µL of PI Stock Solution (1 mg/mL) and incubated for 15 min at room temperature protected from the light. After incubation, 100 µL of Annexin Binding Buffer was added to each tube and cells were analysed within 15 minutes using the BD FACSAccuri. 10.000 cells were collected and analyzed using BD CSampler software.

3.13. Assessment of Cell Cycling Using BrdU Incorporation

BrdU (bromodeoxyuridine) is a synthetic nucleoside (Thymine) analogue, which is incorporated during the S phase of the cell cycle into newly synthesized DNA of dividing cells. Cell cycling was assessed using the Phase-Flow BrdU Cell Proliferation Kit (BioLegend, cat no 370704). Briefly, 10^5 cells were cultured in 24-well plates in 2 mL medium and subjected to different stress conditions in the presence or absence of DADLE. At the day of analysis 1 µL BrdU pulsing solution (10 mg/mL) was added to the cell cultures and cells were incubated for an additional 2 hours. After removing of the medium and harvesting of the cells, cells were fixed for 20 min at 4ºC and treated with 50 µL DNAse (400 µg/mL Ca_2+/Mg_2+ PBS) for 1 hour in a 37ºC incubator or water bath. After addition of 5 µL of anti-BrdU-FITC, cells were incubated for an additional 20 min and counterstained with 7-AAD (1 µg/sample). BrdU incorporation was measured using the BD FACSAccuri and analysed using BD CSampler software. Non-specific binding of antibody was assessed by using cells cultured in the absence of BrdU as a negative control. Data collected was processed and analysed in Microsoft Excel.

3.14. Detection of Intracellular Reactive Oxygen Species

The fluorescent dye-based cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a chemically reduced form of fluorescein, that can be used as an indicator for Reactive Oxygen Species (ROS) in cells. Upon cleavage of the acetate

groups by intracellular esterases and oxidation, the nonfluorescent H_2DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF). Cells were subjected to different types of cellular stress (oxydative stress and prolonged hypothermia) and cultured in the absence (control) or presence of DADLE. On the day of analysis, cells were harvested and washed with PBS to remove StemMACS HSC expansion medium by centrifugation for 5 min at 1500 rpm. Then cells were resuspended in pre-warmed PBS containing 10 μM working concentration H_2DCFDA (Invitrogen, cat no D399) and incubated for 30 minutes at the 37^{o}C. After washing 3 times with PBS cells fluorescence was assessed using the BD FACSAccuri.

3.15. Protein Isolation and Western Blot

To obtain total protein lysates, UCB-HSCs were collected after induction of different stress conditions and washed twice with ice cold PBS. Cells were incubated on ice with Pierce RIPA Buffer (Thermofisher Scientific, cat no 89900) containing Complete mini EDTA-free protease inhibitor cocktail (Roche, cat no 04693159001) for 10 minutes. Lysates were collected by centrifuging at 14,000 xg, at 4^{o}C for 15 min and supernatants were transferred to a new eppendorf tube. Protein lysates were stored at -80^{o}C until further use. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermofisher Scientific, cat no 23225) according to the manufacturer's instructions. Total protein lysates were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) which was performed using the 10% TGX Stain- Free Fastcast Acrylamide kit (Bio-Rad, cat no 1610183) for the gels and the proteins on the gel were transferred to a polyvinylidene (PVDF) membrane (Millipore, cat no P2938) using the Trans-Blot® Turbo™ System (BioRad, USA) and Trans-Blot® Transfer System (Bio-Rad, USA). After transfer, the membrane was washed with washing buffer (PBS with Tween-20, PBS-T) and after blocking for 1 hour with blocking solution (5% skimmed milk powder in PBS-T), incubated with the primary antibody and treated with secondary antibody at the appropriate dilution according to manufacturer's instructions. To assess induction of ER stress, polyclonal anti-P-PERK (Santa Cruz Biotechnologies, cat no sc-32577), ATF4 (Gentex, cat no GTX101943) and BiP/Grp78 (Santa Cruz Biotechnologies, cat

no sc-13968) primary antibodies were used. To assess induction of apoptosis in HSCs, a monoclonal antibody against the anti-apoptotic Bcl-2 protein (Thermofisher Scientific, cat no 13-8800) and monoclonal antibodies against pro-apoptotic proteins Bad (Thermofisher Scientific, cat no MA5-14800) and Bax (Thermofisher Scientific, cat no MA5-14003) were used. As secondary antibody, Goat Anti-Rabbit IgG-HRP (Abcam, cat no ab97051) was used. The protein bands were then visualized by the gel imaging FluorChem FC3 System (Protein Simple, USA) using a chemiluminescence kit (Thermo Fisher Scientific, Super Signal West Femto Maximum Sensitivity Substrate Kit).

3.16. Total RNA Isolation, cDNA Synthesis and RT-qPCR

To determine changes in gene expression of the PDK1, PCNA, SMARCA2, FOXO3, DICER1 genes by HSCs in response to different stress conditions, total RNA was isolated using the miRNeasy minikit (Qiagen, cat no 217004). After induction of ER stress, wells containing a mininum of 10^5 HSCs were treated with 700 µL Qiazol Lysis Reagent (Qiagen, cat no 79306) and incubated at room temperature for 5 minutes. Samples were treated with chloroform (Applichem, cat no A3633) and mixed well. Samples were then incubated for 3 min at room temperature and centrifuged at 12.000xg for 15 min at 4°C. After centrifugation, the aqueous phase was collected and 100% Ethanol was added. Samples were loaded onto the columns and centrifuged for 30 seconds at 10.000xg. Samples were subsequently washed and treated with RWT Buffer, DNase, RWT Buffer and finally RNA was eluted and stored at -80°C until use. RNA concentrations were assessed using the NanoDrop 2000 (Thermo Scientific, USA) and measured at A260/A280 and A260/A230. Using the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific, Germany) mRNA samples were reverse transcribed into cDNA. Quantitation was performed using a LightCycler® 480-II system (Roche, Germany) with the selected appropriate primers (Table 3.1.). Housekeeping gene ACTB was used for normalization of gene expression. Fold changes in gene expression were calculated using relative ($2^{-\Delta\Delta Ct}$) quantitation.

4. RESULTS

4.1 CD34+ HSCs Can Be Obtained From UCB with High Efficiency

In total 18 UCB samples were collected and processed. For each test isolated MNCs from 3 different donors were pooled and used for CD34+ selection. The results are presented in Table 4.1. An average of 78.38 CFU-GM and 8.2 BFU-E colonies were observed per thousand plated CD34+ cells (n=4) (Figure 4.1.).

Table 4.1. MNCs and CD34+ numbers isolated from UCB

UCB (mL)	MNCs x10^6	CD34+ cells x10^6	Recovery (%)
80,0 ± 7,5	203,6 ± 138,8	5,9 ± 2,3	1,3 ± 1,2

Figure 4.1. Colony forming capacity of UCB CD34+ cells. Images of clonogenic potential of umbilical cord blood samples. Left: BFU-E (burst-forming unit-erythroid): clonogenic progenitors that represent mature erythroid progenitors; Middle: CFU-GM (colony forming unit-granulocyte/macrophage), Right: CFU-GEMM (colony forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte). Images were taken at magnification of x20.

The CD34+ percentages of cells after seletion using magnetic separation were evaluated by flow cytometry. Results are presented in Table 4.2.

Table 4.2. CD34/CD38 percentages after magnetic selection from UCB

CD34+ x10^6/mL	CD34+/CD38- (%)	CD34+/CD38+ (%)	CD34-/CD38+ (%)
5,86 ± 2,31	10,3 ± 17,8	67,2 ± 21,1	15,6 ± 11,8

4.2. CD34+ HSCs Express Low Levels of DOR on Their Surface

The expression of DOR was evaluated in freshly isolated CD34+ HSCs (n=8) using co-staining for CD34 and CD38. While 22,4% DOR expression was observed in a single UCB sample, DOR surface expression in all other CD34+ cells was generally very dim and found to be on average 1,0 ± 0,8%. However, DOR expression was detected at a much higher rates in CD34- cells 9.8 ± 8.1%, indicating upregulation of DOR expression during differentiation. Dot plots of a representative sample are given in Figure 4.2.

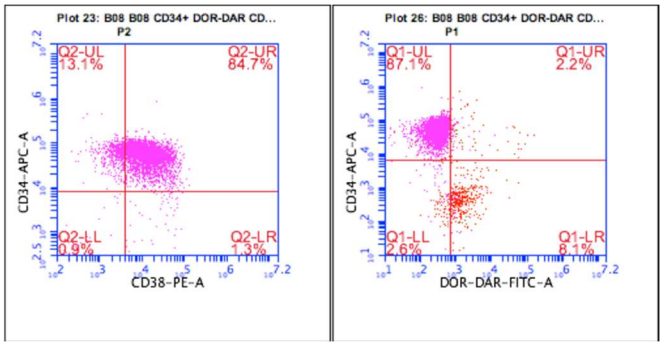


Figure 4.2. DOR expression on CD34+ and CD34- negative cells. The mononuclear fraction of three different UCB samples was pooled and used for CD34 selection using anti-CD34+ magnetic beads. Left: dot plot showing high efficiency of CD34 selection from a representative UCB sample, right: Cells were incubated with rabbit-anti-human Delta Opioid Receptor (DOR) primary antibody, followed by incubation with donkey-anti-rabbit (DOR)-FITC. Whereas CD34+ cells showed only dim expression of DOR, CD34- cells showed increased surface expression of DOR.

4.3. DADLE Protects CD34+ HSCs from Low Level Oxidative Stress

In order to define the culture conditions for further experiments, CD34 positive cells were cultured in 96-well plates in serum-free HSC expansion medium and the effects of DADLE on CD34+ cells, cultured for 48 hours in presence of TPO, SCF, SCF/TPO and SCF/TPO/Flt3-ligand (STF), were assessed using WST-1 proliferation assays (Figure 4.3.). Based on these data a dose of 300 nM DADLE and, for short term cultures, the use of TPO only, rather than SCF or SCF/TPO or STF, was found to be optimal. To induce oxidative stress in CD34+ HSCs, the optimal dose of H_2O_2 for use in cell culture was tested in cultures supplemented with TPO only in the presence or absence of H_2O_2. H_2O_2 doses of 100, 300 and 1000 µM were tested during 48 hour

cultures (Figure 4.4.). At a dose of 300 nM DADLE was shown to exhibit a protective effect on CD34+ cells after treatment with 100 and 300 µM H_2O_2. However, a dose of 1000 µM resulted in immediate and overwhelming cell death. Based on extrapolation of these data, a H_2O_2 dose of 500 µM would have caused approximately a 50% reduction in metabolic activity and was therefore used in following experiments.

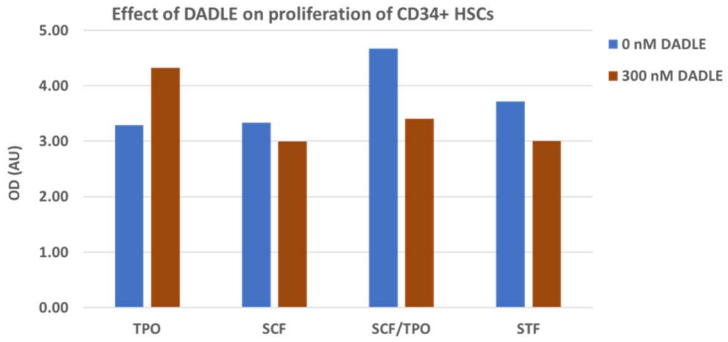


Figure 4.3. Effect of DADLE on proliferation of CD34+ cells. CD34+ HSCs were seeded in 96-well plates in serum-free expansion medium supplemented with TPO, SCF, SCF/TPO or SCF/TPO/Flt3-ligand (STF) and cultured in presence or absence of 300 nM DADLE. After an incubation period of 48 hours, 1:10 WST was added and incubated for 4 hours after which the metabolic activity of the cells was measured as arbitrary units (AU) on a spectrophotometer (optical density, OD) (n=1).

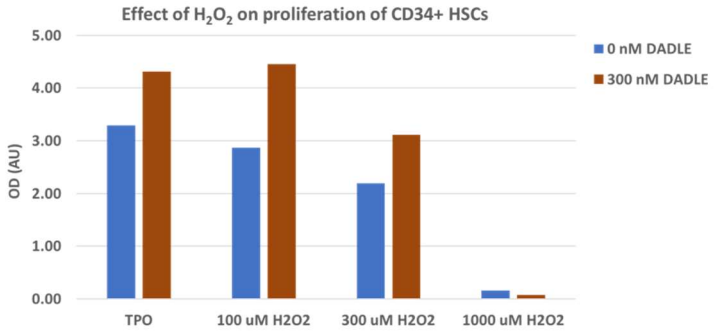


Figure 4.4. Effects of H_2O_2 on proliferation of CD34+ cells. CD34+ HSCs were seeded in 96-well plates and H_2O_2 was tested at different doses. After an incubation period of 48 hours, 1:10 WST was added and incubated for 4 hours after which the metabolic activity of the cells was measured as arbitrary units (AU) on a spectrophotometer (optical density, OD) (n = 2).

To counteract the effect of the synthetic DOR agonist DADLE, the DOR antagonist Naltrindole (ND) was used. In these experiments, cells were first treated

with Naltrindole, 45 minutes later DADLE (D) was added and another 24 hours later cells were exposed to a dose of 500 µM H_2O_2 to induce oxidative stress. The results are shown in Figure 4.5.

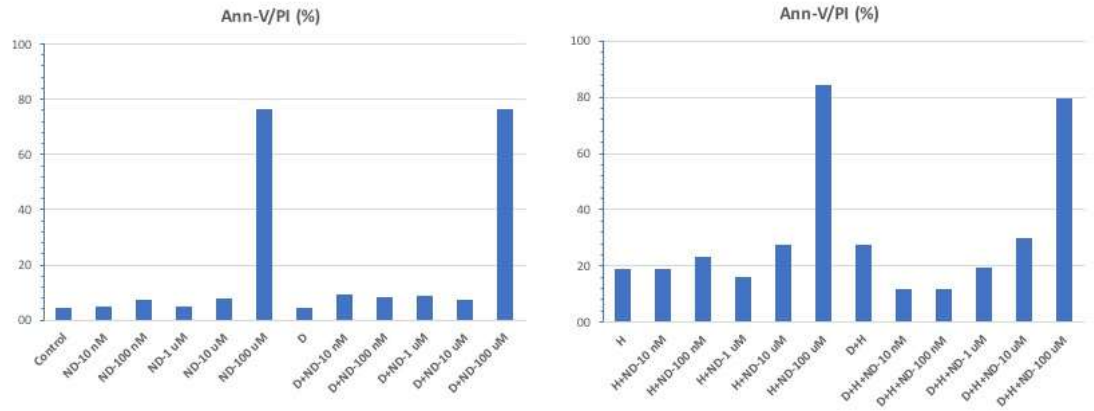


Figure 4.5. Effects of the DOR antagonist Naltrindole and DOR agonist DADLE on H_2O_2-induced oxidative stress-related apoptosis of CD34+ cells. Left: CD34+ HSCs were treated with 10 nM - 100 µM Naltrindole (ND) and/or 300 nM DADLE (D) for 48 hours. Right: CD34+ HSCs were treated with 10 nM - 100 µM Naltrindol (ND) and/or 300 nM DADLE (D) in the presence of 500 µM H_2O_2 (H) for 48 hours (n=1).

In the absence of H_2O_2, 100 µM ND was found to be extremely toxic. A dose of 300 nM DADLE did not adversely affect cell viability, but could also not inhibit the toxic effects of Naltrindole. ND did not show any important protective or aggrevating effect on H_2O_2-induced oxidative stress in CD34+ cells. However, at the dose of 500 µM H_2O_2 tested, DADLE also did not display an important cell-protective effect on CD34 + cells. When 500 µM H_2O_2 was used to induce oxidative stress, in both WST proliferation tests and apoptosis assays measuring the percentages of Annexin-V (Ann-V) positive cells, the proliferation and cell viabilty of cells was found to be decreased. However, the proliferation suppressive effects and apoptosis-inducing effects of 500 µM H_2O_2 were so strong, that neither ND or DADLE treatment was sufficient to achieve any effect at that dose (Figure 4.6. and Figure 4.7.). Nevertheless, at a dose of 100 µM H_2O_2 DADLE does appear to decrease levels of apoptosis and increased cell viability of CD34+ HSCs.

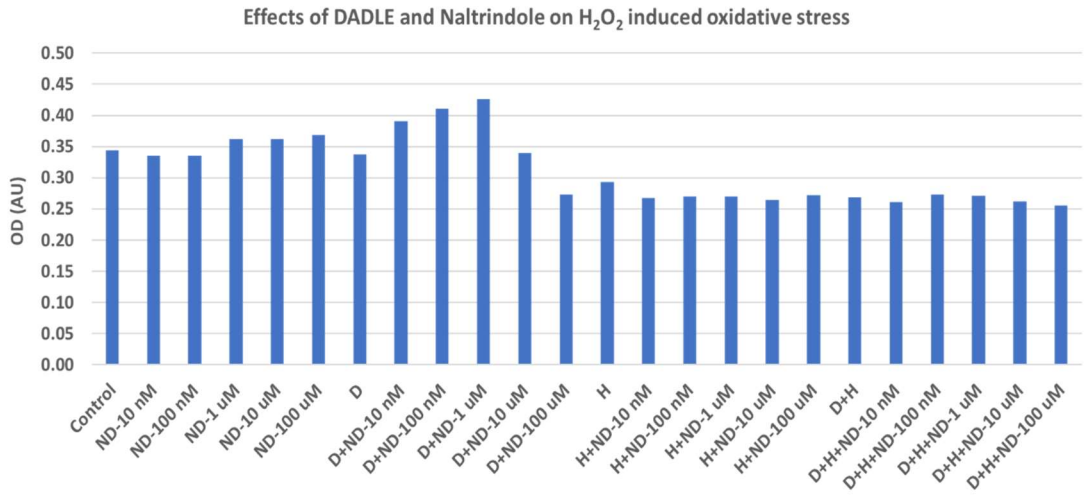


Figure 4.6. Effects of DADLE and ND on proliferation of CD34+ cells after induction of oxidative stress. CD34+ HSCs were treated with 10 nM - 100 µM Naltrindole (ND), 300 nM DADLE (D) and/or 500 µM H_2O_2 (H) for 48 hours. 500 µM H_2O_2 negatively affected proliferation of all cells, independent of the presence of ND or D. Average of two separate experiments (n=2).

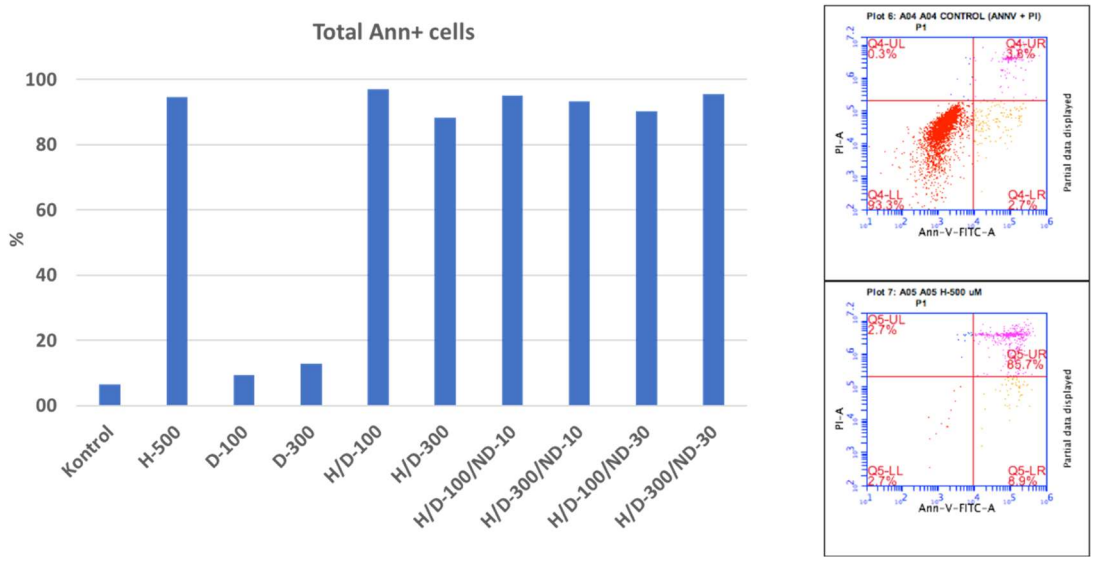


Figure 4.7. Effects of DADLE and ND on apoptosis of CD34+ cells after induction of oxidative stress. Left: CD34+ HSCs were treated with 10 nM or 30 µM Naltrindole (ND), 100 nM or 300 nM DADLE (D) and 500 µM H_2O_2 (H) for 48 hours. 500 µM H_2O_2 induced high levels of apoptosis as measured by total percentage of Annexin-V positive cells (Ann-V+/PI- and Ann-V/PI+ fractions together). Tested doses of ND and D were not sufficient to protect cells from oxidative-stress related cell death. Average of two separate experiments. Right: dot plot of untreated control cells and cells after treatment for 48 hours with 500 µM H_2O_2 (n=1).

The cell-permeant, non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) in

presence of ROS. To confirm induction of oxidative stress in HSCs, doses of 100 µM, 300 µM and 500 µM H_2O_2 were tested in presence of 300 nM and 600 nM DADLE (Figure 4.8.). However, doses exceeding 100 µM were found to be extremely toxic to the cells, and increasing the doses of DADLE from 300 nM to 600 nM was not found to increase the protective effect of DADLE. Therefore, the dose of 100 µM H_2O_2 was found to be optimal to induce oxidative stress and test the cytoprotective effects of DADLE on apoptosis, ROS production and effects on proliferation of CD34+ HSCs. Thus, these data confirm that DADLE can protect CD34+ HSCs from low level oxidative stress, induced by H_2O_2 by reducing production of ROS, thereby decreasing apoptosis and increasing cell viability.

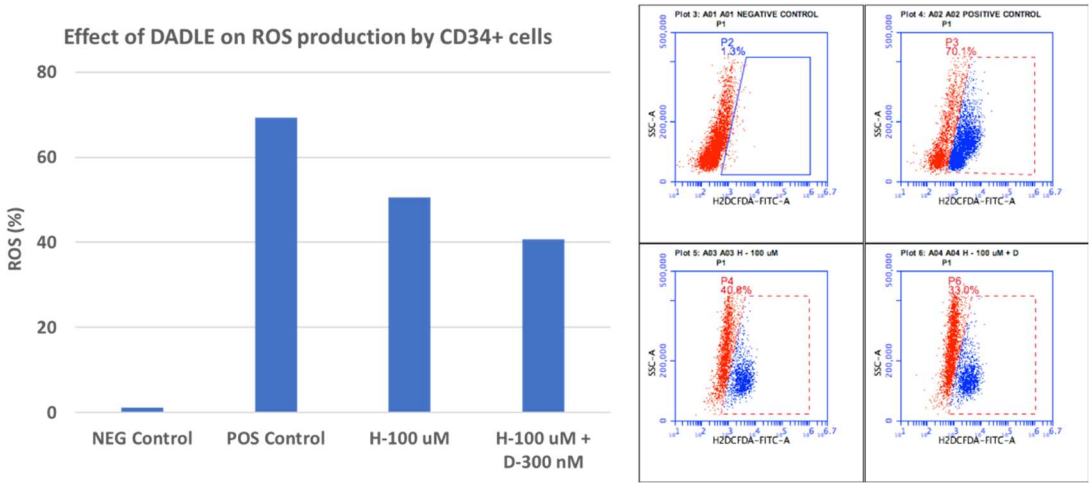


Figure 4.8. Effect of DADLE on ROS production by CD34+ cells in reponse to H_2O_2-induced oxidative stress. Left: CD34+ HSCs were cultured in serum-free medium with TPO and treated with 100, 300 or 500 µM H_2O_2 (H) for 48 hours with or without 300 nM or 600 nM DADLE (D). However, due to extensive cell death in the 300 or 500 µM H_2O_2 groups, ROS could not be determined. Average of two separate experiments. ROS production was measured using a H_2DCFDA kit. Right: representative dot plots (n=1).

4.4. DADLE Protects CD34+ HSCs from Endoplasmic Reticulum Stress

To induce endoplasmic reticulum (ER) stress in HSCs, the ER stress-inducing chemicals Tapsigargin (TG, Sigma) and Tunicamycin (TM, Tocris), were used. TG and TM doses used were first determined using the WST-1 and apoptosis assays (Figure 4.9. and Figure 4.10.). TG or TM-induced ER stress was evaluated on the 4th

or 7th day of CD34+ HSCs culture. Because these experiments are longer, CD34+ HSCs were cultured in serum-free expansion medium supplemented with STF to prevent cell death due to starvation.

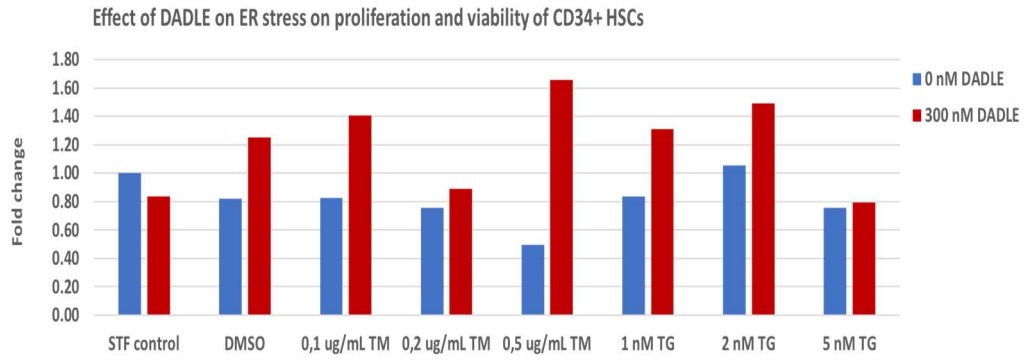


Figure 4.9. DADLE supports proliferation of CD34+ HSCs under ER stress. CD34+ HSCs were cultured in serum-free medium supplemented with STF in presence of 0.1 µg/mL – 0.5 µg/mL Tunicamycin (TM) or 1 nM – 5 nM Thapsigargin (TG) with (red) or without 300 nM DADLE (blue). After 4 days, 1:10 WST was added and incubated for 4 hours after which the metabolic activity of the cells was measured as arbitrary units on a spectrophotometer and normalized according to their respective controls. Data are the average of two separate experiments (n=2).

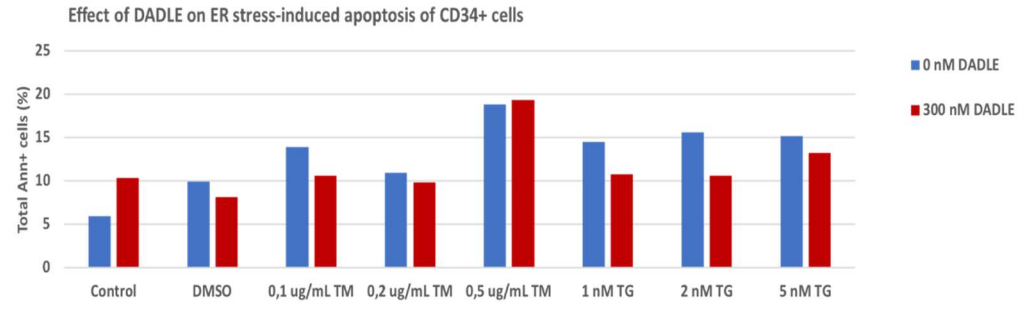


Figure 4.10. DADLE protects CD34+ HSCs from ER stress. CD34+ HSCs were cultured in serum-free medium supplemented with STF in presence of 0.1 µg/mL – 0.5 µg/mL Tunicamycin (TM) or 1 nM – 5 nM Thapsigargin (TG) with (red) or without (blue) 300 nM DADLE. After 4 days, cells were collected and stained with Annexin-V-FITC and Propidium Iodide. Data given are the total number of Annexin positive cells (Ann+/PI- and Ann+/PI+ fractions). Cells was measured using a FACSAccuri (n=1).

Treatment of CD34+ cell cultures with TM resulted in the induction of ER stress, as evident by a decrease in cell proliferation or viability. However, the doses of TG tested induced only a minimal effect on CD34+ cell proliferation. Nevertheless,

addition of DADLE to both the TM and TG-treated cultures resulted in general in a higher proliferative index and lower levels of apoptosis, indicating increased viability of CD34+ HSCs in presence of DADLE. In literature TG is usually used at higher doses (>25 nM) although TG may induce ER stress at different doses in distinct cells. Based on previous data in mesenchymal stem cells, we found that the effects of TG on MSCs started at doses as low as 1 and 2 nM and therefore these doses were used in the first experiments. Although there was no apparent negative effect of TG on proliferation at the tested doses, TG did appear to induce some level of apoptosis in CD34+ cells.

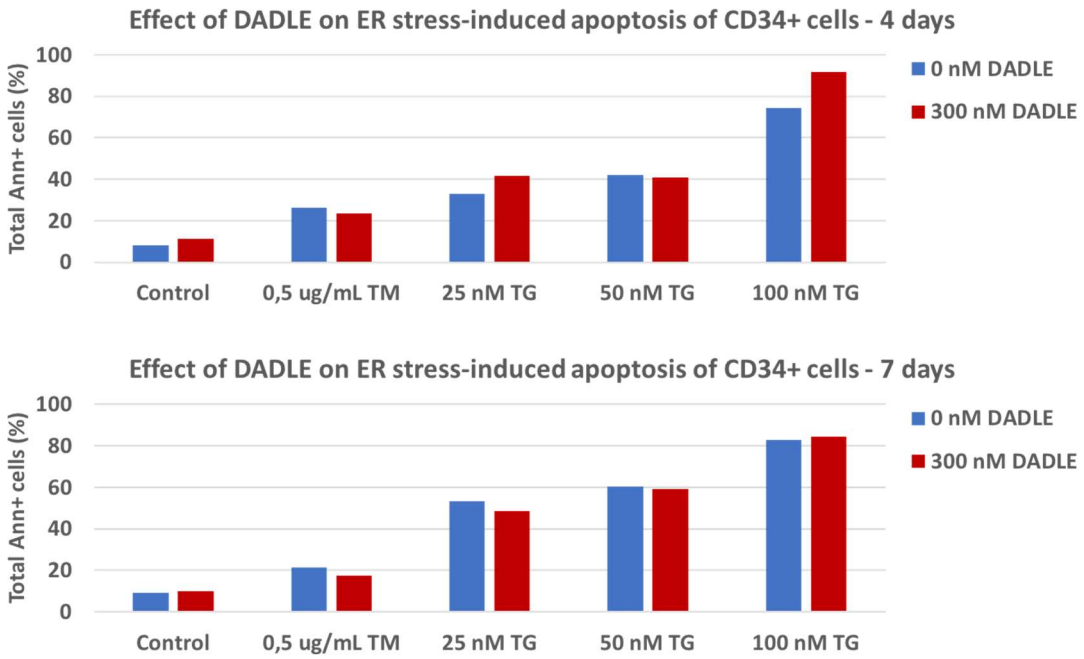


Figure 4.11. Effects of DADLE on ER stress-induced apoptosis of CD34+ cells. CD34+ HSCs were cultured in serum-free medium supplemented with STF in presence of 0.5 µg/mL Tunicamycin (TM) or 25 nM – 100 nM Thapsigargin (TG) with (red) or without (blue) 300 nM DADLE. After 4 days, cells were collected and stained with Annexin-V-FITC and Propidium Iodide. Data given are the total number of Annexin positive cells (Ann+/PI- and Ann+/PI+ fractions). Cells was measured using a FACSAccuri (n=2).

When doses of TG were increased to see if DADLE would still exert any cytoprotective effects, no differences were observed between the TG-treated cells with and without DADLE (Figure 4.11.). Thus, DADLE protects CD34+ HSCs from ER

stress, but similar to the effects as observed with oxidative stress, when a treshold level in ER stress is exceeded, DADLE cannot further support CD34+ cell survival.

When 25 nM TG was used to induce ER stress, total Annexin-V positive cell numbers increased from 10% to approximately 50% (Figure 4.11.), indicating considerable cell death as a result of ER stress, accordingly when the levels of anti-apoptotic protein Bcl-2 were measured using Western Blot, a small decrease in Bcl-2 expression was observed. Addition of DADLE had minor effects on levels of Bcl-2 expression and was not sufficient to protect cells from ER stress-induced apoptosis (Figure 4.12.). Interestingly, expression of the pro-apoptotic protein Bax also decreased in response to 25 nM TG, and appears to normalize after addition of DADLE. The tested dose of 0.5 µg/mL TM was not sufficient to measure significant differences in Bcl-2 or Bax expression levels using Western Blot.

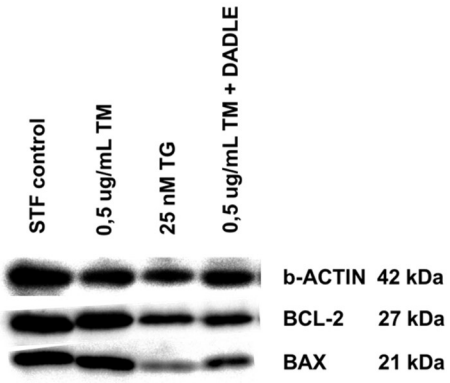
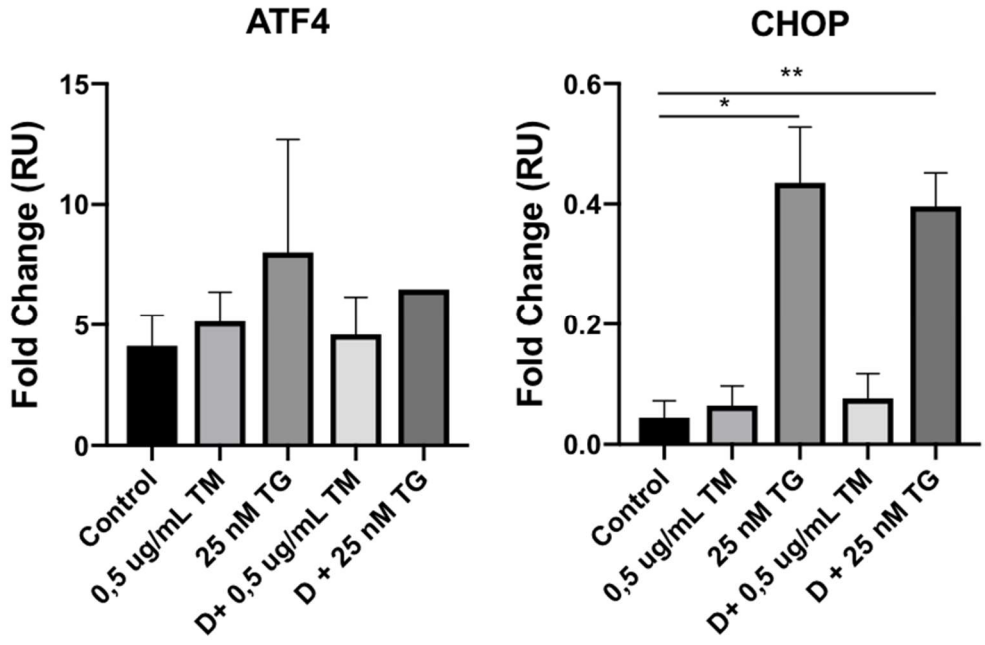


Figure 4.12. Effects of DADLE on expression of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax in HSCs subjected to ER stress. CD34+ HSCs were cultured in serum-free medium supplemented with STF in presence of 0.5 µg/mL Tunicamycin (TM) or 25 nM Thapsigargin (TG) with (300 nM) or without DADLE. After 7 days, cells were collected and proteins isolated. 25 ug protein was loaded onto gels and transferred to membranes. Membranes were then stained with an antibody against the anti-apoptotic protein Bcl-2 (27 kDa) or pro-apoptotic protein Bax (21 kDa). As control anti-b-actin (42 kDa was used).

To confirm induction of ER stress using TM and TG, gene expression of *ATF4* and *CHOP* was assessed after culture of HSCs in serum-free medium (Figure 4.13.).



4.13. DADLE decreases TG-induced ER stress in CD34+ cells. CD34+ HSCs were cultured in serum-free medium supplemented with STF in presence of 0.5 µg/mL Tunicamycin (TM) or 25 nM Thapsigargin (TG) with (300 nM) or without DADLE. After 7 days, cells were collected and total RNA isolated. Expression of ER stress-induced genes CHOP (left) and ATF4 (right) is shown. As a normalizer expression of *ACTB* was used. Data given are the average + SD of 3 different experiments, that were performed in triple. * $p<0.05$; ** $p<0.01$.

Treatment of CD34+ HSCs with a dose of 0.5 µg/mL TM resulted in the induction of minimal ER stress and no significant increase in the expression of neither *CHOP* or *ATF4*. However, treatment with 25 nM TG was very effective in induction of ER stress, with gene expression levels of *CHOP* being significantly increased ($p<0.05$). Although a similar increase in the expression of *ATF4* was observed, this increase was not significant. Treatment with DADLE resulted in a decrease of expression of both CHOP and ATF, albeit not to significant levels. Nevertheless, it supports the data above that DADLE does protect CD34+ HSCs from low levels of ER stress.

Increased expression of FOXO3 has also been associated with increased ER stress. However, induction of ER stress did not affect levels of genes involved in stem cell quiescence or proliferation (Figure 4.14).

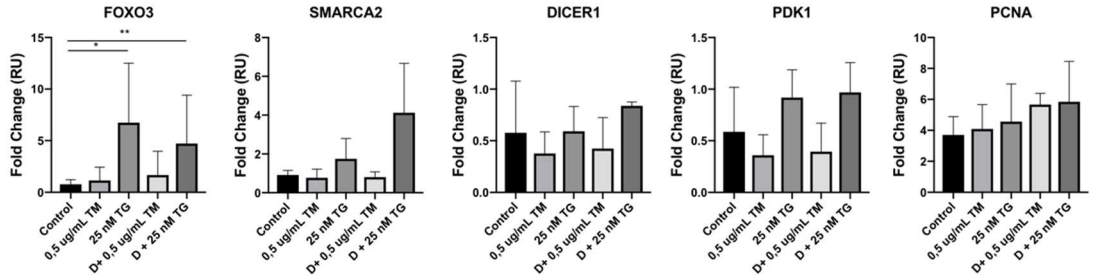


Figure 4.14. FOXO3 gene expression is increased in CD34+ HSCs subjected to ER stress. CD34+ HSCs were cultured in serum-free medium supplemented with STF in presence of 0.5 µg/mL Tunicamycin (TM) or 25 nM Thapsigargin (TG) with (300 nM) or without DADLE. After 7 days, cells were collected and total RNA isolated. Expression of the quiescent stem cell signature genes *FOXO3, SMARCA2, DICER1* and *PDK1* is shown. In addition, marker of proliferation *PCNA* gene expression was assessed. As a normalizer expression of *ACTB* was used. Data given are the average + SD of 3 different experiments, that were performed in triple. * $p<0.05$; ** $p<0.01$.

4.5. DADLE Exerts no Cytoprotective Effects on CD34+ HSCs Exposed to Hypothermic Stress

CD34+ cells were first culture expanded for 7 days in serum-free medium, supplemented with STF. DADLE was directly added at day 7 to the cultured CD34+ HSCs at concentrations of 30, 100 and 300 nM. The wells were sealed with parafilm in an airtight manner and stored at +4°C for 3, 7, 10 and 14 days without changing the medium. Following hypothermic storage, the parafilm was removed, the culture dishes of the cells were placed in a humidified incubator at 37°C and allowed to recover for 4 hours. After incubation, the proliferative status was analysed using WST-1 (Figure 4.15.).

Based on WST-1 evaluations, there was no significant cytoprotective effect of DADLE on CD34+ HSCs during hypothermic stress, independent of the dose of DADLE tested or the duration of the hypothermic storage conditions. When under the same culture conditions apoptosis percentages (with Annexin-V/Propidium Iodide staining) and cell cycle status (BrdU incorporation/7-AAD staining) were determined (Figure 4.16.), apoptosis levels were shown to reach a plateau after 1 week of hypothermia, with 90% apoptosis in each group, and 100% apoptosis-induced cell death after 11 days, independent of the culture conditions used.

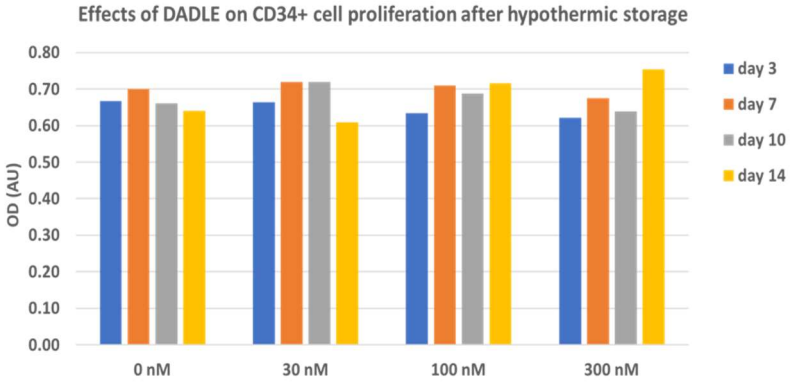


Figure 4.15. Effects of hypothermia on proliferation of CD34+ cells. CD34+ HSCs were cultured in serum-free medium containing STF for 7 days, after which 30, 100 or 300 nM DADLE was added to the wells and the plates were sealed airtight without any medium replacement. Plates were left at +4°C for 3, 7, 10 or 14 days. On the experimental days, the plates were removed and placed in the incubator. After 4 hours, 1:10 WST was added and the results were spectrophotometrically assessed (n=2).

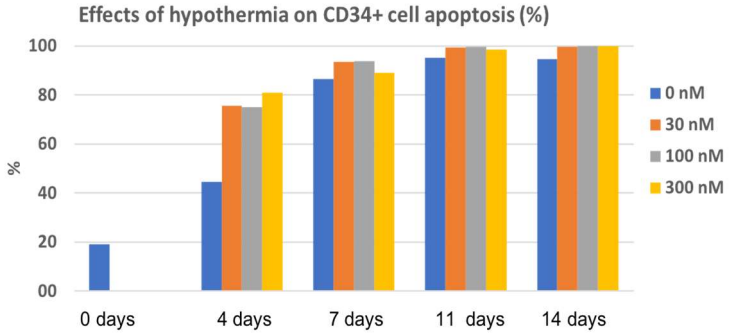


Figure 4.16. Effects of hypothermia on apoptosis of CD34+ cells. CD34+ HSCs were cultured in serum-free medium containing STF for 7 days, after which 30, 100 or 300 nM DADLE was added to the wells and the plates were sealed airtight without any medium replacement. Plates were left at +4°C for 3, 7, 10 or 14 days. On the experimental days, the plates were removed and placed in the incubator. After 4 hours, cells were stained with Ann-V/PI and evaluated for apoptosis (n=2).

When cell cycle analysis was performed of these cultures, up til day 7 initially cells appear to aim to protect cell viability by entering into hibernation (G_0/G_1 of the cell cycle), however, during prolonged hypothermia this mechanism is insufficient to protect cells and due to extensive apoptosis, cell cycle analysis of the few remaining cells could not be satisfactorily analysed (Figure 4.17.). Similarly, when the effects of

DADLE on ROS production by CD34+ cells was tested after 4-14 days of hypothermia, cell death was so extensive that only few cells remained for analysis (Figure 4.18.). However, no differences were observed between the DADLE-treated group and the control culture. Thus, DADLE appears not to have any protective effect on cell death induced by hypothermic stress.

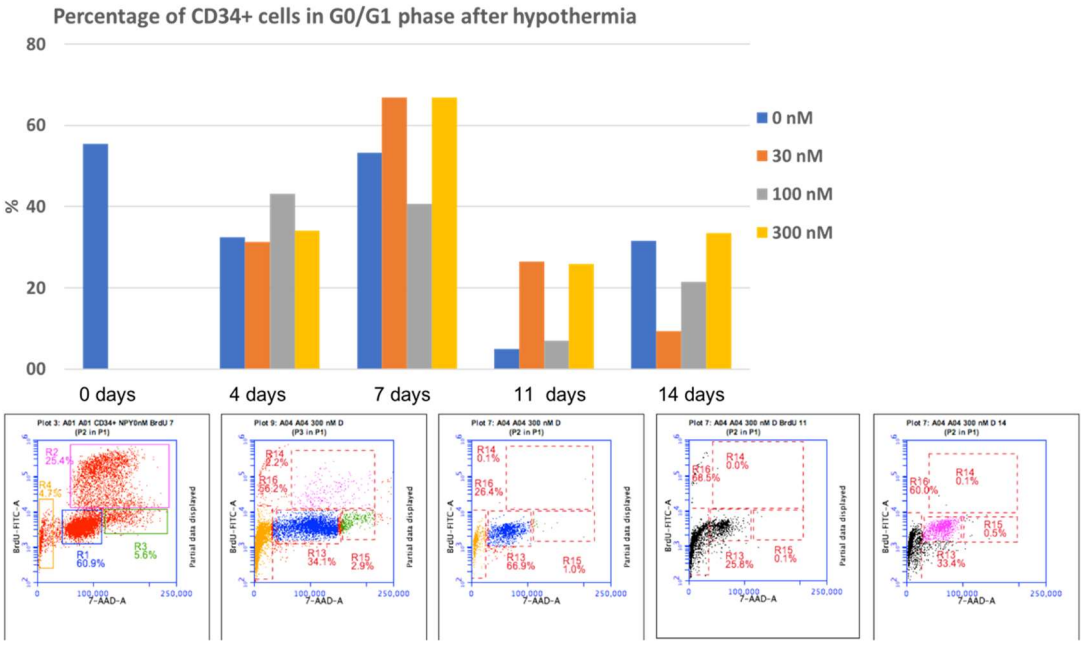


Figure 4.17. Effects of hypothermia on cell cycle of CD34+ cells. CD34+ HSCs were cultured in serum-free medium containing STF for 7 days, after which 30, 100 or 300 nM DADLE was added to the wells and the plates were sealed airtight without any medium replacement. Plates were left at +4ºC for 3, 7, 10 or 14 days. On the experimental days, the plates were removed and placed in the incubator. After 4 hours, BrdU was added to the cells and after 2 hours of incubation cells were stained with anti-BrdU-FITC and counterstained with the DNA dye 7-AAD (n=1).

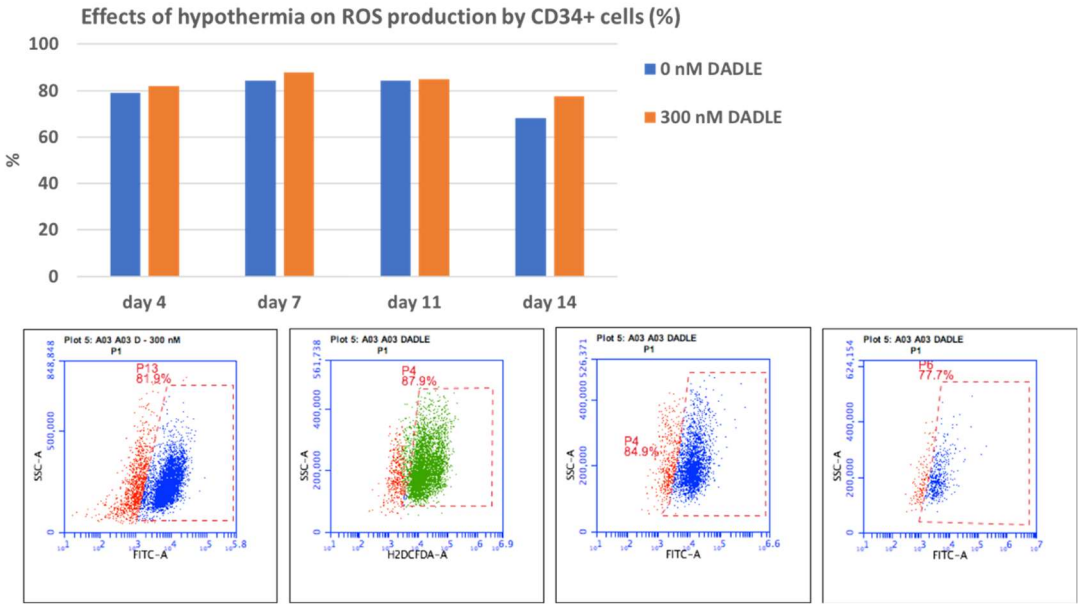


Figure 4.18. Effects of hypothermia on ROS production by CD34+ cells. CD34 + HSCs were cultured in serum-free medium containing STF for 7 days, after which 300 nM DADLE was added to the wells and the plates were sealed airtight. Plates were left at +4ºC for 3, 7, 10 or 14 days. On the experimental days, the plates were removed and placed in the incubator. Cells were incubated with H_2DCFDA, which in presence of ROS produces becomes fluorescent green (n=1).

5. DISCUSSION

Hematopoietic stem cells (HSCs) are among the most studied and widely used adult stem cells. They have the ability to self-renew and differentiate into all mature blood cells and, in the adult, can be obtained from BM, mobilized PB and UCB (8, 55). When HSCs are used for transplantation, they are often exposed to short-term cell culture and/or freezing procedures. These procedures are used for expansion, storage or transportation of HSC products until the patient is ready. However, HSCs can get damaged during these processes and survival/cell viability may be affected due to the use of cryoprotective chemicals required for freezing, such as DMSO, cell culture medium and physical conditions (56). Particularly reactive oxygen species (ROS), which may result from oxidative stress and endoplasmic reticulum (ER) stress during freezing/thawing, may occur during cell culture and can negatively affect the viability and function of HSCs (43). In addition, various bone marrow syndromes and niche disorders may be caused by oxidative stress and/or endoplasmic reticulum stress. DADLE has been tested for its efficacy during hypothermic conditions in several cell lines and whole organs and may be able to induce a similar reversible protection of HSCs. Therefore, we aimed within the scope of this master thesis project to investigate the protective effects of the synthetic opioid peptide DADLE on HSCs during *in vitro* culture on induced oxidative stress, ER stress and hypothermic conditions.

Opioids bind to G-protein mediated receptors and play an important role in cell survival, proliferation, migration and self-regeneration. The role of endogenous opioids and morphines in the regulation of the BM niche and control and maintenance of HSCs, has been relatively unappreciated. A recent review on the role of the neurological involvement in the regulation of the BM niche has highlighted that opioid peptides play a key role in the modulation of the immune system and function as cytokines (Aerts-Kaya *et al*, 2019). Opioid receptor expression has been detected on many hematopoietic cells, including DOR expression by T-cells, B-cells, macrophages and BM dendritic cells (DCs) and BM-MSCs (62, 63); KOR expression by lymphocytes, PB-CD34+ cells (64), BM neutrophils (65), BM stromal cells BM macrophages (66, 67); and MOR expression by lymphocyte subsets,

monocytes/macrophages, granulocytes (68) and PB-CD34+ (64) and UCB-CD34+ cells (69). Agonists of KOR, MOR and DOR were shown to inhibit chemotaxis of BM neutrophils (65) and enkephalins themselves have been shown to display chemotactic properties on DOR+ dendritic cells (62). Agonists of KOR increased numbers of colony forming unit-granulocyte/macrophage (CFU-GM) in synergy with GM-CSF (67), whereas DOR agonists decreased numbers of CFU-GM) (70). Mice subjected to stress induced by immobilization, were shown to develop BM hyperplasia, which could be completely prevented by injection of DOR agonists (71). Furthermore, MOR-deficient mice displayed increased proliferation of hematopoietic progenitor cells in both BM and spleen (72).

D-Ala2, D-Leu5 Enkephalin (DADLE), the synthetic delta opioid peptide ligand of DOR has been shown to stimulate mechanisms of cell repair and prevents cell death due to ischemia (2, 3). DADLE has also been found to prolong survival of organs stored under hypothermic conditions, such as lungs, heart, liver and kidneys and used for transplant purposes (4). DADLE was found to preserve the viability of cultered primary mouse fetal dopaminergic neuronal cells in a dose-dependent manner and transplantation of DADLE-treated dopaminergic cells into the brain of Parkinson's rats resulting in a two-fold increase. Similarly, DADLE reverses and blocks dopaminergic terminal damage by inducing methamphetamine (meth) (35). Addition of DADLE to *in vitro* cultures of PC12 neuronal cells, was shown to completely precent cell death caused by serum deprivation. DADLE, via activation of DOR, was shown to protect neurons in a dose-dependent way from neuronal damage caused by hypoxia and hypoxia like oxygen–glucose deprivation (OGD) (5). The effects of DADLE were shown to be mediated by increases in ERK phosphorylation and the stimulation of p38 phosphorylation and could be blocked by the DOR antagonist Naltrindole (33, 38). Therefore, DADLE has been proposed as a novel neuroprotective, therapeutic protective agent in ischemic and hypothermic conditions.

Prolonged culture or storage of HSCs, may affect the quality of the cells as a result of serum starvation, relative ischemia, hypoxia, hypothermia and adverse physical conditions, related to cell culture, thus affecting homing of the HSCs to the BM and subsequent engraftment. Therefore, it is very important to develop new strategies to increase the survival rate of cultured or frozen/thawed HSCs. Here, we

aimed to show the protective effects of DADLE on HSCs, subjected to a number of stress-inducing conditions. The presence of DOR on HSCs was not previously known. Although we used both flow cytometry and immunofluorescent staining of CD34+ HSCs on slides, we could only detect marginal expression of DOR by HSCs. However, DOR was in line with the literature, highly expressed by mature hematopoietic cells (73). Nevertheless, the absence of DOR on HSCs does not mean that DADLE is ineffective. It has been previously shown that DADLE can rapidly diffuse through the cell and nuclear membrane and directly bind to perichromatin fibrils, which represent the structural counterpart of pre-mRNA transcription and early splicing (74). It was therefore suggested that DADLE might interfere directly with nuclear transcription and regulate other cell functions, such as protein synthesis and cellular quiescence (75).

When we assessed the effect of DADLE on oxidative stress, we found that at a dose of 300 nM DADLE was shown to exhibit a protective effect on CD34+ cells after treatment with 100 and 300 μM H_2O_2. Addition of DADLE resulted in increased proliferation of CD34+ HSCs and a small reduction in apoptosis. When ROS levels were assessed, we found that DADLE was able to decrease ROS production in CD34+ HSCs after treatment with H_2O_2 thereby decreasing apoptosis and increasing cell viability.

Assessment of the protective effects of DADLE on CD34+ HSCs against ER stress, were tested using the inducers of ER stress Tunicamycin (TM) and Thapsigargin (TG) (76). The effects of TM and TG on CD34+ HSCs were assessed at a range of doses in order to find a dose sufficient to induce reversible levels of ER stress. Using WST-1 proliferation assays and Annexin-V, we showed that a dose of 25 nM TG induced considerable ER stress, since total Annexin-V positive cell numbers increased from 10% to 50%. Induction of ER stress was confirmed using RT-PCR and showed an increase in gene expression levels of the ER stress-related genes *CHOP* and *ATF4*. Addition of DADLE supported proliferation and decreased apoptosis of CD34+ HSCs under low level ER stress induced by TM and TG. When the levels of anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax were measured using Western Blot, a small decrease in both Bcl-2 and Bax expression was observed in cells

treated with 25 nM TG, but no effect on levels of these proteins in cells treated with TM. However, we couldn't isolate sufficient protein levels from wells treated with TG+DADLE and therefore we cannot conclude that DADLE would have had an effect here. Further experiments will be needed using at least two fold more cells to assess the effect of DADLE in this setting. Nevertheless, whenever doses of TM exceeded 0.5 μg/mL or doses of TG exceeded 25 nM, DADLE was not sufficient by itself to prevent the cytotoxic effects of ER stress.

DADLE has previously been shown to be able to induce hibernation in certain animals, as well as activate a pattern similar to hibernation in several stem cells, including neuronal stem cells (77, 78). In addition, DADLE has been shown to have an important protective effect on hypothermia. Some of the protective effects of DADLE on hypothermia have been suggested to be mediated by induction of hibernation (79). Therefore, we decided to also assess the effects of DADLE on hibernation of HSCs. HSCs were treated for 7 consecutive days with ER stress inducing chemicals TM and TG in the presence or absence of DADLE. Comparison of microarray data from quiescent HSCs, satellite muscle stem cells and hair follicle stem cells revealed a common quiescent stem cell transcriptome signature (80). Based on this paper, we chose a range of genes that represented this quiescent stem cell signature, including *FOXO3, DICER1, SMARCA2* and *PDK1*. These genes were all found to be significantly upregulated in quiescent cells. In addition the *PCNA* gene was found to be significantly downregulated in quiescent cells. When we assessed expression of these genes in CD34+ HSCs after treatment with TM and TG, with or without DADLE, we found a significantly increased expression of the transcription factor *FOXO3*.

Forkhead box (FOXO) proteins are a family of transcription factors that control the expression of genes effective in apoptosis, cell growth, development, proliferation and survival. The main defining feature of FOXO proteins is the forkhead box region, which can bind to the DNAs of target genes, also known as the winged helix of 80-100 amino acids. FOXO3 expression is upregulated in quiescent stem cells and is involved in transcriptional regulation and stem cell fate decisions and controls HSC quiescence by regulating ROS levels (5,9). In addition, FOXO3a was shown to protect

quiescent cells from oxidative stress (81). In proliferating cells, protection from cell death is mediated by activity of the PI(3)K/PKB signalling pathway, which is dependent on the presence of glucose (82). In the absence of PI(3)K/PKB signalling, FOXO3a is activated, which (83-85) in most cell types, leads to cell-cycle arrest and quiescence, but not apoptosis. Molecularly, the UPR is characterized by the activation of one or more of the three ER transmembrane sensors, known as the pancreatic ER kinase (PKR)-like ER kinase (PERK), the inositol-requiring enzyme 1 (IRE1), and the activating transcription factor 6 (ATF6) . It was previously shown that PERK can act directly on FOXO to increase FOXO activity and AKT activation (86). Activation of PERK by the UPR results downstream in an increase in the levels of ATF4, which functions as a transcription factor, increasing levels of CHOP (87). Here, we showed that treatment of CD34+ HSCs with TG resulted in the induction of ER stress and activation of the PERK pathway, resulting in an increase in *FOXO3*, *CHOP* and *ATF4* expression. Treatment with DADLE resulted in a decrease of *CHOP* and *ATF4* expression, as well as a decrease in *FOXO3*. Therefore, we can conclude that DADLE supports viability of CD34+ HSCs and protects these cells through modulation of the PERK UPR pathway.

Despite its previously published effects on protection of several stem cells and cell lines from hypothermic stress, in our experiments DADLE did not exert any signficiant cytoprotective effects on CD34+ HSCs exposed to hypothermic stress. This may have been related to the extreme sensitivity of HSCs to hypothermic conditions, but may have also had to do with the fact that HSCs did not express DOR. In the hypothermia experiments, CD34+ cells were first culture expanded for 7 days in serum-free medium, supplemented with STF. DADLE was directly added into the wells at day 7 to the cultured CD34+ HSCs without further medium changes and cells were stored at 4^{o}C for up to 14 days. There is no current information on the half-life of DADLE *in vivo* or in culture conditions. However, other opioids have been shown to have half-lifes ranging from 30 minutes up to 55 hours. In the hypothermia experiments, we tried to minimally affect conditions by not changing any media and storing the cells in an air-tight modus. This may have affected the effects of DADLE and it is conceivable that the activity of DADLE may not have been sufficiently long to test its effect in the hypothermic setting. Extensive cell death in all culture

conditions, with or without DADLE, prevented a good assessment of cell cycle analysis and ROS production. Therefore, in order to get a better picture of the working mechanisms of DADLE, it is important to measure metabolic activity of the HSCs, use larger numbers of HSCs, modify the culture and storage protocol, change doses of DADLE tested and/or test the effects of DADLE in combination with other cytoprotective agents.

In conclusion, DADLE has been shown to protect many different cell types after ischemia/reperfusion and support viability of these cells under hypothermic conditions. However, in the framework of this thesis we found supportive evidence that DADLE may protect HSCs from different stress conditions, including oxidative stress and ER stress, but not from hypothermia-induced stress.

6. CONCLUSIONS

When HSCs are used for transplantation, they are often exposed to short-term cell culture and/or freezing procedures. However, HSCs can get damaged during these processes and survival/cell viability may be affected due to the use of cryoprotective chemicals required for freezing, such as DMSO, cell culture medium and physical conditions. Particularly reactive oxygen species (ROS), which may result from oxidative stress and endoplasmic reticulum (ER) stress may occur during cell culture and can negatively affect the viability and function of HSCs.

DADLE has been tested for its efficacy during hypothermic conditions in several cell lines and whole organs and may be able to induce a similar reversible protection of HSCs. Therefore, we aimed within the scope of this master thesis project to investigate the protective effects of the synthetic opioid peptide DADLE on HSCs during *in vitro* culture on induced oxidative stress, ER stress and hypothermic conditions.

Here, we found that 1) although CD34+ HSCs express low levels of DOR on their surface, they are still susceptible to its effects, most likely through direct diffusion through the cellular and nuclear membrane; 2) DADLE protects CD34+ HSCs from low level oxidative stress through lowering the levels of intracellular ROS; 3) the effects of TG-induced ER stress in HSCs are mediated through activation of the PERK pathway and downstream activation of the transcription factors *FOXO3*, *ATF4* and *CHOP*; 4) DADLE decreases expression of *FOXO3, ATF4* and *CHOP* by CD34+ HSCs and protects these cells from low level endoplasmic reticulum stress, as evident from an increase in viability and a decrease in apoptosis; 5) Under the tested conditions DADLE does not appear to provide a protective effect on CD34+ HSCs from the cytotoxic effects of prolonged hypothermia, however using different conditions or addition of other cytoprotective agents, the effect of DADLE maybe more pronounced.

Although DADLE showed significant effects on the protection of CD34+ HSCs against certain stress conditions, it is clear that the effects of DADLE as a single agent are not sufficient to provide full protection. Therefore the use of DADLE in

combination with other cytoprotective agents, which may function through different pathways may result in better effects.

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Mother Tongue: Azerbaijani (fluent)

Second Languagues: Turkish (fluent)

English (advanced)

Russian (advanced)

IV. Laboratory Experience and Interests

Cell Culture Techniques (Cell Culture, Cell Passage, Freezing and Thawing Techniques, Cell Isolation from Tissue and Organ, Mononuclear Cell Isolation, Hematopoietic Stem Cell and Mesenchymal Stromal Cell Cultures Methods, CD34+ Cell Selection Methods, Cell Proliferation Techniques, Coculture), Flow Cytometry, Western Blot Technique, Protein Isolation, Gel Electrophoresis, Elisa, qPCR, RNA Isolation, cDNA Synthesis, Immunofluorescence Staining Techniques, Transfection and Transduction Techniques, Lentivirus Production Techniques

V. Academic and Professional Activities

Publications

- Aerts-Kaya F, Ulum B, **Mammadova A**, Köse S, Aydın G, Korkusuz P, Uçkan-Çetinkaya D. Neurological regulation of the bone marrow niche. *Adv Exp Med Biol – Cell Biology and Translational Medicine.* 2019;25. (online 25.07.2019) DOI: https://doi.org/10.1007/5584_2019_398

- Baris Ulum, **Aynura Mammadova,** Özgür Özyüncü, Duygu Uçkan-Çetinkaya, Tülin Yanık, Fatima Aerts-Kaya. Neuropeptide Y is involved in the regulation of quiescence of Hematopoietic Stem Cells. *Submitted to Neuropeptides.*

Projects

- Assesment of the Role and Antioxidative and Antiapoptotic Effects of Delta-Opioid Peptide D-Ala2-Leu-5-Enkephalin on the Viability of Hematopoietic Stem Cells, Hacettepe University Scientific Research Projects Coordination Unit, Thesis Support Project, Project No: TYL-2018-17435.

- Assessment of optimization of hypothermic storage conditions of Hematopoietic Stem Cells, Supporting by: TUBİTAK, Project No: 118S738, TUBİTAK 1002, Execution Institution : Hacettepe University Graduate School of Health Science, Stem Cell Program, Ankara, Turkey

Conference Abstracts

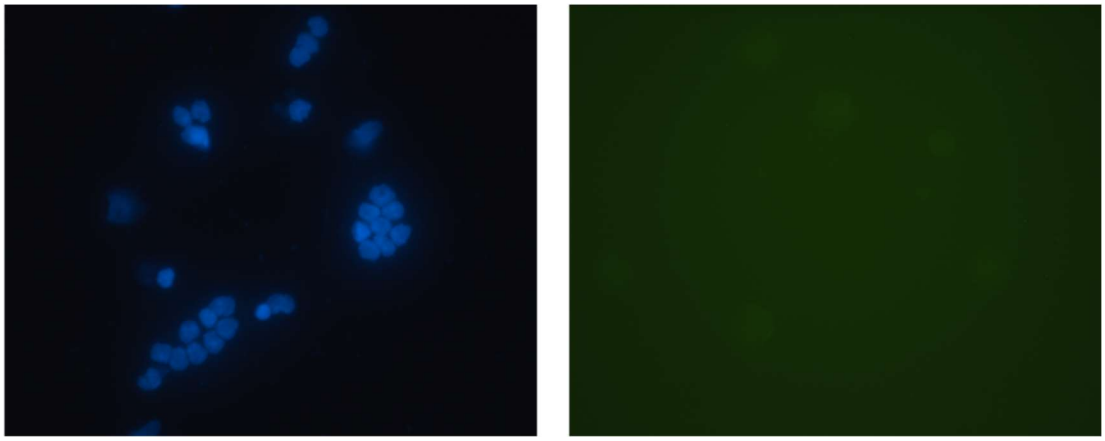
Aynura Mammadova, Barış Ulum, Özgür Özyüncü, Fatima Aerts Kaya. D-Ala2, D-Leu5, Enkephalin Protects Hematopoietic Stem Cells In Vitro Oxidative Stress and Endoplasmic Reticulum Stress. Abstract 3rd International Congress on Stem Cell and Cellular Therapies, Oral Presentation. Istanbul 2019. Abstract Book SS013, p66.

Baris Ulum, **Aynura Mammadova**, Özgür Özyüncü, Duygu Uçkan-Çetinkaya, Tülin Yanık, Fatima Aerts-Kaya. Investigation of the Role of Neuropeptide Y (NPY) in Hematopoietic Stem Cell Regulation. Abstract 3rd International Congress on Stem Cell and Cellular Therapies, short presentation. Istanbul 2019. Abstract book TS008, p137.

This thesis was supported by grants from the Hacettepe University Scientific Research Project Coordination Unit, no TYL-2018-17435 and the Scientific and Technological Research Council of Turkey, no 118S738.

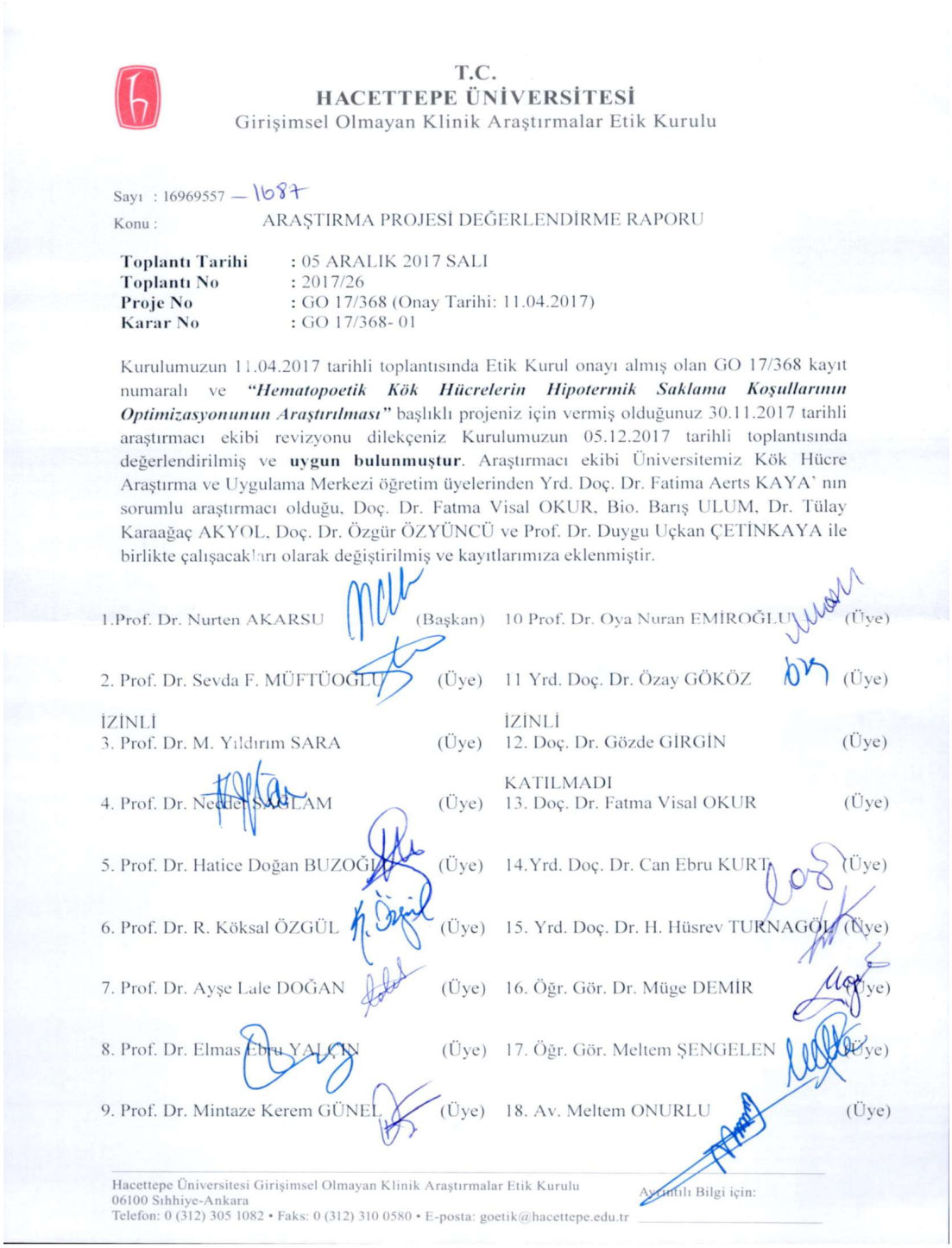
9. SUPPLEMENTS

Supplement 1.



Immunofluorescence images of CD34+ HSCs stained with DAPI and anti-DOR. CD34+ HSCs from the same sample were spun onto different slides and stained with a rabbit anti-DOR antibody and an anti-rabbit IgG-FITC antibody. Nuclei were counterstained with DAPI. Similar to the results from the flow cytometry tests, DOR staining was minimal in CD34+ cells.

Supplement 2.



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HACETTEPE ÜNİVERSİTESİ
Girişimsel Olmayan Klinik Araştırmalar Etik Kurulu

Sayı : 16969557 — 1687
Konu : ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi : 05 ARALIK 2017 SALI
Toplantı No : 2017/26
Proje No : GO 17/368 (Onay Tarihi: 11.04.2017)
Karar No : GO 17/368- 01

Kurulumuzun 11.04.2017 tarihli toplantısında Etik Kurul onayı almış olan GO 17/368 kayıt numaralı ve ***"Hematopoetik Kök Hücrelerin Hipotermik Saklama Koşullarının Optimizasyonunun Araştırılması"*** başlıklı projeniz için vermiş olduğunuz 30.11.2017 tarihli araştırmacı ekibi revizyonu dilekçeniz Kurulumuzun 05.12.2017 tarihli toplantısında değerlendirilmiş ve **uygun bulunmuştur**. Araştırmacı ekibi Üniversitemiz Kök Hücre Araştırma ve Uygulama Merkezi öğretim üyelerinden Yrd. Doç. Dr. Fatima Aerts KAYA' nın sorumlu araştırmacı olduğu, Doç. Dr. Fatma Visal OKUR, Bio. Barış ULUM, Dr. Tülay Karaağaç AKYOL, Doç. Dr. Özgür ÖZYÜNCÜ ve Prof. Dr. Duygu Uçkan ÇETİNKAYA ile birlikte çalışacakları olarak değiştirilmiş ve kayıtlarımıza eklenmiştir.

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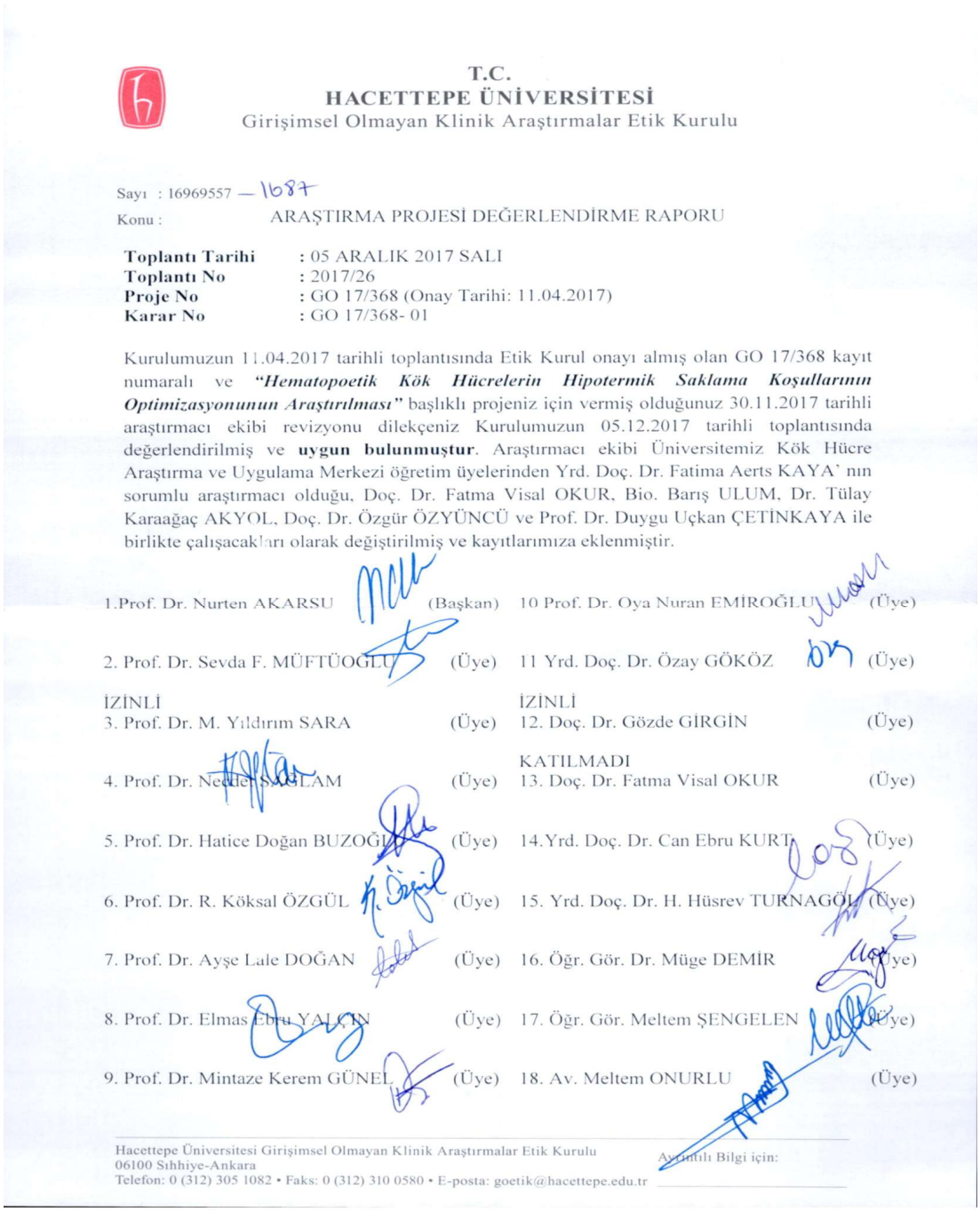

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