Approaches to Reverse Red Cell Sickling

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Medical Sciences - Laboratory Medicine and Pathology
University of Alberta

Abstract

There is evidence that the symptoms associated with sickle cell disease can be relieved by using plant extracts containing anthocyanin (AC) agents. It has been established that ACs were able to reverse sickle cell morphology to a biconcave disc shape. However, no specific form of AC has been investigated in detail. In this thesis, a purified manufactured compound, delphinidin, was selected for study due to its antioxidant property. This study hypothesized that delphinidin chloride (Delph-CL) can reverse the sickle cell morphology by inhibiting hemoglobin S (Hb S) polymerization through a hydration and/or antioxidation biomechanism. Furthermore, the effect of Delph-CL on membrane integrity, Hb S solubility, and O_2 binding were evaluated. Exposure to 100 μg/mL Delph-CL caused some sickle cells to transform into an elliptical shape ($P = 0.04$), possibly as a transition stage to discoid cells. Hb S polymerization also decreased which resulted in more O_2 binding and a low P_{50}. However, Delph-CL acted negatively on the red cell membrane by causing hemolysis. Neither one of the proposed biomechanisms were achieved and further work is necessary to establish the mechanism of sickle cell reversal.

Preface

This thesis is an original work by Asmaa Basonbul. The research project, of which this thesis is a part, received research ethics approval from Canadian Blood Services, Project Name "Investigating the Effect of Anthocyanins on Sickle Red Blood Cells" No. 2013.020, 2013/07/23 and the University of Alberta Research Ethics Board, Project Name "Investigating the Effect of Anthocyanins on Sickle Red Blood Cells" No. Pro00039981, 2013/08/01.

Acknowledgments

This project would have been impossible without many supportive and collaborative people.
First of all, I would like sincerely to thank my supervisor, Ms. Kristi Lew, for encouragement and extraordinary support through my program in her presence and in her away time.
Special thanks to my co-supervisor Dr. Jason Acker for having me in his lab and financial support. In addition, I appreciate his advice and impressive guidance through my degree.
Warm thanks to committee member, Dr. Rodrigo Onell, for his time and clinical advice regarding sickle cell patients.
Thanks to all of the sickle cell patients who believed in the research and agreed to participate in this study.
Great appreciation for Terri Uytterhagen in the apheresis unit at the University of Alberta hospital for giving me the chance to meet the sickle cell patients. The same as well for blood bank lab staff Suzanne Miller, Hilda Gaal, and Sheri Hudson-Tarnawsky for providing the sickle cell samples for this research.
I am so thankful for Dr. Acker's lab research assistants Adele Hansen, Anita Howell, April Xu, Jayme Kurach, and Tracey Turner for collaboration when I needed it. The same as well for lab members Abdulrahman Alshalani, Andrew Jordan, Dr. Beatriz Bicalho, Diana Yu, Kellar Klein, Luciana Da Silveira, and Ruqayyah Almizraq for sharing experiences.
Exclusive thanks are sent back home to my parents, brothers, and sister to pray for me all of the time. Also, for my husband's support in difficult times.
Finally, I would like to thank the Saudi Arabian Cultural Bureau for financial support in my graduate studies.

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ACs, anthocyanins

Au, aurantinidin

Ca^{2+}, calcium

CBS, Canadian Blood Services

Cl^-, chloride

CO_2, carbon dioxide

Cy, cyanidin

Delph-CL, delphinindin chloride

DeoxyHb, deoxygenated Hb

DHA, docosahexaenoic acid

dH_2O, distilled water

Dp, delphinidin

2,3-DPG, diphosphoglycerate

EDTA, ethylenediaminetetraacetic acid

EI, elongation index

EIA/RIA, enzyme immunoassay/radio immunoassay

EI_{max}, maximum elongation index

EPA, eicosapentaenoic acid

Eu, europinidin

FDA, Food Drug Administration

Fe^{3+}, ferric

Fe^{2+}, ferrous

H, hydrogen

Hb, hemoglobin

Hb A, adult hemoglobin

Hb F, fetal hemoglobin

$HbFe(II)O_2$, oxygenated Hb

HbFe(IV)=O, deoxygenated Hb

Hb S, hemoglobin S

Hct, hematocrit

HDL, high density lipoprotein

$HNaO_4S$, anhydrous sodium bisulfate

HPLC, high performance liquid chromatography

HREB, Health Research Ethics Board

HSCT, hematopoietic stem cell transplants

Hu, hydroxyurea

H_2O, water

H_2O_2, hydrogen peroxide

K^+, potassium

Lt, luteolinidin

MCHC, mean cell hemoglobin concentration

MCV, mean cell volume

metHb, methemoglobin- HbFe(III)

MCF, median cell fragility

MPs, microparticles

Mv, malvidin

Na^+, sodium

NO, nitric oxide

OFT, osmotic fragility test

OxyHb, oxygenated Hb

O_2, oxygen

$O_2^{\bullet-}$, superoxide anion

OCH_3, methoxyl

$^{\bullet}OH$, hydroxyl radical

OH, hydroxyl group

PAL, phenylalanine ammonia-lyase

P Atm, pressure atmosphere

PBS, phosphate buffer saline

PCV, packed cell volume

Pg, pelargonidin

Pn, peonidin

PRBC, packed red blood cells

Pt, petunidin

PVP, polyvinylpyrrolidone

Pw, water vapor pressure

P_{50}, partial pressure

RBCs, red blood cells

RCE, red cell exchange

ROS, reactive oxygen species

Rs, rosinidin

RPM, rotations per minute

SCA, sickle cell anemia

SCD, sickle cell disease

SMBS, sodium metabisulfite

WBCs, white blood cells

VCAM-1, vascular cell adhesion molecule-1

Vit C, vitamin C

1 Introduction

1.1 Sickle Cell Disease

The adult human body contains over 5 L of blood, which is composed of cellular and aqueous phases. Erythrocytes, leukocytes and platelets form the cellular phase (1), while the aqueous phase is comprised of plasma filled with organic molecules, proteins and salt. Each cell in the cellular phase is distinct in appearance and biological function. Erythrocytes, or red blood cells (RBCs), comprise the majority at 45 % (5×10^{12}/L) of whole blood (2). RBCs contain a tetramer polypeptide protein (globin) with 4 heme molecules called hemoglobin (Hb) (3). Hb is formed by one pair of globin chains located on the β-globin gene cluster and another pair on the α-globin gene on the short arm of chromosome 11 and 16, respectively (4, 5). These 2 pairs assemble as $\alpha_2\beta_2$ to produce adult Hb (Hb A), making up 96–98 % of total Hb (3).

When a single nucleotide substitution in the 6th position of the β-globin chain takes place, an abnormal Hb is created: Hb S. Delving further, the substitution involves the replacement of a glutamic amino acid to a valine amino acid (GAG → GTG) (6). This condition is a genetic disease known as sickle cell disease (SCD), which was first reported in 1910 when Herrick described a young Grenadian man with recurrent pain, anemia and sickle-shaped red corpuscles in his blood (7). The disease is common in Africa, the Mediterranean and South Asia (5).

Sickle cell disease is homozygous when the mutation occurs in both copies of the β-globin chains (Hb SS) and is heterozygous when the mutation occurs in one copy of the β-globin chain, leaving the other intact (Hb AS). Patients with Hb SS have moderate to severe symptoms and have short life spans. On the other hand, patients with Hb AS are asymptomatic. In addition, Hb S heterozygotes might present with another abnormal Hb genotype, such as Hb C, β-thalassemia, Hb E, hereditary persistence of fetal Hb (HPFP) or a rare abnormal Hb, such as Hb D Los Angeles, Hb G Philadelphia or Hb O Arab (6).

1.1.1 Sickle Cell Disease Pathophysiology

Deoxygenated Hb A, oxygenated Hb A and oxygenated HbS are extremely soluble, but when Hb S is deoxygenated, it is directly transformed into viscous and semisolid gel polymers. This is

a consequence of the hydrophobic bond that forms between valine and the nearby amino acids: alanine, phenylalanine and leucine in the β-globin chain (8). Therefore, the mean cell hemoglobin concentration (MCHC) of Hb S becomes increased and produces a high concentration of Hb at 380 g/L compared to the normal 320 g/L and involves polymer initiation. Polymer formation is delayed 100 seconds when intracellular Hb S is 200 g/L, though faster (10 milliseconds) at a concentration of 400 g/L (7). The polymerization of Hb S affects O_2 transport. The main function of Hb is to transport O_2 from the lungs to the tissues and return to the lungs with carbon dioxide (CO_2) from the tissues. Nonpolymerized Hb S has similar to normal Hb A-O_2 binding affinity and is characterized by allosteric properties, meaning that binding one O_2 molecule activates the binding of additional molecules to achieve the optimum oxygenated state with 4 molecules. However, when a polymer is formed, Hb S has difficulty binding to the O_2 molecule, resulting in red cell hypoxia. Therefore, higher partial pressure (P_{50}) is required for Hb S to be 50 % oxygenated at 35.4 mmHg as compared to normal Hb, which is only 26 mmHg at pH 7.4 and 37 $^{\circ}$C (5, 9). Moreover, other factors affect Hb S–O_2 binding: temperature, pH value and 2,3-diphosphoglycerate (2,3-DPG). Increasing the body temperature to 40 $^{\circ}$C will decrease O_2 binding with increased P_{50}. Changing the pH value influences Hb binding to O_2 in the lungs and its release to the tissues. The organic phosphate molecule 2,3-DPG, considered an intermediate product of the RBC glycolysis, favors the binding of deoxygenated Hb. Hence, polymerized Hb S deoxygenation most of the time will stimulate 2,3-DPG binding (10).

Depending on the rate of deoxygenation, Hb S polymerization promptly alters RBC morphology from a standard biconcave disc to various sickle cell shapes, as in Figure 1.1 (A). The sickle cell shapes are varied from: granular, holly leaf-shaped, classical sickle, and smoother irreversible sickle. The final shape is created based on the organization of the Hb S polymer domains, as in Figure 1.1 (B). Microscopically, the smoother, irreversible sickle cell is a classic diagnostic shape, and stimulates the disease pathogenesis. It is formed after long deoxygenating exposure with a high MCHC (7).

The sickle cell is characterized by poor deformability (7). RBC deformability is defined as "responding to applied forces by extensive changes of their shape, with the degree of deformation under a given force" (11). The biconcave disc shape of normal RBCs (8.4 μm in size and 88 fL in volume) is flexible and deformable. The flexibility of RBCs allows them to

pass through very small capillaries of only 3 μm diameter (2). The major effect on the deterioration of sickle cell deformability is MCHC or the Hb S polymer, followed by biconcave disc geometry. The dehydrated, rigid sickle cell is less deformable because of the elevated MCHC viscosity. Therefore, this leads to decreased cations and H_2O permeability for maintaining the cytoplasmic viscosity (11). This occurs through Ca^{2+} influx and K^+, Cl^- and H_2O efflux via the Ca^{2+}-activated K^+ (Gardos) and K–Cl^- co-transport channels (6, 7) as a result of the Ca^{2+}-dependent signal for maintaining cell volume, membrane composition and rheological properties (12). On the other hand, overhydrating also produces less deformable RBCs because it increases the volume without altering the surface area. Therefore, cell geometry is important, where the ideal deformability of normal RBCs maintains a constant cell volume with an expanding surface area (11).

In addition, Hb S has a particular instability characterized by a redox potential rate (auto-oxidation) of 40 %. The auto-oxidation process ends with oxidative stress radicals, or reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$), along with methemoglobin (metHb). Methemoglobin is the oxidized inactive form of Hb that carries iron atoms in ferric form (Fe^{3+}) instead of the normal ferrous form (Fe^{2+}) (7). Typically, H_2O_2 is converted to H_2O through 2 NADPH molecules in a pentose phosphate shunt via glutathione peroxidase by maintaining glutathione in its reduced form (4). Conversely, metHb is reduced to the active functional form by the metHb reductase enzyme in a Luebering–Rapoport shunt (3). However, in SCD, the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase and nitric oxide (NO) are too low to sufficiently scavenge ROS (13). Thus, ROS participates in SCD pathogenesis by accelerating hemolysis, bringing about hypercoagulability and endothelial dysfunction (14).

In terms of hemolysis, the sickle RBC life span is changed from the normal 120 days to only 10–20 days (6) as a consequence of ROS targeting the RBC membrane and injuring the membrane lipid bilayer via the lipid peroxidation mechanism (7, 14). Consequently, the integral membrane protein band 3, which binds to the spectrin/actin cytoskeleton, is affected by losing its bond with the ankyrin protein. Dislocation of the ankyrin protein leads to the release of the spectrin/actin cytoskeleton from the membrane in the form of vesicles known as RBC-derived microparticles (12). The microparticle is an intact vesicle ranging in size from 0.2–2 μm (15).

Releasing the microparticle leads to loss of the asymmetrical distribution of phospholipid across the lipid membrane bilayer and exposes phosphatidylserine (PS) to the RBC surface (16). The presence of PS acts as a biomarker to destroy the RBC in its pre-mature stage, which is one of the causes of the short life span of the sickle cell (12). In addition, the exposed PS leads to activation of the coagulation pathway, resulting in thrombin formation, depletion of anticoagulant factors and white blood cell (WBC) and platelet activation (15). Consequently, the blood cells are enhanced to adhere to the endothelial cells, causing endothelial dysfunction (6, 7). This process occurs with the participation of adhesion molecules such as intercellular adhesion molecule-4 (ICAM-4), an erythroid-specific membrane molecule with multiple ligands for leukocytes, platelets and endothelial cells. Moreover, CD47 binds to thrombospondin to begin an intracellular signal that specifically increases sickle cell adhesion (17).

Most hemolysis occurs intravascularly, with 30 g Hb lost daily (6). The lysed RBCs are removed through a complex route by one of the following mechanisms: erythrophagocytosis, fragmentation, trapping or osmotic lysis (7). Yet, free Hb remains present in the plasma to react with NO and produce nitrate. Increased hemolysis in patients with SCD results in the consumption of NO. Nitric oxide modulates vascular tone and elicits vasodilation. A severe drop in NO causes vasoconstriction and the accumulation of irreversible sickle cells, manifesting in a clinical symptom known as vaso-occlusion (5, 6, 18). However, metHb levels in a healthy individual comprise 3 % of the total Hb, although metHb formation is elevated in SCD at 1.7 times higher rate because of hemolysis (7, 14). Methemoglobin is not considered a gas transporter because it preferentially binds to H_2O instead of O_2 or CO_2 (19). Hence, a limited O_2 concentration is available to the tissues, causing hypoxia. Additionally, Hb binding to H_2O within the RBC in deoxygenated conditions induces sickling (13).

Therefore, the polymerized Hb causes deterioration in RBC morphology by creating an abnormal crescent-shaped sickle cell, which is characterized by rigidity, low water content and low deformability. In addition, the increased Hb viscosity impairs the RBC–O_2 transport function. Lastly, Hb polymerization increases the Hb oxidation rate, which causes cell membrane damage and hemolysis.

1.1.2 Symptoms of Sickle Cell Disease

The severity of the clinical course in SCD varies with the Hb genotype the patient carries (7). Patients with SCD suffer from various symptoms simultaneously and have reduced life expectancy, estimated to be around 48 years for women and 42 years for men (6). The following major symptoms are briefly outlined.

1.1.2.1 Hemolytic Anemia

A patient with homozygous SCD has severe hemolysis compared to patients with other hemoglobinopathies, such as β-thalassemia. The Hb level is stabilized in a steady state after the first 5 years from the beginning of disease history. On the other hand, the patient may episodically experience unexpected acute decreased Hb below the steady state level. This condition is known as acute exacerbation of anemia, which occurs as a consequence of hyperhemolysis, acute splenic sequestration and aplastic crises. In hyperhemolysis, the rate of RBC destruction increases due to unknown causes. However, there are 3 identifiable hemolysis causative factors: disease complication, following blood transfusion through alloantibodies and induced treatment. On the other hand, in terms of acute splenic sequestration, hemolysis occurs after spleen enlargement through intrasplenic accumulation of blood cells. In fact, this is one of the main explanations for early death in patients with SCD. The final cause of acute exacerbation of anemia is aplastic crisis. In this case, the bone marrow attempts to compensate for RBC destruction and low Hb levels by increasing erythropoiesis. As the erythropoiesis process is easily disrupted, severe anemia may result if the patient has parvovirus B19 infection or if inflammation develops (20).

1.1.2.2 Multi-organ Dysfunction

Sickle cell disease affects the major organs. The major cause of multi-organ dysfunction is vaso-occlusion of the large blood vessels (macrovascular) when vascular intimal hyperplasia takes place. Occlusion of large blood vessels supplying a major organ, such as the heart, gastrointestinal tract, liver, muscles, skin, brain, eyes, and lungs, will alter the blood flow rate and ultimately lead to the onset of organ damage (21). Table 1.1 describes the most common clinical manifestations of each affected organ (6, 20, 22).

1.1.2.3 Painful Episodes

Painful episodes are considered a predictor of early death in adult patients with SCD (23). The pain varies between acute, subacute, chronic and neuropathic. An acute pain episode occurs after tissue ischemia as a result of a vaso-occlusion crisis in the microcirculation (20, 21). The occlusion occurs in small blood vessels after endothelial cell activation, which enhances the adhesion of the poorly rheological sickle cells. Further, the WBCs are involved by increasing RBC aggregation and adhesion by releasing chemotactic substances. Therefore, there is a positive correlation between acute painful crisis and high WBC counts (21). On the other hand, chronic pain manifests with disease complications, as mentioned earlier. The characteristics and sharpness of the pain differ among patients because they depend on the location and severity of the tissue damage (7, 20). However, all affected tissues generally release inflammatory mediators that lead to the pain sensation (6).

1.2 Sickle Cell Disease Therapy

Treatment of SCD may increase patient longevity. Both herbal and non-herbal therapies have been used to treat patients with this incurable disease. Hydroxyurea (Hu), blood transfusions, stem cell transplantations, gene therapy, supplement agents and herbal extracts are examples of the treatments used for SCD (6). Here, the most commonly used herbal and non-herbal therapies are briefly highlighted.

1.2.1 Non-herbal

1.2.1.1 Hydroxyurea

Hydroxyurea (Hu) treatment was approved for use in adults patients with SCD in 1998 by the United States Food and Drug Administration (FDA), but has not been approved for children to date (6, 24). This treatment is considered successful for improving sickle cell deformability and reducing pain incidence and hospitalization (6, 25, 26). It functions according to the Hu mechanism theory, which is based on deoxyribonucleic acid (DNA) inhibition of S-phase cell cycle arrest. Specifically, Hu purportedly functions as a free radical targeting the ribonucleotide reductase enzyme, which is important in DNA synthesis. In addition, Hu is believed to act as a radiation sensitizer agent that impairs reparation of the DNA damage mechanism (27). The overall outcome boosts Hb F production, resulting in reduced intracellular concentrations of HbS

that consequently decrease polymerization (24). After induction treatment, the average proportion of Hb F in patients with SCD is around 8 %, and it can result in a significant increase in life span (28). Moreover, a high level of Hb F leads to increased O_2 binding affinity, where Hb F has a serine amino acid instead of histidine in position 143 on the γ chain, which is less tightly bound to 2,3-DPG (10). Furthermore, Hu increases NO production and decreases the expression of cell adhesion molecules such as vascular cell adhesion molecule-1(VCAM-1), which contribute to vaso-occlusion. Therefore, the incidence of organ dysfunction in these patients declines (6). For example, patients who receive Hu have better splenic functioning following a decrease in the number of Howell-Jolly bodies, RBCs with nuclear remnants usually appear after splenic atrophy as compared to the placebo group (3, 24, 28, 29). On the contrary, at a certain dose Hu treatment may affect hematopoiesis, resulting in cytopenias. In addition, it causes severe neutropenia, thrombocytopenia and reticulocytopenia, which increase the chances of bacteremia or sepsis, bleeding and anemia, respectively. Moreover, it potentially prompts skin dryness and hyperpigmentation. In male patients, sperm abnormalities may occur, including decreased sperm number, forward motility and percentage of living sperm, and abnormal morphology (6, 22, 24).

1.2.1.2 Blood Transfusion

Blood transfusion is an important part of SCD care for managing complications (30). It prevents stroke and reduces the incidence of acute chest syndrome, chronic renal failure, congestive heart failure, pulmonary hypertension and pain crisis (31-33). Fresh units of blood aged 5–7 days are favorable for preventing tissue hypoxia. High levels of Hb with 2,3-DPG encourage RBCs to release O_2 easily to the tissues (10). The blood unit must be negative for Hb S and leukoreduced to prevent human leukocyte antigen immunization and febrile nonhemolytic transfusion reactions. Further, proper phenotype matching is needed for RBCs, especially Rh, E, C and K antigens, to prevent alloimmune hemolysis (34). The reason is that around 20–30% of patients with SCD produce alloantibodies for E, C, K, D, S, Fy^a and JK^b antigens stemming from chronic blood transfusion (35, 36). It becomes more difficult to find compatible blood donors when a patient has formed alloantibodies (35). Long-term blood transfusion therapy, defined as ≥8 transfusions per year or one at least every 7 weeks, causes significant iron overload and organ dysfunction. Further, autopsies of patients with SCD have demonstrated iron deposition in the liver, spleen, kidney, and lymph nodes from chronic blood transfusion (31).

1.2.1.3 Red Cell Exchange

Red cell exchange (RCE) is "a procedure in which a machine removes a patient's abnormal RBCs using a centrifuge to separate the blood into its various parts. These abnormal cells are replaced with several RBC units from healthy volunteer blood donors" (37). The duration of the procedure varies with the amount of abnormal cells to be removed, but usually takes approximately 1–2 hours. RCE is a highly recommended treatment to manage vaso-occlusion in patients with SCD. The iron overload that usually accompanies simple blood transfusions and causes organ dysfunction is not present in RCE-treated patients. In addition, it is effective for reducing the number of erythrocyte- and platelet-derived microparticles (38). Monthly RCE for 6 months in a group of patients improved sickle cell deformability by increasing cell elasticity (39). RCE also decreases the number of circulating endothelial cells, involved in vaso-occlusive painful crisis, and decreases vascular bone necrosis, cerebrovascular accidents and unhealed leg ulcer outcomes (40). However, RCE requires twice as many packed RBC units versus a simple transfusion to decrease Hb S to < 30 % (41). For example, at King's College Hospital, London, it was demonstrated that blood usage increased from 15 % in 2000 to 19 % in 2009. Clearly, the average blood units used per patient increased from 11 units in 2000 to 21 units in 2009. The exchange was used to manage limited disease complications in 2000, such as stroke, end-stage renal failure and symptomatic anemia, but was broadly expanded in 2009 to include frequent acute pain, recalcitrant leg ulcers, renal impairment, anemia, hepatopathology, priapism and lung disease with pulmonary hypertension (25). RCE is also very expensive compared to simple transfusions, and the procedure should be performed every 3–5 weeks to maintain Hb S levels (41).

1.2.1.4 Stem Cell Transplantation

More than 72,000 people in the United States of America suffer from SCD, although only 500 have successfully undergone stem cell transplantation (42). Hematopoietic stem cell transplants (HSCT) can be obtained from the bone marrow, peripheral blood and cord blood. Bone marrow stem cells are collected from the posterior iliac crest using large-bore needles. On the other hand, peripheral blood stem cells are collected using an apheresis machine through an intravenous line. Similar to blood transfusion, the transplantation procedure involves simply transfusing the stem cells to the recipient patient. The selection of the stem cell source for treatment is based on availability and graft rejection risk (43). Increased erythropoiesis and improvement in the

function of affected organs such as the central nervous system and lungs are a sign of cure. However, patients may develop complications such as hypertension, vasculopathy-driven hemorrhagic or ischemic strokes and progressive renal or pulmonary dysfunction (42). HSCT is limited to patients who meet specific criteria in terms of age, health status and donor matching, among others. It is mostly recommended for children who have a high risk of disease complications, such as stroke, and multiple episodes of acute chest syndrome or recurrent vaso-occlusion crises. Yet, there is potential for side effects such as graft rejection and infection (44). To prevent graft rejection, a suitable human leukocyte antigen matched sibling donor is recommended; however, less than 14 % of patients are matched and have a chance for transplantation (42). In addition, prior to HSCT sickle patients receive a high dose of chemotherapy, such as cyclophsphamide and busulfan to suppress their own bone marrow and prevent graft rejection (45). However, there are significant risks in with myeloablative conditioning, including graft versus host disease and irreversible pancytopenia (46).

1.2.1.5 Gene Therapy

Gene therapy was widely explored during the mid-1990s when human immunodeficiency virus-1 (HIV-1)-based lentiviral vectors were utilized as carriers to introduce a functional copy of the β-globin gene. Adding a new gene to β-globin via the above mentioned vector is a sensitive process. Only the mutant point in the β-globin protein should be changed without increasing the production of the total amount of Hb (normal Hb A and sickle Hb S) (47). Successful gene intervention was achieved in a human sickle cell line by "a vector that combined a γ-globin gene with a small hairpin RNA targeting the sickle β-globin messenger RNA (mRNA)" (47). The sickle cell was recovered after increasing Hb F and decreasing sickle β-chain synthesis (47, 48). Moreover, it was also accomplished in mouse models by improving the anti-sickling effect of the β S-Antilles-D Punjab phenotype (47, 49). However, no clinical studies on patients with SCD have been reported yet. The main concern is obtaining different SCD phenotypes similar to α-thalassemia due to the increased level of β-globin chains exceeding that of α-globin chains. Currently, the change in disease phenotypes is restored when the lentivirus is combined with ankyrin T9W. Consequently, the "sickling vs. functional Hb proportion is modified without changing Hb content" (47). However, uncontrollable gene addition outcome causes gene replication or cell cycle alteration, which result may in cancer.

1.2.1.6 Supplementation

Patients with SCD take supplementary vitamins, antioxidant agents, iron chelation agents, and anti-adhesion agents to improve their quality of life. Patients who take fish oil supplements containing omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid have reduced clinical vaso-occlusive events, severe anemia, and blood transfusions (50). In addition, 100 mg/day vitamin E (α-tocopherol) for 6 weeks enhances packed cell volume (PCV), Hb concentration, Hb F percentage and resistance of the cell to lysis in children with SCD. On the other hand, it diminishes MCHC and the percentage of irreversible sickle cells (51). The use of a cocktail of antioxidant therapies was first attempted in 2000 (52). This treatment consists of lipophilic vitamin E (1200 IU), hydrophilic vitamin C, aged garlic extract and natural products. Aged garlic extract contains thioallyl, phenolic and flavonoids that have been demonstrated to have considerable antioxidant activity. Applying this cocktail therapy to a small group of patients with SCD improved hematocrit (Hct) values and reduced the occurrence of sickle cell crises. Incidentally, the Hct levels were much better than that obtained after using Hu treatment. Lastly, oral iron chelators prevent iron overload, the result of multiple and long-term blood transfusion. Desferrioxamine and deferasirox are the most popular iron chelators in use. The mechanism of desferrioxamine involves increasing iron excretion through the urine and bile ducts by interacting with hepatocellular and extracellular iron. The drug should be administered subcutaneously or intravenously for 8–12 hours/day for 5–7 days a week to achieve a powerful effect. It is not suitable for young patients who have lower body iron stores because it may result in serious neurotoxicity, cartilage formation abnormalities and other serious adverse effects. Deferasirox is the most recent treatment approved by the FDA, and is safe and effective for all age groups, even 2 years old children (6, 31, 53).

1.2.2 Herbal

Herbal therapy, or phytotherapy, is “a medicinal derived from a plant source” (54). It was initially discovered by the African populations, in which the disease is widely distributed, as seen in Figure 1.2 (5, 55). The limited sources of treatment in this poor population spurred their interest to further explore this form of treatment. They found that medicinal plants are cheap and easy to obtain from markets, especially in Kinshasa, the Democratic Republic of Congo, where 2% of the population suffers from SCD. Historically, the population in this area has used 18

different plant species to manage SCD (55). They found that it reduced the disease crisis with no adverse effects reported (32), which provided the clue that phytotherapy could be an alternative treatment for SCD that is natural and safe to use (54).

Phytotherapy was scientifically evaluated initially by Abu et al. in 1981 after analyzing the root bark aqueous extract of *Fagara xanthoxyloides* plant by column chromatography (56). Investigation of the aqueous extract demonstrated that all but one fraction contained metabisulfite, which induced sickling *in vitro* in Hb S homozygous RBCs; the remaining fraction contained alkaloids that actually induced an anti-sickling effect (54, 56). Since then, the African population has used a number of methods to obtain plant extracts, such as decoction and maceration, while sometimes utilizing crude consumption. People in the Democratic Republic of Congo use *Centella asiatica*, *Thomandersia hensii* and *Ricinodendron heudelotii* plant extracts to manage SCD. Chemical screening has indicated a high normalization effect on sickle cell morphology at a rate of 75 % (55). The source of sickle cell reversing therapy has been broadly explored through fruit extracts. Alkaline extraction of papaya and alcohol extraction of crude avocado juice caused a significant drop in sickle cell numbers *in vitro* after 24 hour incubation (57). In-depth studies of herbal extracts mostly detected the presence of flavonoid and phenolic acid compounds, which are produced during photosynthesis (58).

In the case of SCD, the Niprisan drug is extracted from *Piper guineense* seeds, *Pterocarpus osun* stems, *Eugenia caryophyllum* fruit and *Sorghum bicolor* leaves. This treatment was first explored in 2010, and a significant, direct interaction with the Hb S molecule that inhibited polymerization was discovered (54). Using Niprisan treatment in patients with SCD (12 mg/kg) for 6 months was statistically significant for reducing disease crises, pain severity, absenteeism from work and hospital admission frequency. It was also indicative of reduced frequency of leg ulcers, bone/joint pain and catarrh/sore throats. Niprisan was approved by the FDA because it is safe to use, and no serious adverse effects on the kidney or liver function in patients with SCD have been recorded (59). Ciklavit is also a drug produced from *Cajanus cajan* plant extract. It has been clinically shown to diminish painful crises from the anti-sickling effects, referring to the recovery of the abnormal crescent sickle cell to a normal biconcave disc morphology (57).

1.3 Phytotherapy

The photosynthesis mechanism takes places when a plant absorbs sunlight to produce a high level of O_2, flavonoids and phenolic acid compounds. The shikimic acid pathway is responsible for synthesizing flavonoids and phenolic acid compounds, as depicted in Figure 1.3. The first step is the condensation of erythrose-4-phosphate after its generation via the pentose phosphate pathway. Later, phenylalanine is produced via the shikimic acid pathway, and then catalyzed by the phenylalanine ammonia-lyase (PAL) enzyme, and the phenolic acid and flavonoid compounds are produced with microorganism support (58).

Many studies have investigated the biological activities of phenolic acids in upregulating bile secretion, reducing blood cholesterol and lipid levels and antimicrobial activity against various strains of bacteria, such as *Staphylococcus aureus* (58). The flavonoid compounds are divided into 6 subgroups, as shown in Figure 1.4 (58):

1. Flavones (luteonin, apigenin, tangeretin)
2. Flavonols (quercetin, kaempferol, myricetin, isorhamnetin, pachypodol)
3. Flavanones (hesteretin, naringenin, eriodictyol)
4. Flavan-3-ols (catechins and epicatechins)
5. Isoflavones (genistein, daidzein, glycitein)
6. Anthocyanidins (aglycons) or anthocyanins (glycoside) when bound to sugars such as glucose, galactose, rhamnose, xylose and arabinose at the 3- or 5-hydroxyl sites (60).

The famous subgroup is anthocyanidins, where 500 forms of aglycosides and 23 aglycons are known. However, the aglycoside–anthocyanin (AC) forms are more well represented in nature in 10 well-studied forms: cyanidin (Cy), peonidin (Pn), petunidin (Pt), delphinidin (Dp), malvidin (Mv), pelargonidin (Pg), rosinidin (Rs), europinidin (Eu), aurantinidin (Au) and luteolinidin (Lt) (61). Many studies have focused on ACs because of their beneficial health effects, for example, ACs inhibited proliferation of the human tumor cell line HT29 clone 19 A when black carrot extract was applied (62). Analysis of the extract revealed the presence of the Cy-3-glycoside AC form. In addition, aged red wine demonstrated a potential protective effect against oxidative stress in human RBCs by decreasing intracellular ROS and metHb concentrations (63). This

finding was a consequence of the Mv-3-*O*-glucoside, Cy-3-*O*-glucoside, Pn-3-*O*-glucoside and Dp-3-*O*-glucoside ACs (63, 64). Generally, most studies refer to the AC subgroup with regards to sickle cell reversal. The observed results report the reversal of the abnormal sickle cell morphology to normal biconcave discs (55, 65). *Justicia secunda* Vahl plant extract exerted a reversal effect due to the presence of alkaloids and polyphenols such as flavonoids, leuco-ACs and ACs (65). The authors stated that the reversal effect was caused by the AC extract, but no specific form was mentioned.

1.3.1 Anthocyanins

Anthocyanin is the Greek term for "blue flower", *anthos* meaning flower and *kianos* meaning blue (61). AC compounds form a flavonoid subgroup that contains 10 well-studied forms as previously mentioned (58, 66). All AC compounds share the same basic chemical structure, as portrayed in Figure 1.5. There are 2 aromatic C_6 rings (A and B) and one heterocyclic ring (C) that contains one O_2 atom. Anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or the flavylium ion (A and C rings) (60). Therefore, the difference in the AC form is the R group substitution by a hydroxyl group (OH), hydrogen atom (H) or methanol group (OCH_3). Table 1.2 defines the R group of each form.
Anthocyanins are responsible for the coloring of most flowers, fruits, vegetables and plants. In particular, the resonant structure of the flavylium ion stimulates the color, clarity and intensity (67). Anthocyanin colors vary from red to violet based on the pH value. The red color appears on those with more OCH_3 groups, while AC compounds with more OH groups appear blue/violet (66). In addition, ACs are unstable and are partially degraded by internal and external factors, such as light, temperature, pH, O_2, metal ions, coexisting sugars and concentration and presence of specific enzymes and proteins. In addition, the number of OH and OCH_3 groups affects AC chemical behavior. The presence of OH groups decreases the stability of the pigment excessively compared to compounds with more OCH_3 groups (61, 66).

1.3.2 Sources

Anthocyanin agents can be extracted from flowers, fruits, vegetables, plants and even commercial beverages. An AC forms a blue color in *Heliophila coronopifolia* flowers and a red color in *Rosa hybrid* (66). In fruits, AC agents are present in avocado, orange, papaya, blueberry, bilberry, grape and apple, among others (57, 61, 68). Fruit freshness, storage temperature,

transportation or shipment and light exposure are factors affecting AC concentration content in these fruits. Anthocyanin stability was tested after fresh blueberries were shipped from Poland and Uruguay to Germany. The results suggested the locally cultivated German blueberries had the highest AC concentration at 2761 mg/100 g dry weight (DW) as compared to the blueberries from Poland (2242 mg/100 g DW) and Uruguay (1570 mg/100 g DW) (68). In vegetables, AC compounds are found as constituents of black carrot, red and green cabbage, radish, tomato, potato, spinach and paprika (61, 62, 66). However, the most abundant source of AC agents is in the plant, some of which are more well-known than others. Anthocyanin compounds can be extracted from seeds, stems, leaves and roots. Many studies have investigated AC agents from the following plants: *Euphorbiaceae*, *Zanthoxylum* (*Fagara*) *zanthoxyloides*, *C. cajan*, *P. guineense*, and *J. secunda* Vahl (54, 55, 65). Lastly, various concentrations of AC agents are found in beverages prepared from the raw material of fresh fruits, such as whiskey, sake, Jerez sherries, cava, and Chilean cabernet sauvignon red wine (66). Conversely, the AC content in commercial juices depends on the percentage of fresh juice used in the integrand. The blueberry Nectar juice has 386 mg/L AC when it contains 70 % fresh blueberry juice; however, the AC concentration is decreases to 258 mg/L when only 40 % fresh blueberry juice is used (68).

Sickle cell disease is a genetic disease found worldwide, with high prevalence in Africa, the Mediterranean and South Asia, as seen in Figure 1.2 (5). Around 80 % of African children with sickle cell disease die before the age of 5 years because of poor medical care (55). One in 600 African-Americans is born with homozygous β-globin chain mutation (i.e. Hb S, C and E) and 1 in 400 African-Americans have heterozygous status (7). Although there are many ways to manage SCD, as described earlier in detail, i.e., Hu treatment, blood transfusion, RCE, stem cell transplant and gene therapy, each has its disadvantages and risks. Using phytotherapy as an alternative treatment in the future could be possible, although it is still under investigation.

This study will examine sickle cell reversal and elucidate the biomechanisms involved by focusing on the Dp AC form. This form of AC was selected because it can easily be obtained from bilberry fruit; specifically, the *Vaccinium myrtillus* L. species, in which Dp-3-*O*-galactoside and Dp-3-*O*-glucoside are present at concentrations of 1060 and 1247 mg/100 g, respectively (68). Moreover, Dp-3-*O*-β-glucosides decreased the blood pressure in patients with hypertension and increased high-density lipoprotein (HDL) cholesterol (69). Dp is one of only 3 ACs that does

not have a methylene group in its chemical structure. Six -OH groups are attached, which creates a high polarity compound and strong antioxidant. In addition, it is found widely in nature with 80% in leaves, 69% in fruits and 50% in flowers (61). Utilizing Dp specifically with sickle cells has not yet been demonstrated. This study used a highly purified (≥ 95 %) high-performance liquid chromatography (HPLC)-manufactured Dp chloride (Delph-CL), as shown in Figure 1.6. A manufactured source was chosen to avoid the time consuming extraction process and phytochemical screening for active ingredients from a plant source.

1.4 Hypothesis

This study hypothesizes that Delph-CL can reverse abnormal sickle cell morphology to a normal biconcave disc by inhibiting Hb S polymerization. Two specific biomechanisms are believed to achieve sickle cell reversal:

First: A hydration mechanism by increasing K^+, Cl^- and water uptake and decreasing Ca^{2+} influx through Delph-CL blocking/inhibiting the Gardos channel. To determine sickle cell hydration, the extracellular K^+ electrolyte concentration is measured after sickle cells are incubated with Delph-CL.

Second: An antioxidation mechanism by scavenging or accepting the free radicals of ROS. The free radical is attached to an unpaired electron of the O_2 atom in the heterocyclic ring of the AC chemical structure. Subsequently, a neutralization state is obtained. Decreasing the ROS halts the production of the oxidized metHb (Hb Fe^{3+}) form. In this study, Hb S oxidation is monitored by measuring the metHb levels before and after the application of an oxidative stress agent (i.e., H_2O_2) to determine whether Delph-CL functions as an antioxidant and decreases metHb.

With one or both of these mechanisms increasing Hb S solubility, the following outcomes may be obtained:

A. Recovering sickle cell morphology to a normal biconcave disc
B. Enhancing Hb–O_2 binding affinity
C. Increasing sickle cell deformability
D. Improving sickle cell membrane elasticity and decreasing hemolysis

1.5 Objectives and Study Design

1.5.1 Determining the Concentration Efficacy for Sickle Cell Reversal

Prior to examining sickle cell reversal, the Delph-CL toxicity will be evaluated by measuring hemolysis using the standard Drabkin's method. Later on, the sickle cell morphology reversal will be assessed microscopically using the Emmel test (65). Sickle cells treated with Delph-CL will be incubated with a reducing agent (2 % sodium metabisulfite [SMBS]) in deoxygenated conditions at 37 °C for 24 hours to determine whether there is a potential effect of sickle cell reversal. At the end point, the percentages of sickle/reversed cells in 3 different fields will be calculated. The results compare the percentage of each cell type at each Delph-CL concentration. For numerical data, the mean cell volume (MCV) of treated samples will be evaluated using a Beckman Coulter ACT 8 unit before and after the 24 hours incubation. The dose(s) that reverse the sickle cells to a normal biconcave disc shape effectively will be used for further investigation.

1.5.2 Demonstrating the Effect of Delphinidin Chloride on Sickle Cell Membrane Integrity, Deformability, Hb Solubility, and O_2 Binding

This objective is to identify the effect of Delph-CL on sickle cell impairment. Initially the weakness of membrane integrity will be examined by using the osmotic fragility test (OFT). This test evaluates the ability of sickle cells to adapt to hypotonic saline solutions (NaCl 9–0 g/L) without lysing. The percentage of hemolysis in each concentration will be calculated after measuring the optical density spectrophotometrically. Decreasing hemolysis will indicate that Delph-CL decreases the sickle cell membrane fragility.

For deformability, sickle cell elasticity will be measured by determining the elongation index (EI) via ektacytometry after the application of 0.95–30 Pa shear stress at 37˚C. The maximum EI (EI_{max}) will be considered to ensure that Delph-CL improves sickle cell deformability. The increased EI_{max} of treated sickle cells as compared to normal RBCs is a consequence of elasticity.

Hb solubility and O_2 binding will be assessed to determine the ability of Delph-CL to approach Hb S. The absorbance of Hb solubility will be measured spectrophotometry after exposed from RBC to the extracellular through surfactant. The released Hb is reduced with SMBS. Increased Hb absorbance following Delph-CL treatment may be an effect of inhibited Hb S polymerization and increased solubility. Obtaining a soluble Hb will enhance Hb–O_2 binding, which will be assessed using a Hemox analyzer.

1.5.3 The Biomechanism of Delphinidin Chloride on Sickle Cell Reversal

If Delph-CL reverses the sickle cell morphology and/or improves the sickle cell impairment, the biomechanism will be explored. The hydration and antioxidant biomechanisms are hypothesized in Section 1.4. The two biomechanisms will be evaluated after 24 hours incubation with Delph-CL at 37°C. The hydration biomechanism will be determined by measuring the concentration of extracellular K^+ using a Beckman Coulter analyzer. A decrease in K^+ concentrations will be assumed to be due to Delph-CL effecting hydration by moving K^+ in to the intracellular space. On the other hand, the antioxidant biomechanism will be based on the concentration of oxidized metHb as measured by spectrophotometry. The metHb will be measured before and after H_2O_2-induced oxidation. Inhibited metHb levels could indicate that Delph-CL has antioxidant properties.

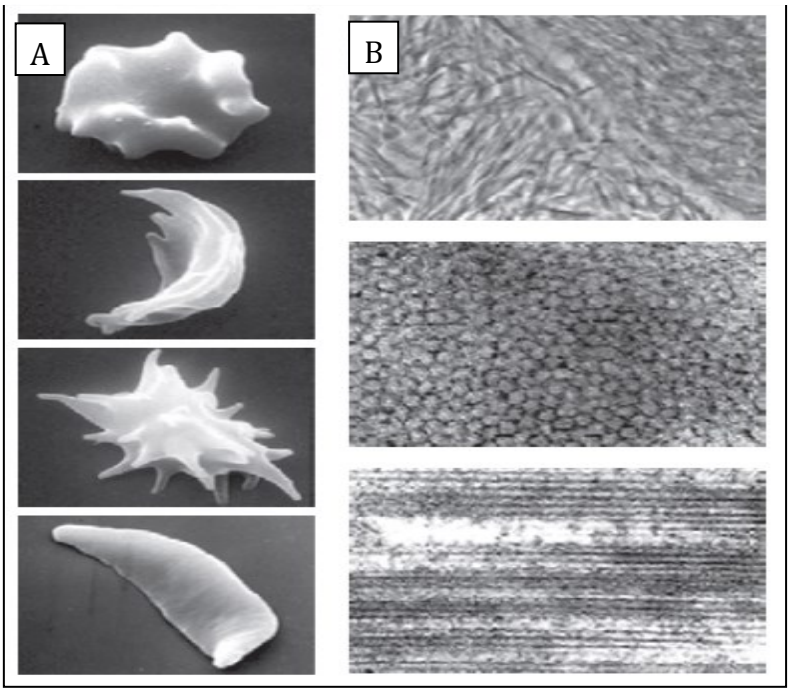

Figure 1.1: The relationship between sickle cell morphologies and Hb S polymer domain organization (A) Sickle cell morphologies (top to bottom): granular, holly leaf–shaped, classic sickle, smoother irreversible sickle; (B) Electron microscopic images of the Hb S polymer domain organization (top to bottom): highly disorganized, highly organized on end, highly organized polymer (Modified from Hoffman R. Hematology. [electronic resource] : Basic Principles and Practice. 6th ed: Philadelphia, PA : Saunders/Elsevier; 2013). (7).

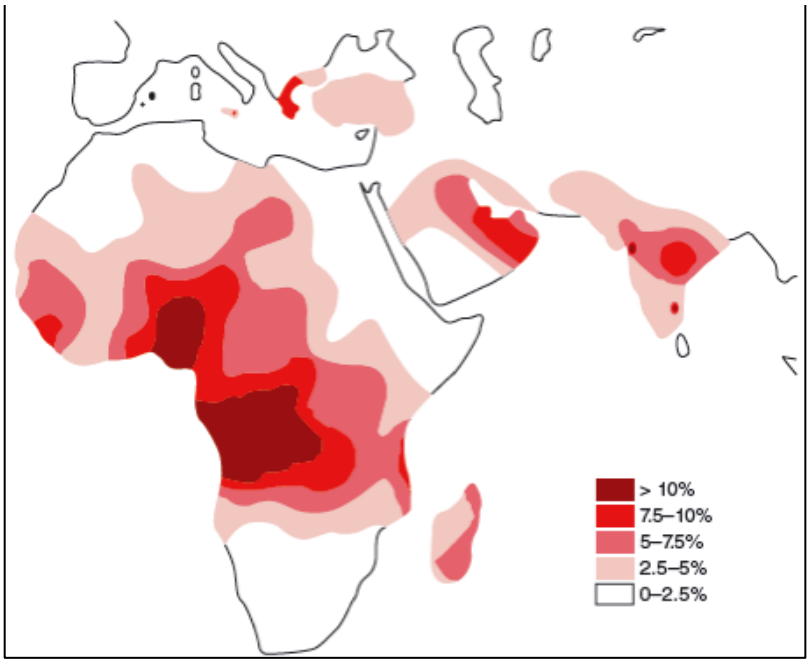

Figure 1.2: The percentage of SCD distributions with high prevalence in Africa, the Mediterranean and South Asia (5).

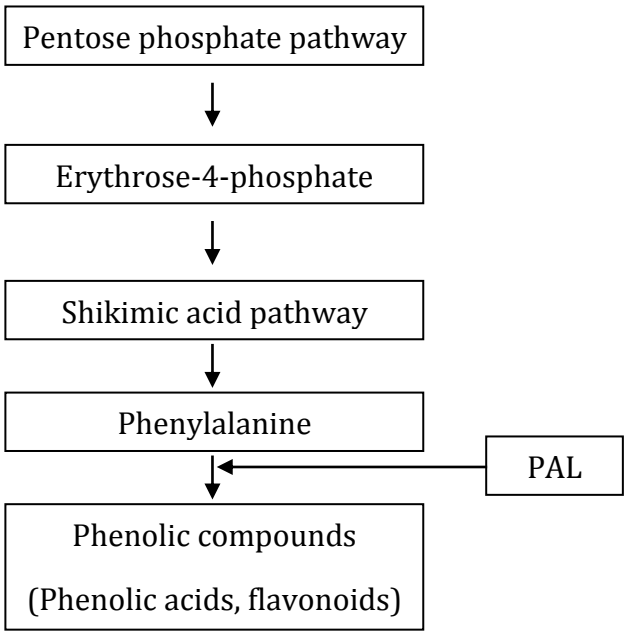

Figure 1.3: The shikimic acid pathway is a major route of phenolic compound biosynthesis in plants (Modified from Ghasemzadeh A and Ghasemzadeh N. Flavonoids and phenolic acids: Role and biochemical activity in plants and human. J Med Plant Res. 2011;5(31)). (58).

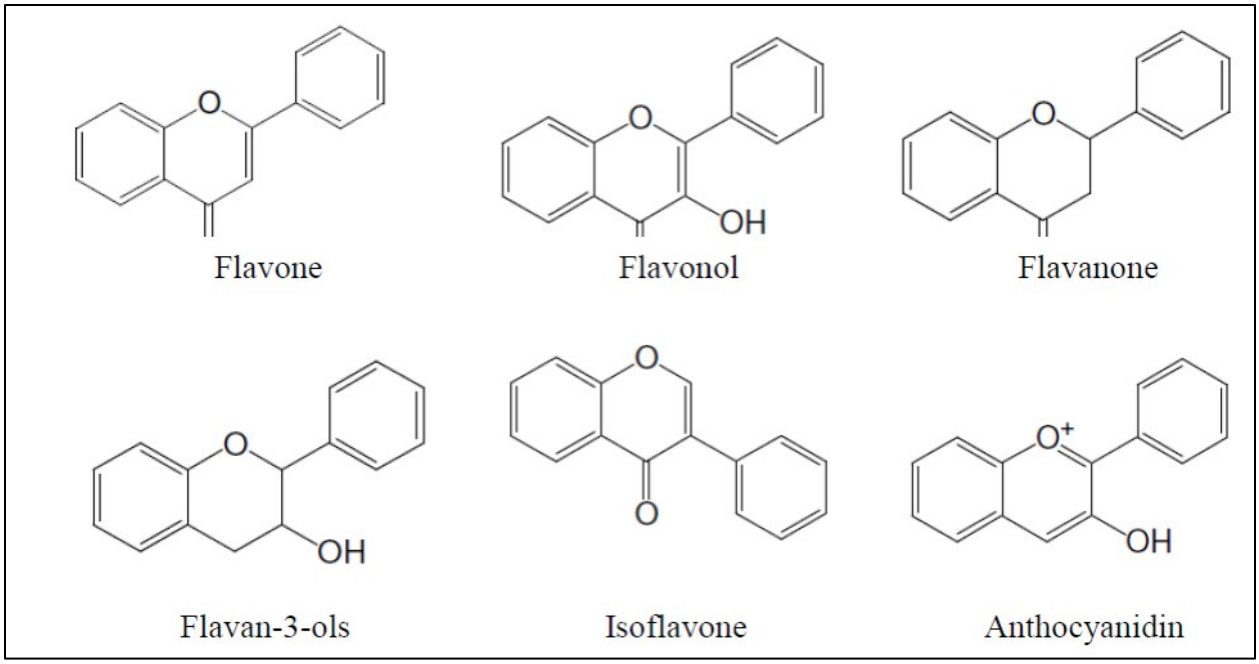

Figure 1.4: The chemical structure of flavonoid subgroups (Adapted from Ghasemzadeh A and Ghasemzadeh N. Flavonoids and phenolic acids: Role and biochemical activity in plants and human. J Med Plant Res. 2011;5(31)) (58).

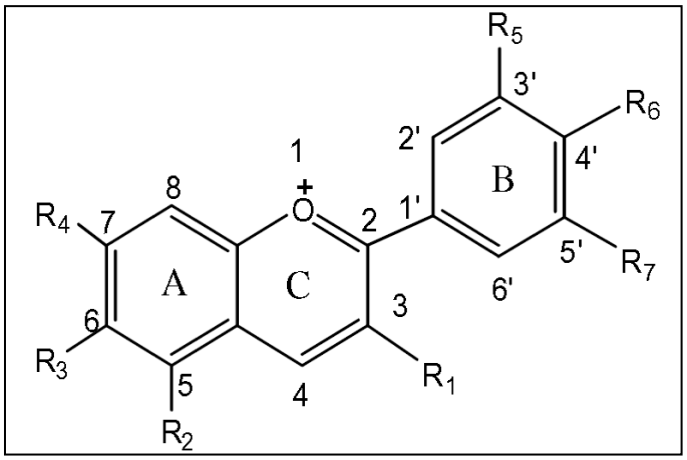

Figure 1.5: The basic structure of AC compounds. (Adapted from Castañeda-Ovando A, Pacheco-Hernández M, Páez-Hernández M, Rodríguez J, Galán-Vidal C. Chemical studies of anthocyanins: A review. Food Chem. 2009;113(4):859-71, Copyright © 2008 Elsevier Ltd. All rights reserved. (61)

Table 1.1: The complications outcomes in different organs/systems after macrovascular occlusion (6, 20, 22) .

Heart	**Gastrointestinal/Hepatobiliary**
• Cardiomyopathy • Cardiomegaly • Congestive heart failure	• Hepatic sequestration • Intrahepatic cholestasis
Muscular/Skeletal/Skin	**Brain**
• Avascular necrosis • Leg ulcer • Osteopenia	• Cerebrovascular accident • Silent cerebral infarcts • Hemorrhagic stroke
Eyes	**Lungs**
• Glaucoma • Proliferative sickle retinopathy • Vitreous hemorrhage	• Acute chest syndrome • Pulmonary hypertension
Renal/Genitourinary	**Spleen**
• Acute renal failure • Hematuria/proteinuria • Priapism	• Acute splenic infarction • Functional asplenia • Hypersplenism

Table 1.2: The substitution of each R-group in 10 forms of AC compounds (61).

Name	Abbreviation	Substitution						
		R_1	R_2	R_3	R_4	R_5	R_6	R_7
Cyanidin	Cy	OH	OH	H	OH	OH	OH	H
Peonidin	Pn	OH	OH	H	OH	OCH_3	OH	H
Petunidin	Pt	OH	OH	H	OH	OCH_3	OH	OH
Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH
Pelargonidin	Pg	OH	OH	H	OH	H	OH	H
Malvidin	Mv	OH	OH	H	OH	OCH_3	OH	OCH_3
Rosinidin	Rs	OH	OH	H	OCH_3	OCH_3	OH	H
Europinidin	Eu	OH	OCH_3	H	OH	OCH_3	OH	OH
Aurantinidin	Au	OH	OH	OH	OH	H	OH	H
Luteolinidin	Lt	H	OH	H	OH	OH	OH	H

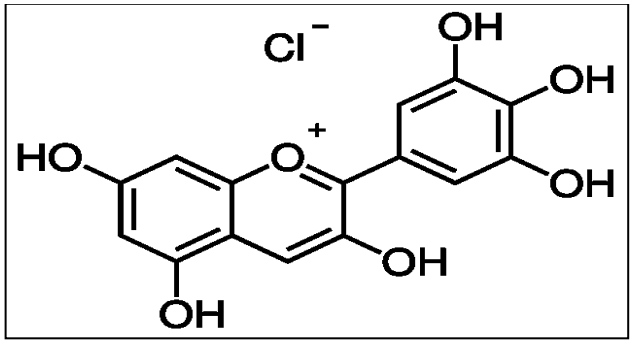

Figure 1.6: Delph-CL chemical structure (70).

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2 Determining the Concentration Efficacy of Sickle Cell Reversal

2.1 Introduction

Sickle cells are characterized by a sickle or crescent shape rather than a normal biconcave disc. This shape is induced by the deoxygenation (hypoxia) of hemoglobin S (Hb S), where Hb S is transformed from a soluble into an insoluble semisolid gel, or further, into fibers termed polymers (1, 2). The abnormal shape of the sickle cell is crucial in the pathogenesis of sickle cell disease (SCD). Sickle cells disrupt blood flow and cause microvascular occlusion. In addition, the smaller than normal surface area of sickle erythrocytes promotes hemolysis based on Weed's geometric relationship (3).

In the blood of a patient with SCD, the morphology of sickle cells can be easily determined with a stained blood smear. In addition, such patients may have target cells, which appear as cells with a deeply staining central zone and peripheral rim. The target cell is seen in Hb abnormalities such as Hb S, C, thalassemias, or iniron deficiency anemia and liver disease. In splenic atrophy cases, Howell-Jolly bodies (nuclear remnants), basophilic stippling and Pappenheimer bodies may also be seen (4-6). The function of the spleen is to remove aged red blood cells (RBCs) from the circulation through the phagocytosis mechanism (7). Accumulation of sickle cells and other blood cells in large numbers leads to splenic enlargement, which adversely affects splenic function and causes splenic atrophy (8). The Emmel test is used to examine the susceptibility of Hb S to polymerization in hypoxic conditions (9, 10). After applying a reducing substance for 24 hours at 37˚C, the deoxygenated condition induces a classical sickle shape (11).

Phytomedicine or herbal treatment was initially explored by African populations as a method of sickle cell reversal or anti-sickling (9). Many studies have investigated the anti-sickling effect of herbal and fruit extracts. Applying a crude juice of papaya in an alkaline extract and avocado in an alcohol extract to sickle cells significantly reduced the number of sickle cells *in vitro* after 24-hour incubation (12). In addition, *Justicia secunda* Vahl, *Zanthoxylum* (*Fagara*) *zanthoxyloides*, *Centella asiatica*, *Thomandersia hensii* and *Ricinodendron heudelotii* plant extracts demonstrated significant sickle cell reversal (9, 13, 14). Most anti-sickling agents contain phenylalanine, which is a substrate of the phenolic acid and flavonoid compounds in the photosynthesis mechanism. The anthocyanin (AC) group is one of 6 flavonoid compound

subgroups that contains 10 popular forms: cyanidin (Cy), peonidin (Pn), petunidin (Pt), delphinidin (Dp), malvidin (Mv), pelargonidin (Pg), rosinidin , europinidin (Eu), aurantinidin (Au) and luteolinidin (9, 15). This study focused on Dp ACs because they can easily be extracted from the bilberry fruit; specifically, the *Vaccinium myrtillus* L. species. Delphinidin ACs are found in the form of Dp-3-*O*-galactoside and Dp-3-*O*-glucoside in high concentrations of 1060 and 1247 mg/100 g, respectively (16). Moreover, this form of ACs was used *in vivo* by hypertensive patients in a cardiovascular risk study without any side effects. The participants took Medox capsules containing Dp-3-*O*-β-glucoside and Cy-3-*O*-β-glucoside AC forms extracted from *V. myrtillus*. After 4 weeks, the authors observed lower blood pressure, from 143/96 ± 13/6 mmHg to 137/85 ± 12/7 mmHg and elevated high-density lipoprotein (HDL) cholesterol in the patients (17). The use of Dp on erythrocytes, specifically sickle cells, has not been demonstrated; therefore, the manufactured form, Dp chloride (Delph-CL), was used in this study. A manufactured source was chosen to avoid the time consuming extraction process and phytochemical screening for active ingredients in the herbal extract.

Since Delph-CL is a new approach to treating sickle RBCs, the agent toxicity will be evaluated by measuring hemolysis. Later on, the ability of Delph-CL to reverse the abnormal sickle cell shape to a normal biconcave disc will be examined. Subsequently, the effective dose during this process will be determined for further investigation.

2.2 Materials and Methods

2.2.1 Biological Samples

Eleven patients with sickle cell anemia (SCA) (6 men and 5 women) aged 19–40 years participated in this study, which was conducted between November 2013–May 2015. Ethics approval was obtained from the University of Alberta Health Research Ethics Board (HREB) and Canadian Blood Services (CBS). All patients were met in the apheresis unit at the University of Alberta Hospital during their red cell exchange (RCE) sessions. The study was explained verbally by the author to each patient. An information letter was provided to each participant and written consent was obtained (Appendix A). Ethylenediaminetetraacetic acid (EDTA) whole blood samples (4 mL) were collected by venipuncture from the patients by a phlebotomist 2 days before the RCE. The blood samples were stored at 1–6°C. The control group was comprised of

11 healthy volunteers (4 men and 7 women) aged 19–35 years. The study was explained verbally by the author to each volunteer. An information letter was provided to each participant and written consent was obtained (Appendix A). Around 4 mL EDTA blood per volunteer was freshly collected by a CBS lab technician. The control samples were collected on the day of the experiment.

2.2.2 Delphinidin Chloride Reagent Preparation

Delphinidin chloride (analytical-standard powder), synonym: 3,3′,4′,5,5′,7-hexahydroxyflavylium chloride, 3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-1-benzopyrylium chloride ($C_{15}H_{11}ClO_7$), was purchased from Sigma-Aldrich (MFCD00016663). This specific AC form was considered from among others because it is an analytical-standard compound within the AC group with ≥95% purity according to high-performance liquid chromatography (HPLC). The molecular weight of Delph-CL was 338.70 g/mol and it was stored at −20˚C before use. A stock solution 10 mg/10mL (1 mg/mL) was prepared using 10 mg Delph-CL dissolved in 10 mL 1× phosphate-buffered saline (PBS) without calcium and magnesium (Mediatech, Inc.); it was stored between 2–30˚C. To obtain a completely dissolved solution, the mixture was warmed at 25°C and stirred continuously on an automated mixer at 110 rotations per minute (RPM) with a single magnetic stirring bar. The reagent became homogeneous after 35 minutes. Working concentrations (2, 20, 200 and 500 µg/mL) were prepared through serial dilutions with PBS. All prepared concentrations were stored at −20˚C.

2.2.3 Blood Sample Preparation

The control and experimental whole blood samples were centrifuged (Eppendorf 5810 rotators) at 2200 × g at 4 ˚C for 10 minutes. After the first centrifugation, the plasma and buffy coat were removed. Next, the packed RBCs (PRBCs) were washed 3 times with PBS. The washed PRBCs were suspended in PBS to achieve 14–28% hematocrit (Hct). An electronic cell analyzer (Beckman Coulter ACT 8 software) was used to determine the Hct. As the ideal Delph-CL testing concentration was unknown, an equal ratio (1:1) of 500 µL Delph-CL and RBC suspension was used for sample treatment. Therefore, the final Hct of the tested samples was 7–14 % and the Delph-CL concentrations were changed to 1, 10, 100 and 250 µg/mL. In addition, the 1:1 treatment ratio excluded the excessive effect of agent treatment on the RBCs and maintained cell viability.

The positive control was normal RBCs suspended in PBS instead of Delph-CL. The control was used to compare the outcomes of normal RBCs with that of abnormal sickle cells. Untreated sickle RBCs suspended in PBS only, labeled as 0 µg/mL, were used to demonstrate the zero effect of Delph-CL on sickle cells.

2.2.4 Correlation Coefficient of Delphinidin Chloride Reagent

The absorbance of Delph-CL concentrations 2, 20, 200, 500 and stock 1000 µg/mL was measured using a spectrophotometer (Spectramax PLUS 384, Molecular Device, software 4.3). In addition, the above mentioned concentrations were treated equally with PBS to obtain the final concentrations (1, 10, 100 and 250 µg/mL). All reagents absorbance were read at the default wavelength 405 nm. The purpose of this experiment was to determine the extinction coefficient. The result will help determine if Delph-CL was fully dissolved in the PBS. Furthermore, it will be determined if this reagent color was detected by the spectrophotometer. Finally, it will be defined if there was a positive correlation between gradually increasing concentration of the samples and the reading of absorbance. From each concentration a volume of 200 µL was pipetted into a specific well of a flat bottom, medium binding 96 well enzyme immunoassay/ radio immunoassay (EIA/RIA) plate (Corning Costar) for reading.

2.2.5 Maximum Detection Wavelength of Delphinidin Chloride Reagent

This experiment was used to define the maximum absorbance wavelength of Delph-CL concentrations (1, 2, 10, 20, 100, 200, 250, 500 and 1000 µg/mL). It was intended to detect interference with the detection wavelength 540 nm, which will be used in determining RBC hemolysis, osmotic fragility, and solubility tests. A volume of 200 µL of each concentration was pipetted into a specific well of a flat bottom, medium binding 96 well EIA/RIA plate (Corning Costar) for reading. A spectrophotometer (Spectramax PLUS 384, Molecular Device, software 4.3) was used to measure the absorbance between 190 and 600 nm.

2.2.6 Hemolysis Assay

The percentage of hemolyzed cells was determined by taking the ratio of the free and bound Hb in the blood sample, which are known as the total and supernatant Hb, respectively. To measure the total Hb, 1 mL of Drabkin's reagent was vortexed with 5 µL of treated blood samples. The tube was incubated at room temperature at least 5 minutes before reading. The Hct after 24 hours of incubation was measured on a Beckman Coulter® ACT 8. This study used the automated

Beckman Coulter® ACT 8 for Hct detection instead of the spun standard method, because the Hct value is influenced by MCV level. Whereas, the Hct value has a direct correlation to the MCV level as seen in the following formula (19):

$$\text{Hct (\%)} = \text{RBC } (10^6/\mu\text{L}) \times \text{MCV (fL)} /10$$

Once the MCV is increased the percentage of Hct is elevated and the opposite is correct (20). The objective of this study was to obtain reversal sickle cell morphology through increasing cell volume after applying Delph-CL treatment. Therefore, using Beckman Coulter® ACT 8 in this situation is more relevant whereas expecting to detect intact full volume sickle cells.

For the supernatant Hb, the treated sample was centrifuged (Eppendorf 5415 rotators) at 2,200 x g at 4 ˚C for 10 minutes to obtain a supernatant. To measure the supernatant Hb, 40 µL of each sample supernatant was dispensed into 160 µL of the Drabkin's reagent. Next, all the tubes were capped and vortexed, and then incubated at room temperature at least 5 minutes before reading. Low, medium, and high Hb commercial controls (Tri-level Hb, Stanbio Laboratory) were stored at 4 ˚C, and were measured with each experiment as total Hb sample. The controls were used for spectrophotometer machine inspection, Drabkin's reagent validity and verify the results. Each sample was measured in triplicate. For reading, 200 µL was aspirated from the controls, total Hb, and supernatant Hb and dispensed into the wells of a flat bottom, medium binding 96 well EIA/RIA plate (Corning® Costar®). In a new plate, 200 µL of dH_2O was dispensed into each corresponding well that was used on the test plate. The percentage of hemolysis was read automatically by the spectrophotometer (Spectramax PLUS 384, Molecular devices, software 4.3) at 540 nm. A high percentage of hemolysis indicates that Delph-CL causes red cell membrane damage; which means is it toxic.

2.2.7 Stained Blood Smear

Before preparing the samples for treatment with Delph-CL, a peripheral blood smear was made to determine the average number of sickle cells in the patients. A slide-to-slide manual technique was used (21). To accomplish this task, thin, long and tail edge smears were made before staining. Subsequently, the blood smears were left on a standing rack to dry at room temperature. A Hema 3 stain set (Fisher Scientific), which includes a fixative solution, Eosin Y (solution I) and Wright's stain (solution II), was used for staining. The Wright's stain was filtered through a

medium-flow filter (VWR Grade 413–18.5 cm) before each use. The slide was dipped in the fixative solution 5 times for 1 second each. Next, the slide was transferred to Eosin Y and Wright's stain consecutively and was dipped three times each. Finally, the slide was washed with tap water to remove excess stain. Stained slides were kept on a standing rack to dry at room temperature for 15–20 minutes. An oil immersion (100x) objective on an inverted microscope (Nikon TE 2000-U) was used for 250x magnification. Sickle cells in 3 fields were counted manually using a Diffcount unit (model 10-312, serial no.318475). Counting on the Nikon TE 2000-U was performed within a 35 mm photo frame termed a photo-mask dial (22). The photo-mask dial appears on the viewing field and is used for sharp focusing and to keep the counting area consistent. The sickle cells and normal RBCs in 3 different fields were counted separately. The percentage of sickle cells in each field was calculated using the formula below. Later on, the average of 3 fields was used.

$$\text{Sickle cell (\%)} = \text{Number of sickle cells/Total} \times 100$$

2.2.8 Anti-sickling Effect

An Emmel test protocol was followed to assess sickle cell reversal (14). Fresh 2% sodium metabisulfite (SMBS) reagent was prepared by dissolving 400 mg anhydrous sodium bisulfate ($HNaO_4S$) (MFCD00167602), measured on a Mettler Toledo AX205 microbalance, in 2 mL distilled water. The sickle RBCs were mixed with an equal amount of 2% SMBS in 1.5 mL microtubes. Then, 5 µL of the mixture was dispensed on a clean glass slide to maintain a consistent volume on all examination slides. The drop was covered with a cover slip and sealed with Vaseline to be stored in deoxygenated conditions. Then, the slide was placed in a wet Petri dish and incubated in a 37˚C incubator (Thermo-Steri Cycle CO_2 HEPA Class 100) for 24 hours. All slides were examined with an inverted microscope (Nikon TE 2000-U) under a 40x objective lens with 100x magnification power. The photo-mask dial was also used during counting for sharp focusing and to keep the counting area consistent. In 3 different fields, each cell type was observed and counted manually with the Diffcount unit (model 10-312, serial no. 318475). In each field, the percentage of each cell type was calculated as previously described. The result was compared to the percentage of each cell type at each Delph-CL concentration.

2.2.9 Mean Cell Volume

The mean cell volume (MCV) was measured to support the microscopic reversal result with numerical data. All sickle cells treated with Delph-CL and the control samples were incubated in 1.5 mL microtubes in oxygenated conditions without 2 % SMBS. The MCV was measured before and after the 24 hours incubation at 37˚C to assess any sickle cell volume differences. The automated Beckman Coulter ACT 8 was used. Each sample was gently mixed by inverting 3 times before testing.

2.2.10 Statistical Analysis

IBM SPSS Statistics 22 was used for data analysis. Nonparametric tests were chosen; the Wilcoxon signed rank test was used to compare the negative control and the treated sickle cells according to Delph-CL concentration. The Mann-Whitney test was applied when comparing the positive control and the treated sickle cells according to Delph-CL concentration.

2.3 Results

2.3.1 Correlation Coefficient of Delphinidin Chloride Reagent

There was a linear relationship between the Delph-CL concentration 1, 2, 10, 20, 100, 200, 250, 500 and 1000 µg/mL and the reading absorbance $R^2 = 0.9913$ at the default wavelength 405 nm, Figure 2.1. The curve linearity demonstrates a positive correlation between the concentration and absorbance.

2.3.2 Maximum Detection Wavelength of Delphinidin Chloride Reagent

Figure 2.2 (A and B) shows the maximum detection wavelengths for Delph-CL concentrations 1, 2, 10, 20, 100, 200, 250, 500 and 1000 µg/mL. The scanning were lengths used to determine the maximum wavelength for each concentration was between ~ 500 - 600 nm. The maximum wavelength for each concentration is demonstrated in Table 2.1.

2.3.3 Hemolysis Assay

Delphinidin chloride did not decrease sickle cell hemolysis in any tested concentrations compared to untreated sickle cell (0 µg/mL). Figure 2.3 indicates a direct relationship between the percentage of hemolysis and the tested concentrations. The 100 and 250 µg/mL

concentrations caused significantly more hemolysis in the treated concentrations than normal RBCs and the untreated sickle cells ($p < 0.05$).

2.3.4 Stained Blood Smear

Before examining the anti-sickling property of Delph-CL, patient sickle cells in 3 microscopic fields were manually counted in the original sample. Figure 2.4 depicts the digital microscopic images of blood smears of the sickle cell samples after Hema 3 staining. Based on 6 non-randomly selected patients, the average of sickle cells in each field was 7.2 ± 4 cells/field.

2.3.5 Anti-sickling Effect

All treated smears demonstrated different cell types: sickle, elliptical, intact and hemolyzed. Each cell type was counted separately in 3 different fields. Figure 2.5 demonstrates that 1, 10 and 100 µg/mL Delph-CL treatment decreased the percentage of sickle cells in a dose-dependent manner to 13.9 %, 12.6 % and 9.7 %, respectively. This was indirectly related with the percentage of elliptocytes, which increased in tandem with the decrease in sickle cell percentage. The elliptical shape is evidence of the reversal process, where the cell size is increased and there is transformation, as seen in Figure 2.6 (C and D). Specifically, 100 µg/mL Delph-CL effected a significant change in cell morphology from the sickle shape into the elliptical shape as compared to cells treated with 0 µg/mL Delph-CL ($p = 0.04$).

The lowest dose, 1 µg/mL, increased the intact cells to 68.5 % as compared to the increase by the 0 µg/mL dose to 61.4 % ($p > 0.05$). Subsequently, the percentage of intact cells gradually decreased. Consequently, the percentage of hemolyzed cells increased. Moreover, the cells were significantly hemolyzed following treatment with 250 µg/mL Delph-CL ($p < 0.05$), as demonstrated in Figure 2.3. Therefore, the shape of the RBCs was unrecognizable, as seen in Figure 2.6 (E); consequently, those cells were not counted.

2.3.6 Mean Cell Volume

Figure 2.7 demonstrates the MCV before and after the 24 hours incubation at 37˚C. Generally, there was a statistically significant increase in the MCV of all groups after the 24 hours incubation ($p = 0.03$). Treatment with 100 µg/mL Delph-CL led to an MCV of 101 fL ± 7 fL as compared to untreated sickle cells, i.e., 0 µg/mL Delph-CL, which had an MCV of 92.32 fL ±

6.2 ($p < 0.001$). On the other hand, 250 µg/mL Delph-CL increased the MCV to 123 fL ± 13 as compared to that of the normal RBCs with an MCV of 92 fL ± 9 ($p = 0.04$).

2.4 Discussion

Delphinidin AC has a blue/violet color due to hydrogen (H) atom in R3 and six hydroxyl groups in R1 – R7 as shown in Table 1.2 (23). Consequently, Delph-CL concentrations showed a positive correlation with spectrophotometric reading absorbance $R^2 = 0.9913$, which means that Delph-CL can be dissolved in isotonic (pH = 7) PBS. Most of the solvents used to dissolve ACs are aqueous mixtures of alcohol, such as ethanol, methanol, or acetone due to the high polarity of AC agents (13, 15). Dissolving Delph-CL in PBS was achievable at 110 RPM at 25 ˚C for 35 minutes. The PBS solvent was chosen to protect the RBCs from swelling or shrinking. In addition, it is ideal to protect the ACs from degradation at pH higher than 7 (24).

The maximum wavelength of each concentration was detected through spectrophotometry. All tested concentrations demonstrated a maximum wavelength far away from 540 nm, except the stock concentration 1000 µg/mL was 550 nm, Table 2.1. This wavelength is used as a standard in most spectrophotometric reading experiments. Therefore, the self-color property of Delph-CL will not significantly interfere with wavelength used in the hemolysis assay.

The Drabkin's method is the most-used method for hemolysis assays. The principle of this method relies on converting Hb and derivatives to metHb by oxidation through alkaline potassium ferricyanide (25). The percentage of resulting hemolysis demonstrated a gradual dose-response effect with Delph-CL. The Delph-CL absorbance color at 540 nm is not detected by the spectrophotometer as found in Section 2.3.2 result, therefore does not elevate the hemolysis reading. The results were due to Delph-CL toxicity. At 250 µg/mL the sickle cells were significantly hemolyzed and their shapes were unrecognizable, as seen in Figure 2.6 (E). Therefore, this concentration will be excluded from further experiments. Elevating the metHb naturally in SCD patients, as described previously in Section 1.1.1, also participates in the Hb oxidation and elevates hemolysis further (1).

The sickle cell percentages in the whole blood samples before Delph-CL treatment aided in the exclusion of factors that may have affected the Delph-CL outcome. The main factor was patient treatment. The average of sickle cell percentages in the patients' blood was 7.2 $\pm$ 4. The

participants underwent regular RCE once every month, which is an effective way to reduce the sickle cell percentage and maintain iron levels and blood viscosity (26). Moreover, most of the patients were on hydroxyurea (Hu) treatment. This treatment is considered a powerful and successful drug for reducing the incidence of pain and hospitalization. These effects are achieved when the production of fetal Hb (Hb F) is increased (27). In healthy adults, the percentage of Hb F is <1 %, but in patients with SCA, the percentage is much higher due to Hu treatment (28).

In this study, the Hb F levels were unknown, but what is known is that elevated Hb F, from 5–10 % to 15–20 % (about 10 g/L), leads to reduced intracellular concentrations of Hb S, which consequently decreases polymerization and protects sickle cells from hemolysis (29). It also causes minor improvement in cell deformability, as studied by Athanassiou et al. (30). This treatment can also reduce the incidence of splenic dysfunction. Patients who receive this treatment have better splenic function, as demonstrated by the decreased Howell-Jolly bodies (29).

Following the Delph-CL treatment, the sickle cell percentage continuously decreased as the Delph-CL dosage was gradually increased, i.e., from 1, 10 to 100 µg/mL. This resulted in transformation of the sickle cells into elliptical shaped cells. We assume that the elliptical shape is a transition stage in the reversal process to achieve the normal RBC shape. The sickle cell percentage was decreased significantly ($p = 0.04$) by 100 µg/mL Delph-CL; therefore, it should be considered for investigation of the biomechanism of sickle cell reversal. As a consequence of the reversal, incubation in 1, 10 and 100 µg/mL Delph-CL elevated the MCV, which is perhaps an effect of Delph-CL on sickle cell dehydration. The MCV was also increased in the study by Ballas, who incubated sickle cells with 400 µg/mL *Pfaffia paniculata* extract and recorded an MCV of 111 ± 12 fL after 4-hour incubation. The *P. paniculata* biomechanism on the sickle cells involved hydration, as the author determined that intracellular sodium (Na^+) ions were increased (31). The MCV value of normal RBCs after 24 hours of incubation (92 fL ± 9) was expected as Ballas's study which is 90 fL ± 9.

The hemolysis and anti-sickling effect in each Delph-CL treatment concentration may refer to the heterogeneity of the sickle cell population: granular, holly leaf, classical and smoother irreversible sickle formed under deoxygenated condition. These cells differ from young

deformable to rigid irreversible based on the degree of Hb S polymerization, which affects the cell membrane viscosity (1, 32).

Elliptical RBCs have cell membrane normal in viscosity that facilitates the diffusion of Delph-CL through both the hydrophilic and hydrophobic layers. As a result, Delph-CL can reach the cytoplasm and enhance Hb S solubility. On the other hand, when sickle cells retain their shape, it could be that Delph-CL is incorporated into the hydrophobic layer and could not reach the cytoplasm because of the high membrane viscosity. Hemolysed cells may have very high membrane viscosity that prevents the diffusion of Delph-CL into the cell, causing it to stay in the outer hydrophilic layer and leading to cell damage (32) .

Using a valid sickle cell reversal treatment such as Medox capsules or Ciklavit as a control during the anti-sickling experiment should be proposed (13). That will help to determine the agent's efficacy, the sickle cell morphology chang and exclude the independent variable such as time, temperature etc. In addition, the cell radius, perimeter and surface area should be measured to better evaluate the change in cell volume, especially at the 1 and 10 µg/mL doses (14).

Delph-CL caused cell toxicity especially at 250 µg/mL. However, a minor reversal of sickle cell shape at 100 µg/mL was achieved. Moreover, Delph-CL did not show strong absorbance at 540 nm, which is the wavelength to be used for further study in this work. As 100 µg/mL resulted in minor sickle cell reversal it will be considered in the subsequent studies to determine if this concentration will improve the sickle cell impairment. The Hb S solubility and O_2 binding affinity, sickle cell deformability and membrane fragility will be assessed.

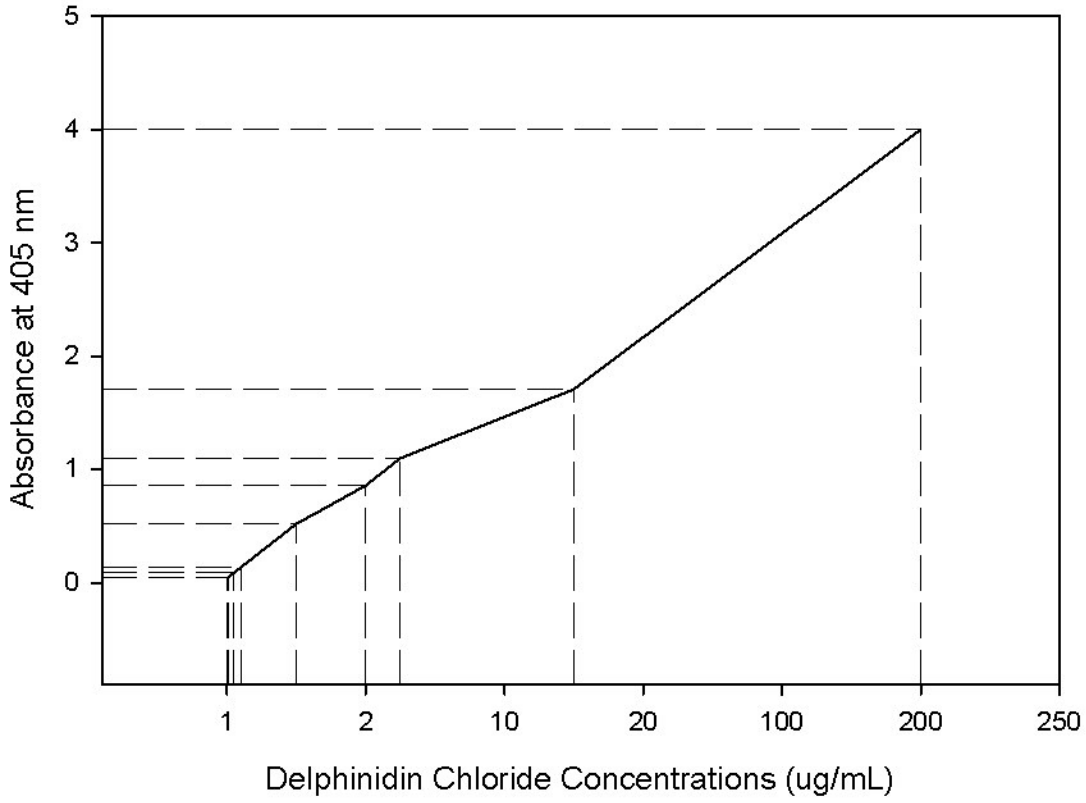
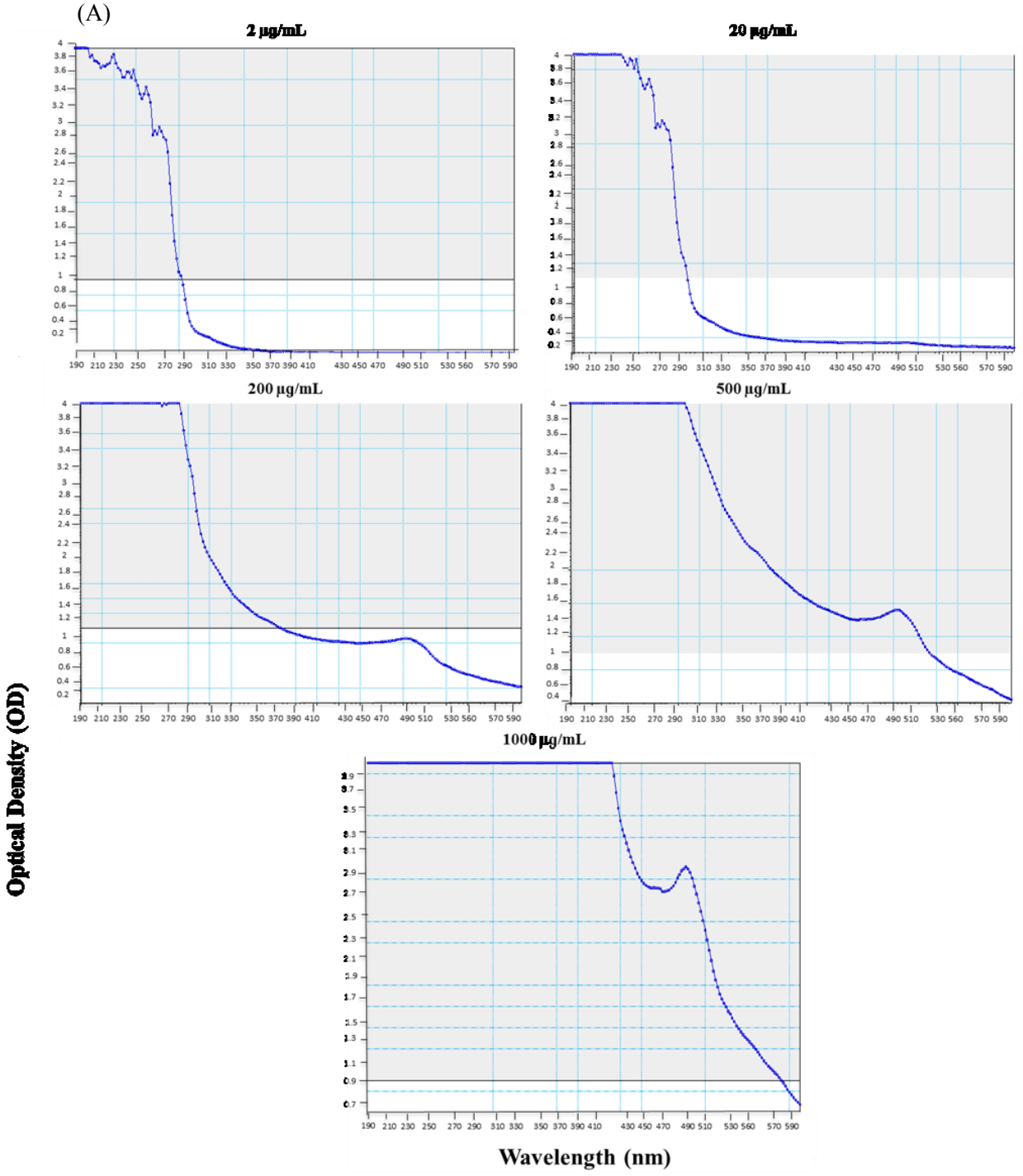


Figure 2.1: The peak absorbance of the Delph-CL concentrations at 405 nm: 1, 2, 10, 20, 100, 200, 250, 500 and 1000 µg/mL.

(A)


2 µg/mL
20 µg/mL
200 µg/mL
500 µg/mL
1000 µg/mL
Optical Density (OD)
Wavelength (nm)

(B)

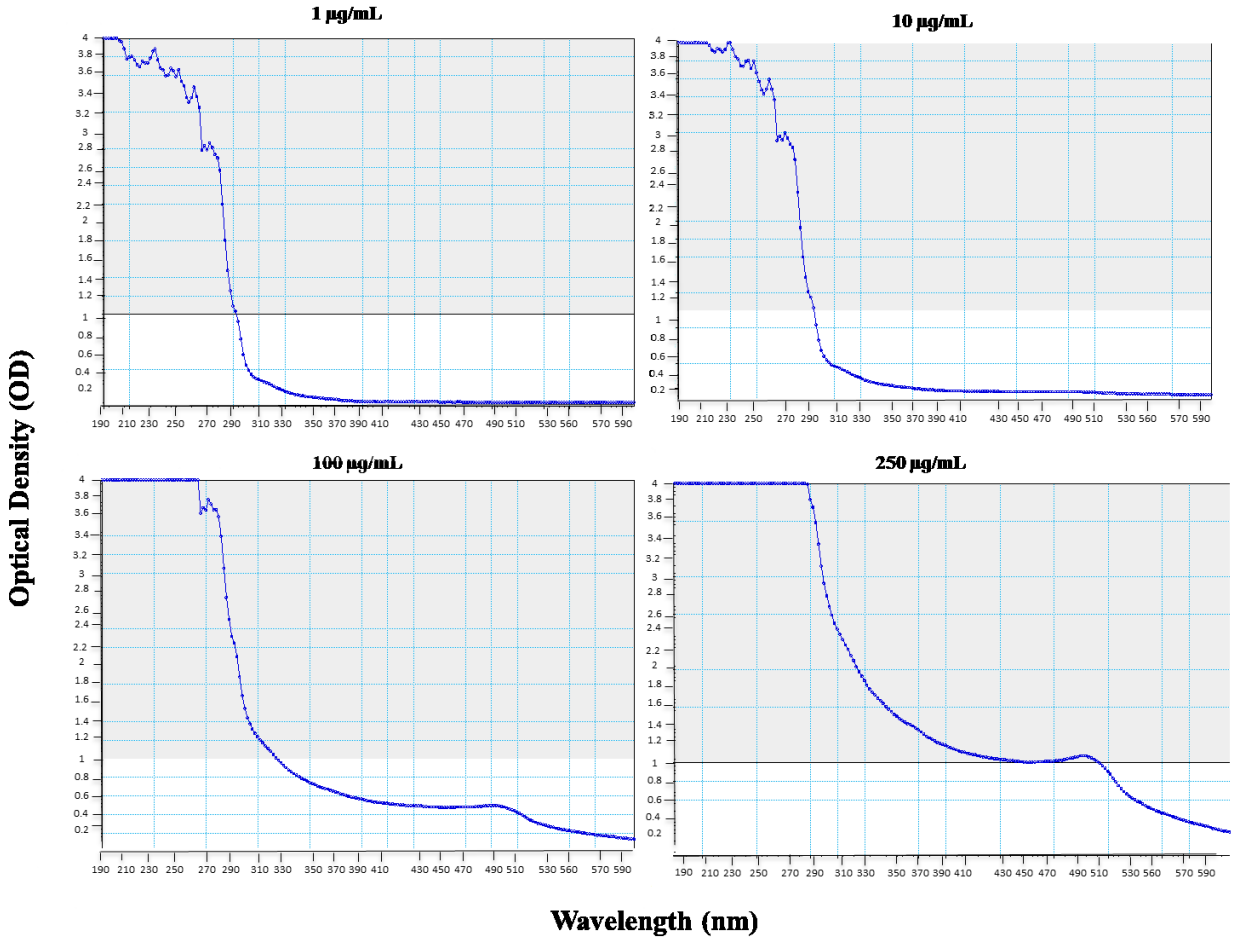


Figure 2.2: The maximum detection wavelength curve of the Delph-CL concentrations at 405 nm, (A): 2, 20, 200, 500 and 1000 µg/mL. (B): 1, 10, 100, and 250 µg/mL.

Table 2.1: The maximum wavelength for different Delph-CL concentrations

Delph-CL Concentrations (µg/mL)	Maximum Wavelength (nm)
1	294
2	294
10	296
20	296
100	326
200	378
250	504
500	524
1000	584

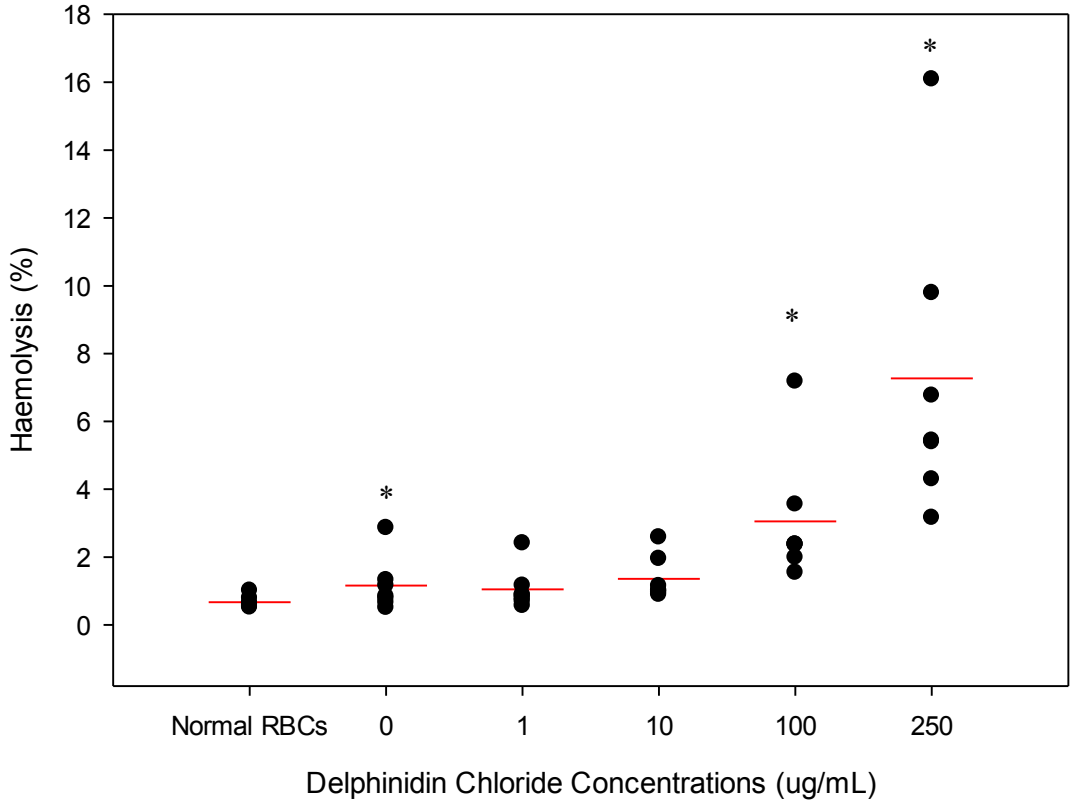


Figure 2.3: The percentage of hemolysis after 24 hours incubation of Delph-CL with sickle cells (n = 7). **(*)** Denotes a significant difference between 100 and 250 µg/mL and 0 (untreated sickle cell), $p < 0.05$.

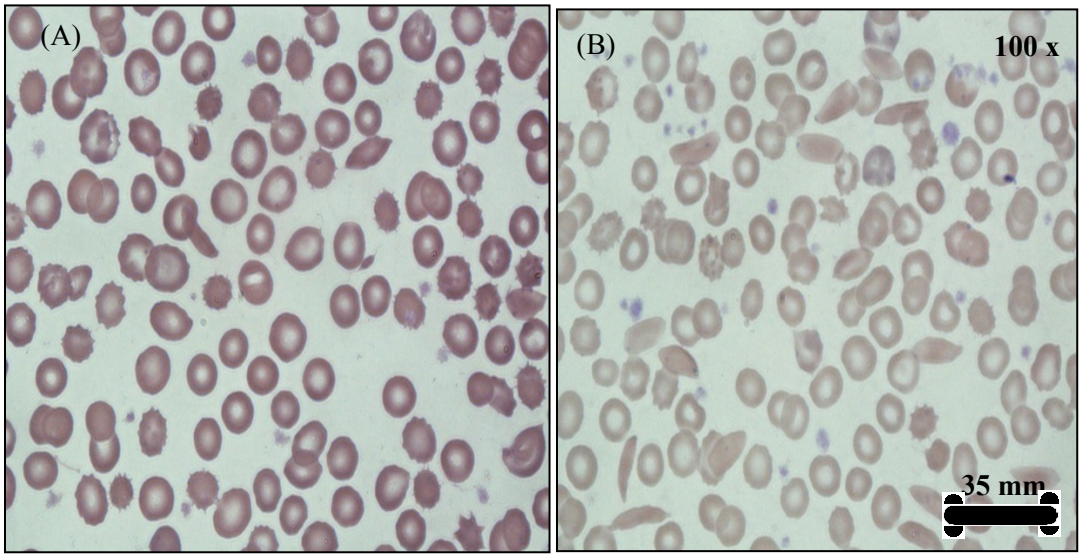


Figure 2.4: Stained blood smear: (A) Female, 23 years old; (B) male, 34 years old.

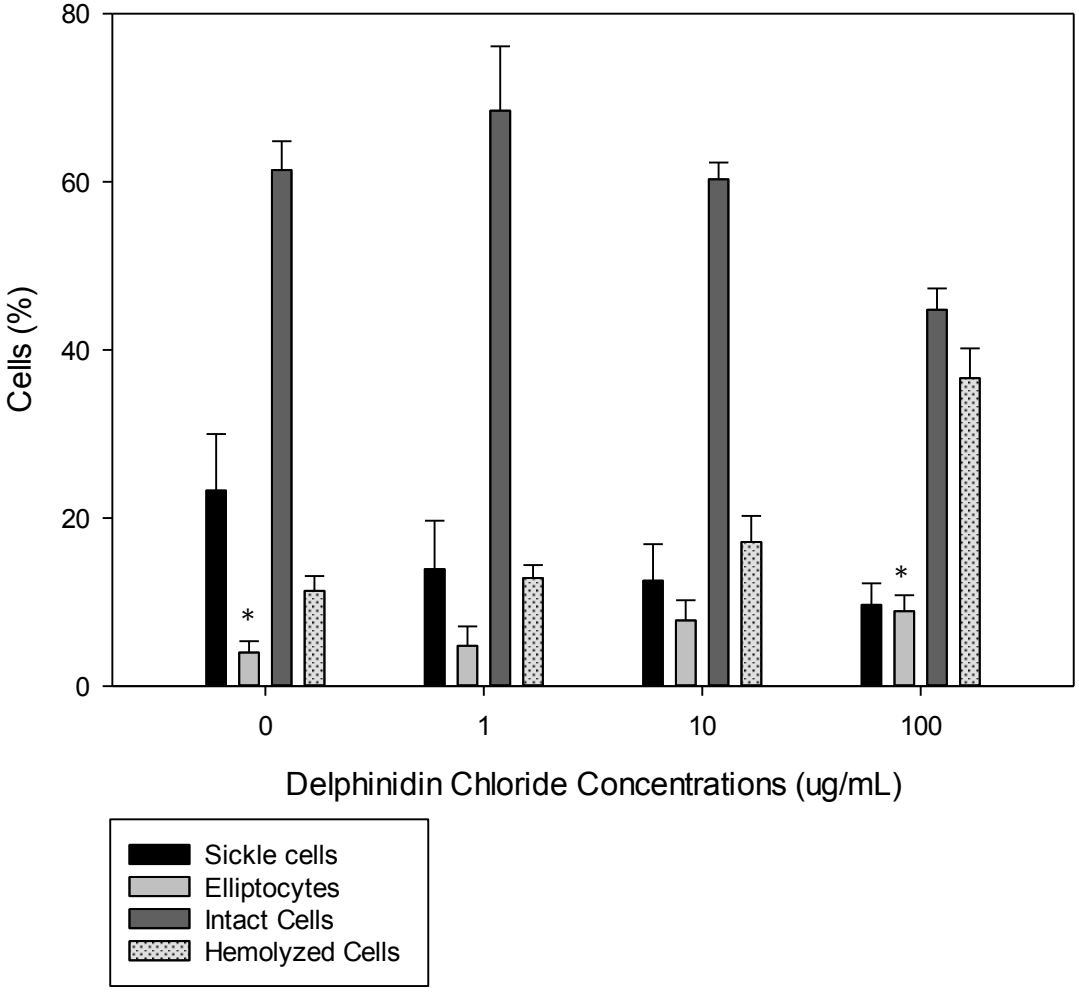


Figure 2.5: The percentage of each cell type in different Delph-CL concentrations (n = 5). (*) Denotes a significant difference between 100 μg/mL and 0 (untreated sickle cell), p = 0.04.

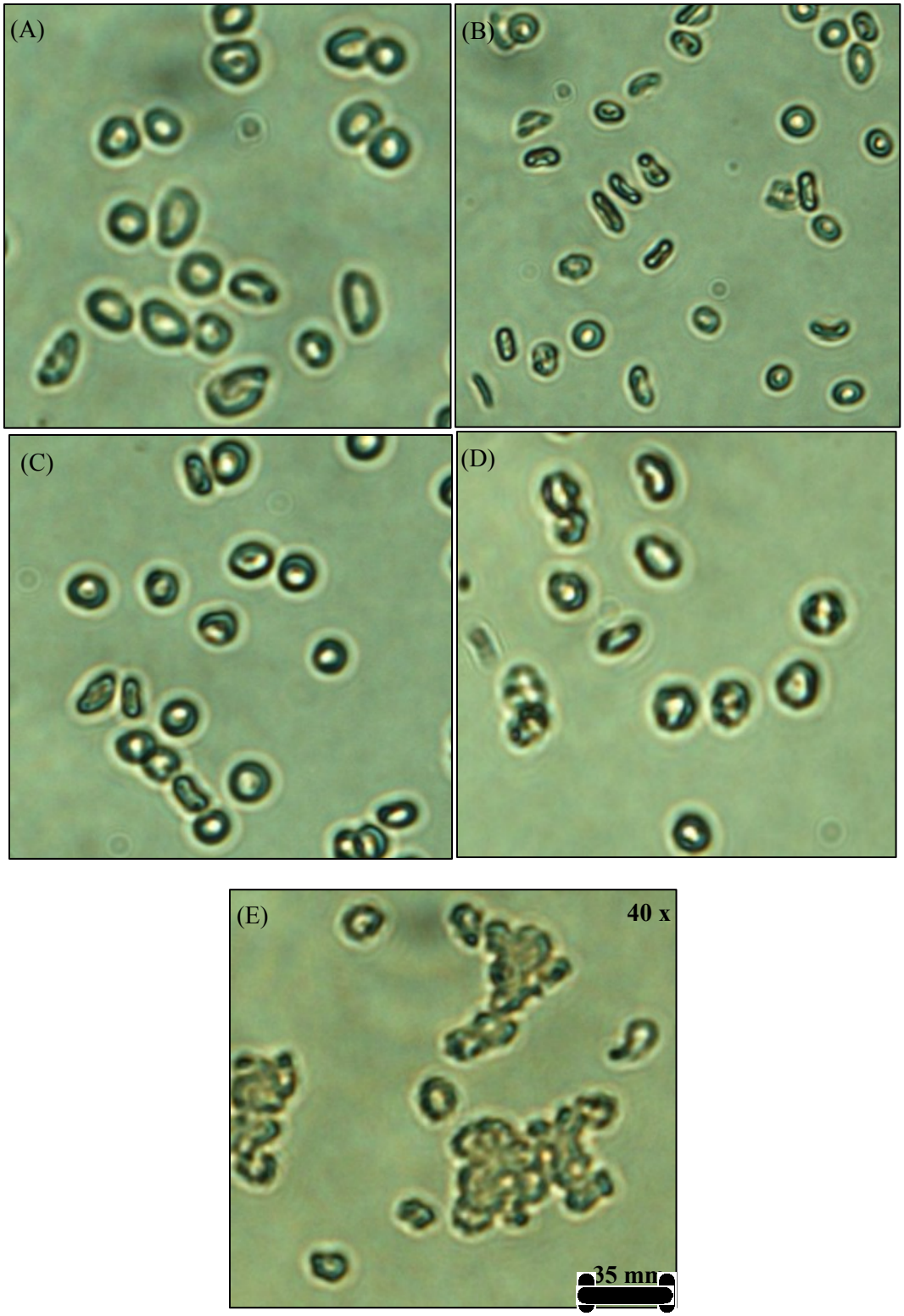


Figure 2.6: Digital microscopic images of sickle cell reversal after 24-hour incubation at 37˚ C in deoxygenated conditions: (A) 0 µg/mL; (B) 1 µg/mL; (C) 10 µg/mL; (D) 100 µg/mL and (E) 250 µg/mL Delph-CL.

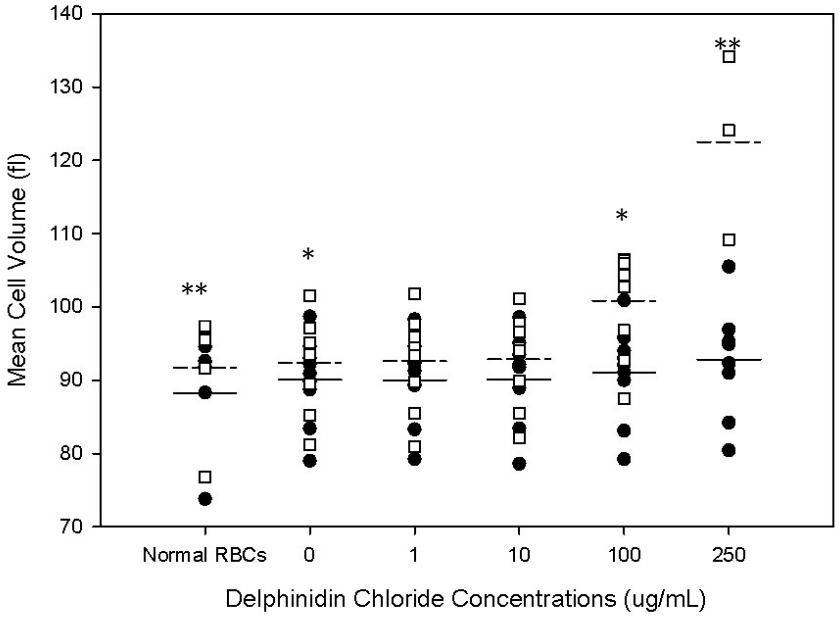


Figure 2.7: The MCV: (●, ---) before; (□,-) after 24 hours incubation at 37 ˚C (n = 9). (*) Significant difference between 100 µg/mL and 0 (untreated sickle cell), $p < 0.001$. (**) Significant difference between 250 µg/mL and normal RBCs, $p = 0.04$.

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3 Effect of Delphinidin Chloride on Sickle Cell Membrane Integrity, Deformability, Hemoglobin Solubility and Oxygen Binding

3.1 Introduction

Sickle cell disease (SCD) is a genetic disorder caused by a single nucleotide substitution that results in a replacement of a glutamic amino acid with a valine amino acid (GAG → GTG) in the 6^{th} position of the β globin chain (1). A hydrophobic bond forms between the valine and the nearby amino acids alanine, phenylalanine, and leucine in the hemoglobin S (Hb S) molecule. This hydrophobic bond in the deoxygenated condition converts Hb S from soluble to fibers or polymers (2). Therefore, Hb S solubility is reduced from 700 g/L for normal adult hemoglobin (Hb A) to 170 g/L (3). The presence of Hb A helps to reduce Hb S polymerization by approximately 100-fold due to replacement of 50% of Hb S by Hb A (4).

Hemoglobin S polymerization concentration also affects the integrity of the red blood cell (RBC) membrane. The polymerization-depolymerization cycle when oxygenated-deoxygenated of Hb reduces membrane function (3) by increasing calcium (Ca^{2+}) influx and decreasing intracellular potassium (K^+), chloride (Cl^-), and water (H_2O). This process occurs through Ca^{2+} activated (Gardos) K^+ and K-Cl^- cation co-transport channels (5). Therefore, the sickle erythrocytes will suffer from dehydration. Dehydration produces rigid and less deformable sickle cells, which results in the inability of these cells to move easily during circulation through narrow blood vessels (1, 5, 6).

The plasma membrane is damaged as a side effect of intracellular Ca^{2+} accumulation. Damaging the sickle cell membrane leads to hemolysis in the early stages of erythropoiesis, which is sometimes called apoptosis (4). Moreover, circulating RBCs with a weak membrane leads to the production of RBC-derived microparticles. Microparticles are intact vesicles generated from cell membrane phospholipid asymmetry in RBCs. In SCD, MPs are present in the chronic or crisis stage and participate in the disease pathology. Chronic destruction of RBCs activates the coagulation pathway, resulting in thrombin formation and anticoagulant consumption (7).

When Hb S is polymerized oxygen (O_2) has difficulty binding. Nonpolymerized Hb S has an O_2 affinity similar to Hb A because the β6 mutation is far away from the O_2 binding position (3, 8, 9). Hemoglobin S requires 35.4 mmHg partial pressure (P_{50}), compared to normal Hb, which requires 26 mmHg, to be 50% saturated (10, 11). During oxygenated and deoxygenated cycles of Hb-O_2 transport, the iron is continuously converted from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) form (4). Oxygenated Hb naturally has an auto-oxidation property resulted with oxidized form of Hb called methemoglobin (metHb) HbFe(III), 0.5-3% per day, and small amount of superoxide or known as reactive oxygen species (ROS): superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) (12). The auto-oxidation rate for Hb S is high around 40% compared to that of normal Hb (6). In addition, the function of the anti-oxidant system in SCD patient is impaired (4). Therefore, ROS and metHb are always high and participated in the disease pathology such as accelerated hemolysis (13). This is a result of a reaction that occurs between metHb and H_2O_2 to produce oxoferrylHb (HbFe(IV)=O) and protein radicals present on the globin chain. Later on, several intracellular reactions take a place to end with products have a hydrophobic affinity for lipid binding. Therefore, the cell membrane is damaged and releases microparticles such as containing (12).

$$HbFe(III) + H_2O_2 \longrightarrow {}^{\bullet}HbFe(IV)=O + H_2O$$

The objective of this chapter is to investigate the effect of Delph-CL on the sickle cell membrane and hemoglobin, specifically through the assessment of membrane integrity, deformability, hemoglobin solubility, and hemoglobin oxygen binding affinity..

3.2 Materials and Methods

3.2.1 Biological Samples

Please refer to Section 2.2.1

3.2.2 Delphinidin Chloride Reagent Preparation

Please refer to Section 2.2.2

3.2.3 Blood Sample Preparation

Please refer to Section 2.2.3

3.2.4 Hemoglobin S Solubility

To assess the Hb S polymerisation after treatment with Delph-CL, a Pacific Haemostasis® kit (Sickle Screen® Sickling Hemoglobin Screening Kit) was used to measure the Hb S solubility (14). The principle of this kit is to lyse RBCs with a surfactant. The released Hb is reduced with sodium hydrosulfite. If Hb S is present the suspension becomes turbid, but it remains clear with Hb A. Four mL of phosphate buffer solution, which was already prepared, was added to one vial of sodium hydrosulfite powder. The test solution was recapped to mix well then kept for 15 minutes to dissolve. A 200 µL aliquot was transferred from each Delph-CL test concentration and the control sample into the test solution. After that, the tubes were recapped again, inverted a few times, and then incubated for 5 minutes at room temperature. For reading, the centrifugation method was used based on the TCS biosciences kit (TCS biosciences Ltd, Sickle-check screening test for hemoglobin – S). The principle of this kit is similar to Pacific Haemostasis®. The samples were centrifuged (Eppendorf 5810 Rotators) at 1000 x g for 5 minutes at 22 ˚C (15). To obtain a clear supernatant, the product was filtered through a medium-flow filter paper (VWR® Grade 413-18.5 cm) as Randolph and Wheelhouse method (16). Finally, 200 µL of each supernatant was aspirated into an appropriate well on a 96-well, flat bottom, medium binding EIA/RIA plate (Corning® Costar®). The absorbance of the Hb was read spectrophotometrically (Spectramax PLUS 384, Molecular devices, software 4.3) at 540 nm. The Hb absorbance value indicates the Hb solubility, which means a high absorbance reading demonstrates a high Hb solubility and low Hb polymerisation. Elevated levels of fetal Hb (Hb F) and severe anemia can also cause false-negatives. Hyperlipidaemia and the presence of abnormal protein can cause false-positives. Recent blood transfusion may cause false positives due to the increased percentage of Hct which leads to an increase in the Hb concentration (14, 15). On the other hand, it may also cause false negatives if Hb S levels are less than 20-25 % (17).

3.2.5 Deformability Test

After 24 hours of incubation at 37 ˚C sickle cells treated with various Delph-CL concentrations were brought to room temperature and mixed well by inversion until the suspension was homogeneous. Frozen deformability suspension media at –20˚C polyvinylpyrrolidone (PVP) (Sigma Aldrich, 360-1KG, 40% W/W at 26˚C) was warmed to room temperature before use. Ten microliters from each treated sickle sample was added to 1 mL of PVP. The sickle cell deformability was measured by ektacytometry using a laser-assisted optical

rotational cell analyser (LORCA), (Mechatronics, The Netherlands). Ektacytometry measures the cell elongation through an automated adjustable shear stress ranging from 0.95 to 30 Pa at 37 ˚C. To ensure the machine was warmed up to 37 ˚C two regular samples were run before the test sample. The elongation index (EI) at each shear stress point was calculated by measuring the vertical (A) and horizontal (B) axes of RBCs as follows (18):

$$EI = A - B/A + B$$

After that, the maximum elongation index (EI_{max}) at the infinite shear stress 30 Pa and the shear stress required to achieve a half of the EI_{max} (K_{EI}) were calculated via Eadie-Hofstee linearization (19). The result was a comparison between each Delph-CL concentration treated sickle cell and normal RBC. The deformability was measured to assess any changes of the sickle cell elasticity after treatment with Delph-CL. Improvements in this outcome mean an increasing EI_{max} and a decreasing K_{EI}.

3.2.6 Hemoglobin S-O_2 Binding Affinity

As a result of Hb S polymerisation, Hb S is not fully saturated with O_2. Therefore, improving the binding affinity between Hb S and O_2 using Delph-CL is crucial. The Hemox analyser (TCS Scientific Corp) was used to determine the 50% O_2 saturation level (P_{50}). The machine was prepared one day before use by changing the electrode membrane (TCS Scientific Corp), cleaning the electrode using a cleaning powder, and then placing one drop of KCL solution onto the O_2 electrode (TCS Scientific Corp). The cuvette was cleaned on the outside with a Kimwipe tissue. After that, the machine was turned on to warm up to 37 ˚C for 30 minutes. A distilled water sample was loaded to deoxygenate through exposure to nitrogen (N_2) gas, until the pO_2 reached 1.9 mmHg. Finally, a commercial control (RNA medical EQUIL Plus with glucose lactate–blood gas control Level 2 (Nova, QC463) was run by dispensing 50 µL of the control in 5000 µL of Hemox buffer (TCS Scientific Corp), 10 µL of anti-foaming agent (TCS Scientific Corp), and 20 µL of 22 % of albumin (Sigma Aldrich) into the dilution vial. The control was used to ensure that the O_2 electrode membrane placed correctly on the electrode and has the ability for gases (O_2 and N_2) to go through to oxygenate and deoxygenate the Hb molecules.

The control was loaded into the Hemox cuvette by using a fill/flush switch. Then, it was oxygenated for 15 minutes through a compressed air tank of pressure 20 PSI. After that, the S1

(gain) and S2 (balance) knobs were adjusted to read2.50. Therefore, the S1/S2 LED should read 0.000 $\pm$ 0.001. Finally, the pO_2 was adjusted to the daily value using the following calculation:

$$pO_2 = ((P\ Atm \times 0.7501) - pw) \times 0.209$$

Where: P Atm = atmospheric pressure (hPa read from the barometer),

0.7501 = altitude correction factor for Edmonton,

Pw = water vapor pressure (47 mm at 37 ˚C), and

0.209 = a multiplier derived from the oxygen content of the air (20.9%).

The control was deoxygenated for approximately 20-40 minutes through exposure to N_2 gas from a compressed N_2 tank with a pressure of 10 PSI. On the computer screen, the deoxygenation curve started to form. The P_{50} value was achieved when the curve was 100% complete, and the pO_2 reached 1.9 mmHg. The reference range of commercial control should be within 24.92-28.40 mmHg. Hemox analyzers need a long time to warm before used. After that, the tested sample was prepared and loaded into the Hemox cuvette for measurement similar to the control sample.

3.2.7 Osmotic Fragility Test

Sickle cells are characterized by a weak and fragile membrane, which results in hemolysis. Osmotic fragility was determined by incubating RBCs in hypotonic saline solutions (NaCl 9–0 g/L). The solutions were prepared and kept at 1- 6 ˚C. A 10 µL aliquot of sickle RBCs treated with Delph-CL for 24-hour at 37 ˚C was dispensed into 1 mL of each concentration. Normal RBCs suspended in PBS were used as controls. Each tube was mixed by inverting several times and allowed to incubate for 30 minutes at room temperature. Next, the samples were centrifuged (Eppendorf 5415 Rotators) at 2000 x g for 5 minutes at 4 ˚C to obtain the supernatant. Finally, 200 µL of each sample supernatant was transferred into appropriate wells of a flat bottom, medium binding 96 well EIA/RIA plate (Corning® Costar®). The spectrophotometer (Spectramax PLUS 384, Molecular devices, software 4.3) was used to read the optical density (OD) of each tube at 540 nm (19). The percentage of hemolysis was calculated at each concentration of NaCl as follows:

Hemolysis (%) = OD of Supernatant tube/ OD of 100% Hemolysis (0 g/L NaCl tube) x100 (20)

The % of hemolysis was plotted verses NaCl saline concentrations to obtain an osmotic fragility curve. Finally, the median cell fragility (MCF) was defined, which is the concentration of the buffer that caused 50% hemolysis (21).

3.2.8 Statistical Analysis

The IBM SPSS Statistics 22 software package was used for data analysis. Nonparametric tests were chosen, Wilcoxon sign-rank test was used to compare between treated and untreated negative control sickle cell samples. On the other hand, the Mann-Whitney test was used when comparing the normal RBCs positive control and the treated sickle cell samples.

3.3 Results

3.3.1 Hemoglobin S Solubility

The Hb absorbance on Y axis refers to the Hb solubility. A high absorbance value obtained demonstrates a soluble low polymerized Hb S. As seen in Figure 3.1, sickle cells treated with 1, 10 µg/mL demonstrate a high Hb absorbance with significant difference from untreated sickle cells ($P < 0.05$). In addition, 1, 10 and 100 µg/mL concentrations showed an insignificant difference from normal RBCs ($P > 0.05$).

3.3.2 Deformability

Figure 3.2 displays the sickle cell elongation index on the X axis vs. the elongation index at specific shear stress points 0.95–30 Pa. The elongation index (EI) of 1 and 10 µg/mL overlie untreated sickle cells (0 µg/mL). On the other hand, increasing the concentration of Delph-CL to 100 µg/mL demonstrated increasing cell rigidity by shifted the curve to the left.

Treating sickle cells with 1, 10, and 100 µg/mL did not achieve the normal RBC EI_{max} of 0.55 ± 0.04 with a significant difference ($p < 0.05$), Figure 3.3. However, 100 µg/mL had a minor improvement ($P= 0.16$) to 0.47 ± 0.06 compared to untreated sickle cells (0 µg/mL) 0.45 ± 0.06.

The other important parameter in deformability terms is K_{EI}, which means the shear stress required to achieve half of the EI_{max} (i.e rigidity). As consequence of low EI_{max} the cells required low K_{EI} to achieve half of EI_{max}. There were insignificant differences between 0, 1, and 10 µg/mL, Figure 3.4. On the other hand, 100 µg/mL was required to achieve a slightly higher K_{EI} to elongate the cell like normal RBCs.

3.3.3 Hemoglobin S–O_2 Binding Affinity

Treating sickle cells with Delph-CL resulted in a decrease in P_{50} at 10 and 100 µg/mL in a dose-dependent manner. Treatment with 100 µg/mL caused a significant difference ($p < 0.05$) in O_2–Hb binding compared to normal RBCs and untreated sickle cells (0 µg/mL), Figure 3.5.

3.3.4 Osmotic Fragility Test

Sickle cells physiologically are more resistant to hemolysis when incubated at decreasing hypotonic saline solutions (NaCl 0-9 g/L). Figure 3.6 (A and B) showed insignificant sickle cell fragility ($P = 0.6$) compared to untreated sickle cells (0 µg/mL). Furthermore, 50% of treated sickle cell with 1 and 10 µg/mL where hemolyzed at hypotonic solution with MCF values = 4.8 $\pm$ 0.2, 4.9 $\pm$ 0.3, respectively (Figure 3.7). On the other hand, the fragility of sickle cells was increased in 100 µg/mL as seen in Figure 3.6 (C) where the curves shifted to the lift. That was demonstrated insignificant difference ($P > 0.05$) of the MCF to normal RBC 5.34 $\pm$ 0.12 g/L.

3.4 Discussion

Delph-CL decreased the polymerization of Hb S at 1 and10 µg/mL (see Figure 3.1). Depolymerizing means that Hb S is converted from a fiber or polymer into a soluble form. This result suggests that Delph-CL can enter and hydrate the sickle cells. The hydration mechanism could be developed through Ca^{2+} activated (Gardos) K^+ and/or K^+Cl^- cation co-transport channels by decreasing Ca^{2+} and increasing K^+, Cl^- and H_2O intracellular (supernatant K^+ measurement will be discussed in Chapter 4). On the other hand, it may a result from Delph-CL's polarity due to the presence of 6 hydroxyl (OH) groups (see Table 1.2) in its chemical structure. A highly polar Delph-CL might decrease the hydrophobicity of valine in Hb S after a bond forms between Delph-CL's O+ in the heterocyclic ring and valine amino acid carboxyl group, Figure 3.8.

Hydroxyurea (Hu) treatment, is used by most of the sickle patients, which may interfere with the result by depolymerized the Hb S through the elevation the of fetal hemoglobin (Hb F) as described previously in Section 1.2.1.1 (4).

Oxygen binds easily with a low P_{50} in 100 µg/mL as an effect of decreasing the Hb S polymerization. Non-polymerized Hb S has a similar to normal Hb A-O_2 binding affinity (8, 9). Unexpectedly, the normal RBC also had very low P_{50}. However, there are experimental factors that may participate in decreasing the P_{50}, such as testing the sample after a long period (24

hours). Blood gas should be tested immediately after blood collection, otherwise the P_{50} is decreased due to elevations of metHb and decreased 2,3-diphosphoglycerate (2,3 DPG) (22). As seen in Figure 3.5, there was not much difference in P_{50} between the normal RBCs and untreated sickle cells, which indicates that metHb is elevated in both of them to similar levels due to the influence of the incubation time. In terms of the sickle sample, the metHb is elevated naturally in SCD patients due to the Hb S auto-oxidation as described previously. It is achievable to have a strong binding between Hb S and O_2 with low P_{50}, but it becomes harder to release the O_2 to the tissue. The consequence of that is tissue hypoxia, which is the main problem in SCD patients.

Sickle cells are resistant to osmotic stress due to their abnormal shape (20). Exposure to 1 and 10 µg/mL showed insignificant difference from the untreated sickle cells, Figure 3.6 (A and B). That indicates most of the sickle cell shape was not reversed. On the other hand, Delph-CL at 100 µg/mL increased the fragility close to normal RBC, as a result of the sickle cell reversing where only 9.7 % of sickle cell was remained after treatment as shown in Figure 2.5.

Delphinidin chloride did not improve sickle cell elasticity at any tested concentration. It caused cell membrane rigidity at 100 µg/mL, and this slightly higher K_{EI} was necessary to elongate the cell as compared to normal RBCs, Figure 3.4. This result is opposite to Ballas' finding that 400 µg/mL *Pafaffia paniculata* is able to increase the deformability index of sickle cells to be closer to normal RBCs (23). The short incubation period of 4 hours rather than the 24 hours, which was chosen as standard time for anti-sickling experiment, of *P. paniculata* may boost the sickle cell elasticity. The deformability was measured by ektacytometry because it is fully automated and needs only a small volume of blood sample. In addition, the machine measures the deformability at 37 ˚C, which is similar to incubation temperature. However, the result is based on an average of all RBCs, after calculating the best elliptical shape after transition from biconcave under increasing shear stress.

To conclude, Delph-CL is able to depolymerise Hb S at 1 and 10 µg/mL. Converting the Hb S to a soluble form allows the Hb to become oxygenated. However, it does not improve the sickle cell elasticity; instead, it produced rigid and less deformable cells. The rigid cells were easy to hemolyze as seen in the osmotic fragility test (Figure 3.6) and hemolysis assay (Figure 2.3).

The inverse relationship between hemolysis and deformability was previously established (24) as obtained here. Low deformability was related to a high hemolysis rate in the sickle cells. The released Hb reacted directly with Delph-CL to become soluble and oxygenated Hb. As there was a sickle cell reversal and improvement in Hb S solubility and O_2 binding in 100 μg/mL of Delph-CL,this concentration will be considered for the biomechanism investigations to follow.

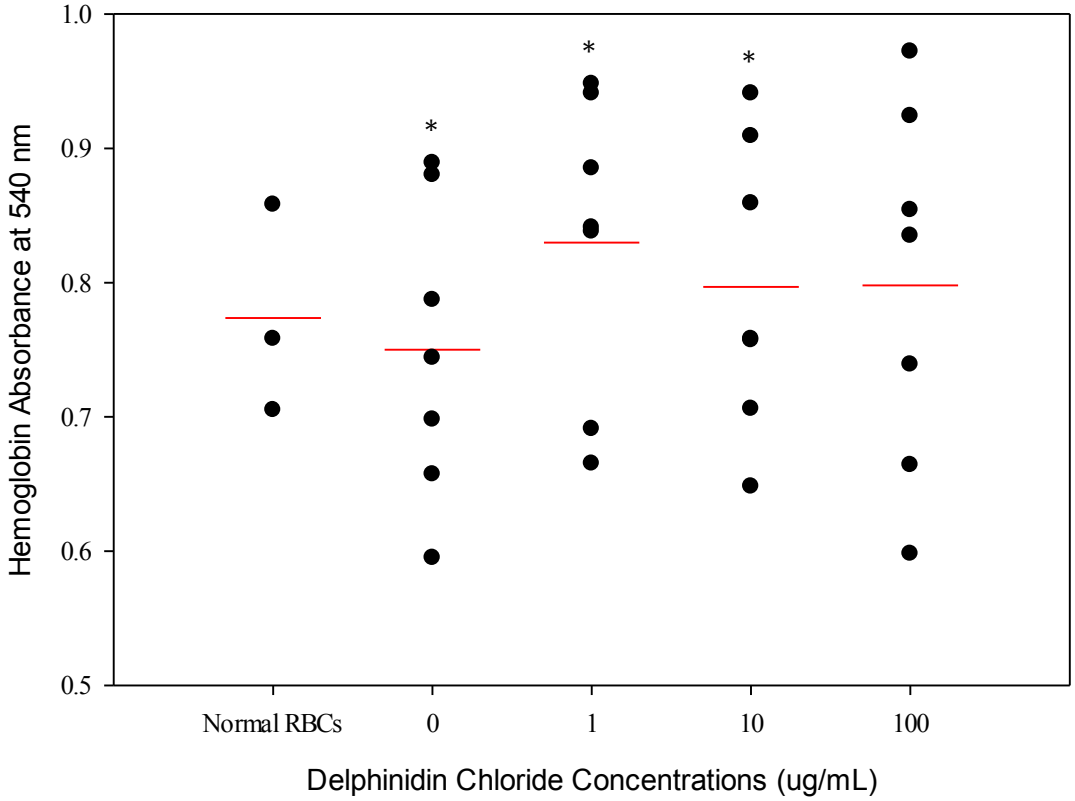

Figure 3.1: The hemoglobin solubility of normal RBC (Hb A) and sickle cell (Hb S) at 540 nm (n = 7). (*) Denotes a significant difference between 1 and 10 µg/mL and 0 (untreated sickle cell), $p < 0.05$.

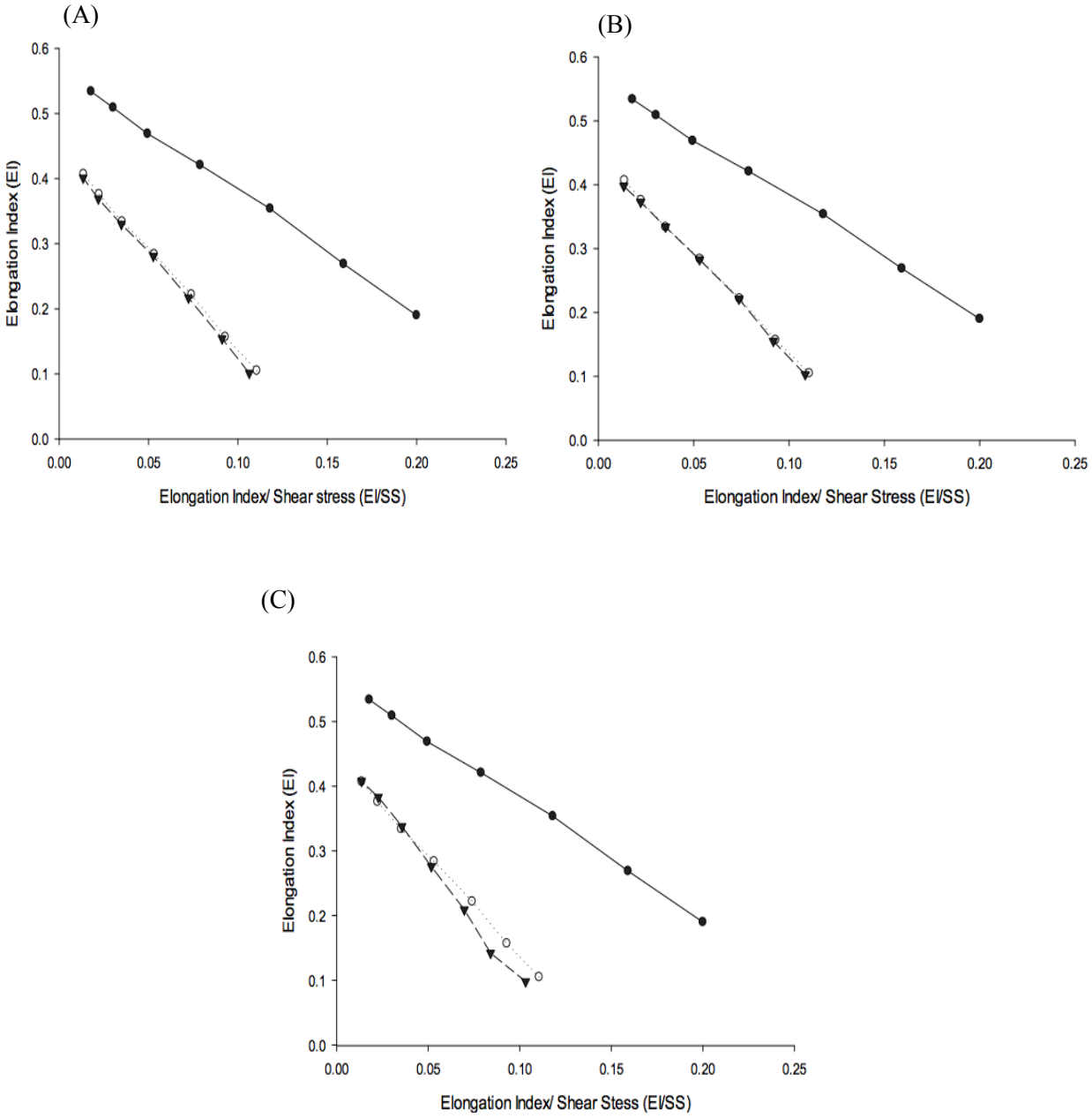

Figure 3.2: The deformability of sickle cells treated with Delph-CL after incubation for 24 hours at 37˚C: (●) Normal RBCs; (○) 0 µg/mL; and (▼) A: 1, B: 10 and C: 100 µg/mL.

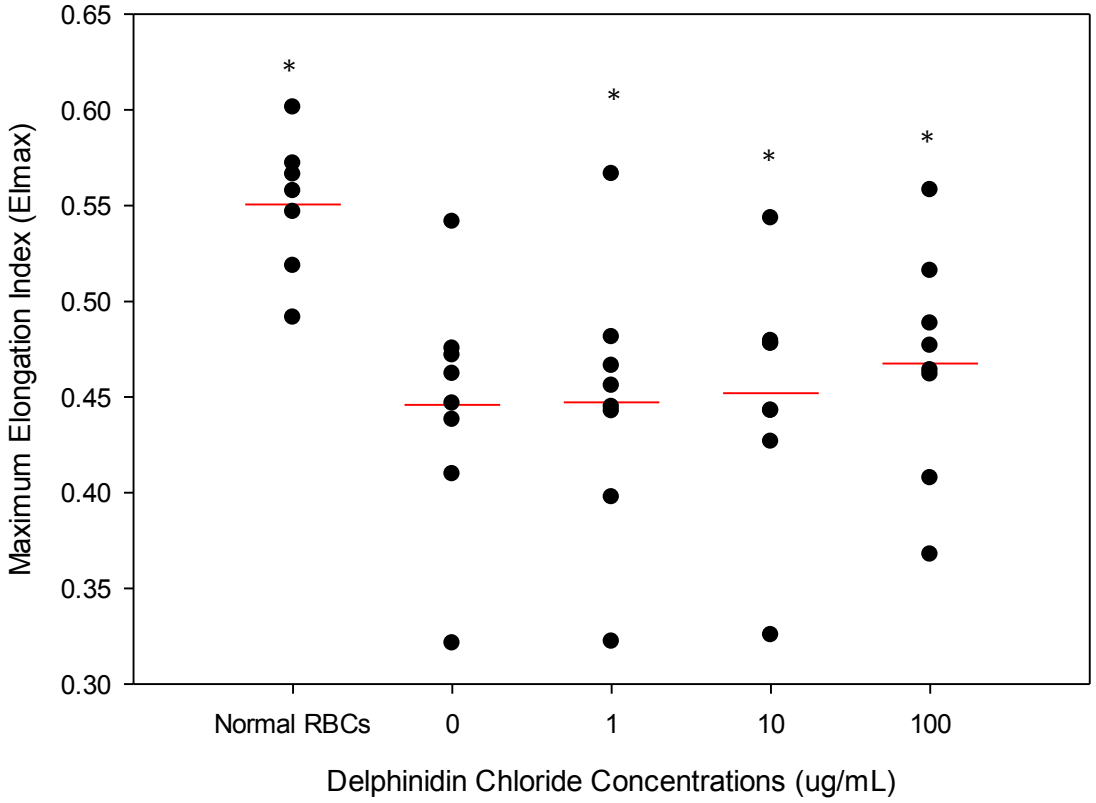

Figure 3.3: The maximum elongation index (EI_{max}) at infinite shear stress 30 Pa of sickle cells treated with Delph-CL. The EI_{max} values were calculated via Eadie-Hofstee linearization.

(*) Denotes a significant difference between 1, 10 and 100 µg/mL and normal RBCs, $p < 0.05$.

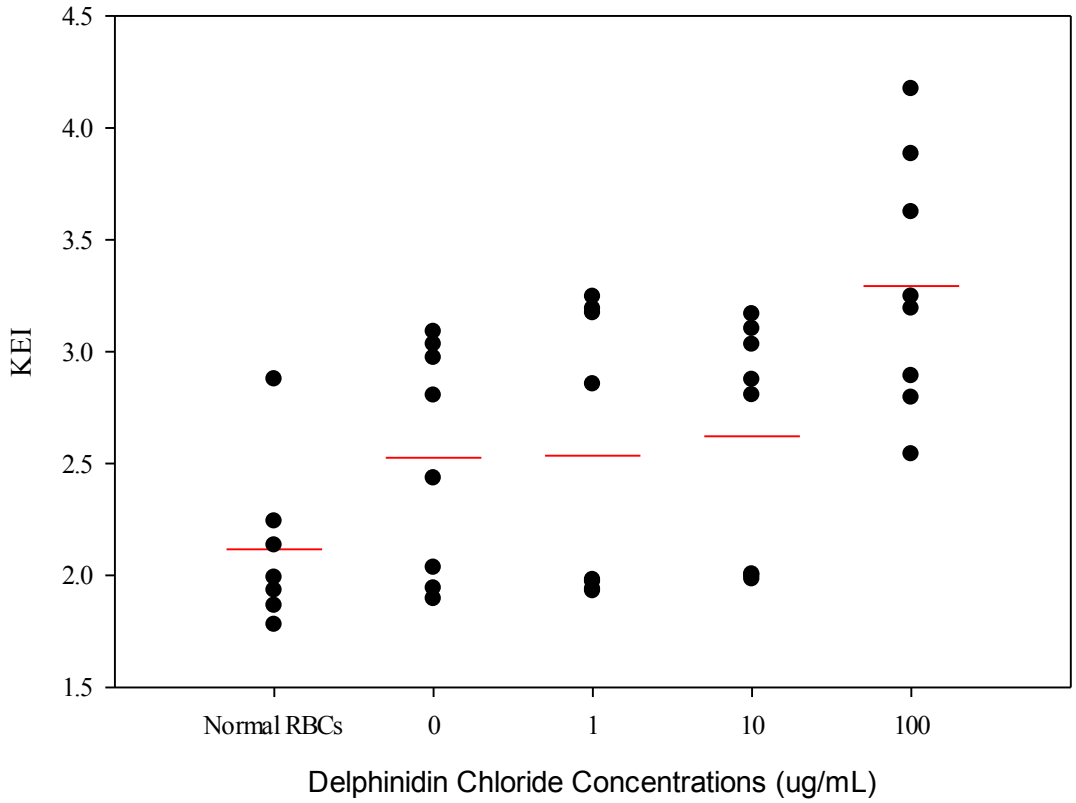

Figure 3.4: The K_{EI} values of sickle cells treated with Delph-CL were calculated via Eadie-Hofstee linearization (n = 8).

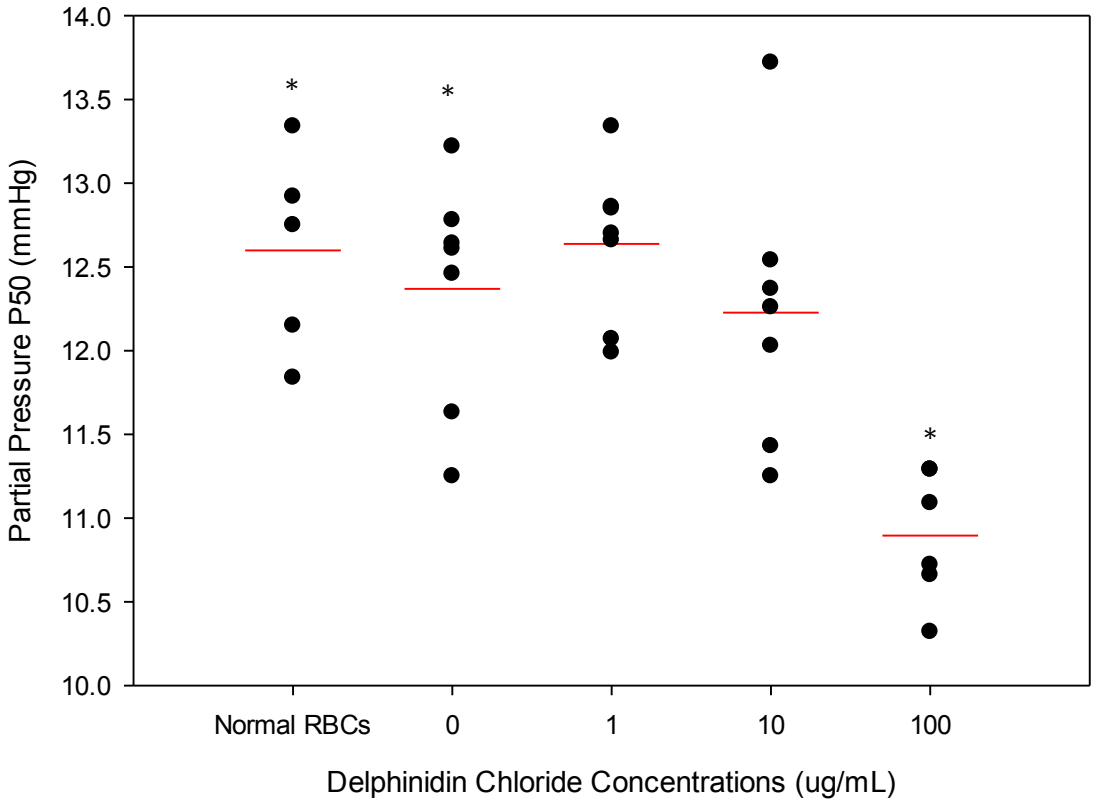

Figure 3.5: The partial pressure (P_{50}) of sickle cells after exposure to different concentrations of Delph-CL (n = 7). (*) Denotes a significant difference between 100 µg/mL and both 0 (untreated sickle cell) and normal RBCs, $p < 0.05$.

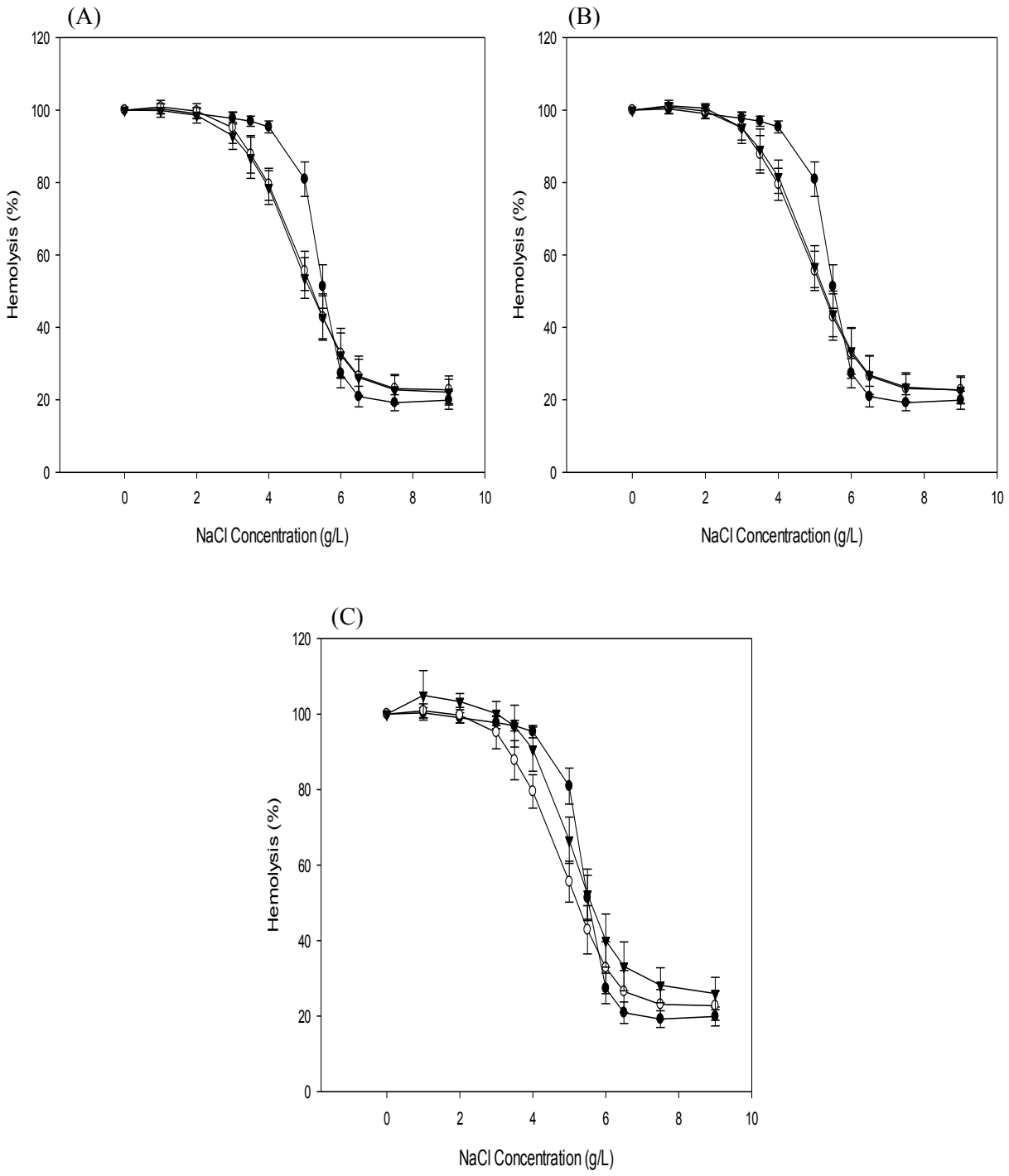

Figure 3.6: The osmotic fragility curve of sickle cells after treated with Delph-CL for 24 hours at 37 ˚C **(**n = 8): (●) Normal RBCs; (○) 0 µg/mL; and (▼) A: 1, B: 10 and C: 100 µg/mL.

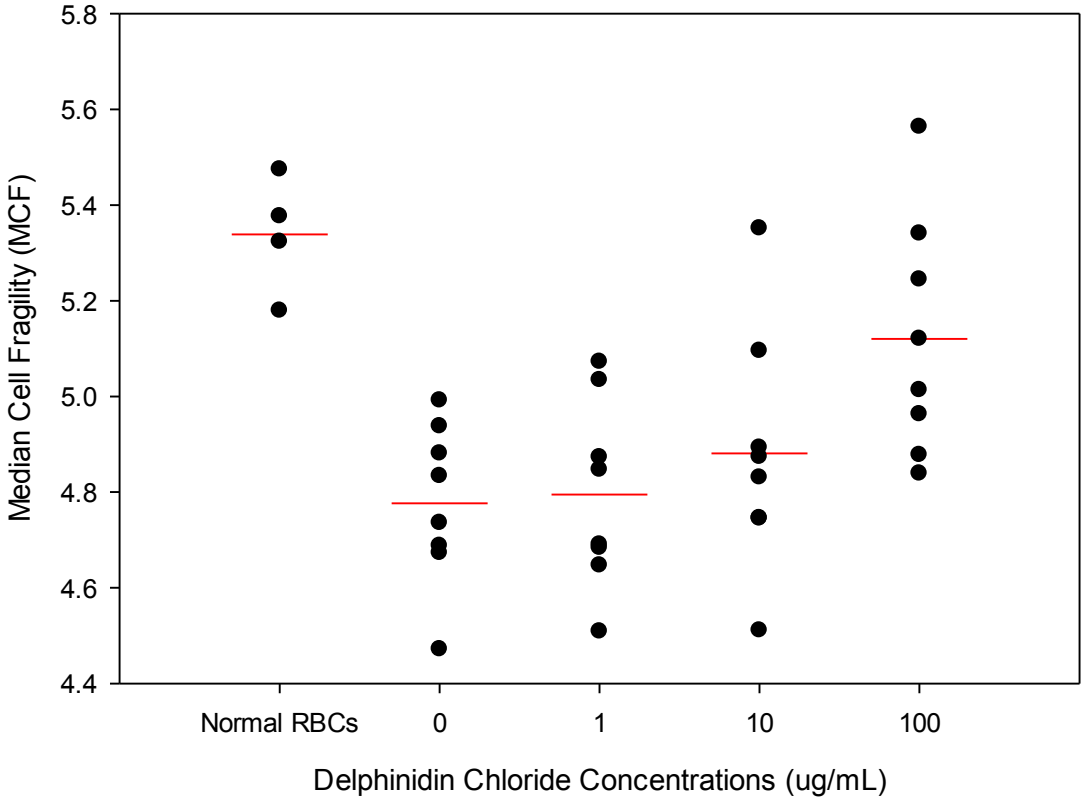

Figure 3.7: The median cell fragility of sickle cells treated with Delph-CL for 24 hours at 37 ˚C (n=8).

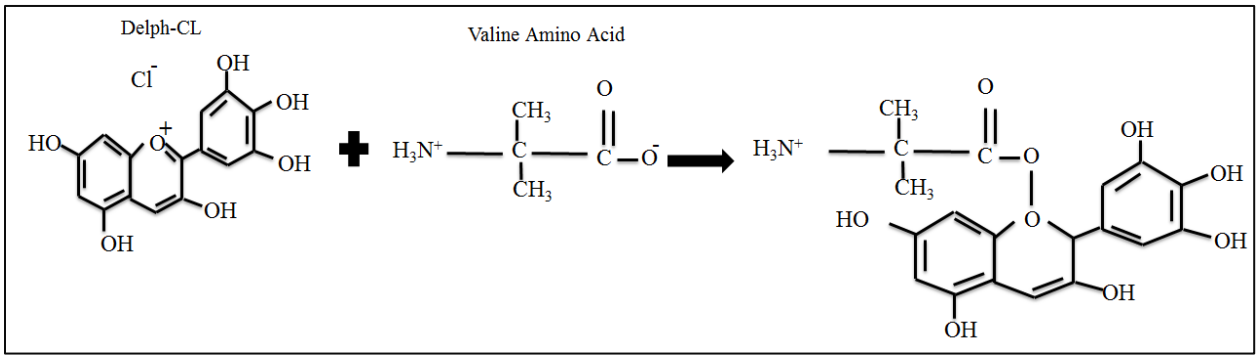

Figure 3.8: The reaction between Delph-CL and valine.

3.5 References

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4 The Biomechanism of Delphinidin Chloride on Sickle Cell Reversal

4.1 Introduction

Sickle cell reversal following delphinidin chloride (Delph-CL) treatment may occur via 2 different biomechanisms. Reversal of the sickle cell to the normal biconcave disc shape could be achieved through a hydration biomechanism. In sickle cell disease (SCD), replacing the glutamic amino acid with the valine amino acid forms a hydrophobic bond between valine and the nearby amino acids alanine, phenylalanine and leucine. In deoxygenated conditions, this hydrophobic bond converts hemoglobin S (Hb S) from soluble to fibers or polymers (1, 2). After polymerization, the density of Hb is increased by elevating the mean cell hemoglobin concentration (MCHC) to a range of 230–500 g/L (3). This high-viscosity Hb promotes sickle cell dehydration. Moreover, the membrane Ca^{2+}-activated K^+ (Gardos) and/or K–Cl co-transport channels are also involved (4). Activation of these channels increases calcium (Ca^{2+}) and decrease potassium (K^+) and water (H_2O) intracellularly (3, 4). Inhibitor treatment of the Gardos and K–Cl co-transport channels is a potential treatment that is currently under clinical investigation (5). Herbal remedies have been tested to ascertain whether they can properly hydrate sickle cells. The powder of *Pfaffia paniculata* root improved SCD symptoms (6). The finding was presumed to be the result of sickle cell hydration with increases in the mean cell volume (MCV) and decreases in the MCHC. These were accomplished after significantly increasing the intracellular sodium ion (Na^+) concentration. On the other hand, polyphenol and anthocyanin (ACs) extracts from honeysuckle were hypothesized to hydrate the hydrophobic portion of the red blood cell (RBC) lipid membrane, however, it was not achieved (7). Therefore, herbal treatment most likely targets RBC cation hemostasis rather than affecting the cell membrane.

The second biomechanism by which sickle cell reversal may occur is through antioxidation. The AC chemical structure is composed of 2 aromatic rings (A and B) and 1 heterocyclic ring (C) containing one oxygen (O_2) atom. The electron deficiency in that ring is responsible for the AC antioxidant activity (Figure 1.5) by accepting unpaired electrons from free radicals of reactive oxygen species (ROS) (8, 9).

Several studies have reported that ACs have an antioxidant effect on normal and sickle RBCs by decreasing the concentration of ROS through lipid peroxidation inhibition, or hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$) scavenging (10-12). As is presently known, Hb S is unstable and is oxidized easily based on its redox potential (auto-oxidation) rate of 40% as compared to that of normal Hb (3). The auto-oxidation of oxygenated Hb [$HbFe(II)O_2$] and deoxygenated Hb [HbFe(IV)=O] through H_2O_2 produces methemoglobin (metHb) [HbFe(III)] and ROS.

$$HbFe(II)O_2 + H_2O_2 \longrightarrow HbFe(IV)=O + H_2O_2 \longrightarrow HbFe(III) + O^{\bullet-}{}_2 + H_2O \quad (13)$$

In SCD, the antioxidant enzymes superoxide dismutase, catalase, glutathione peroxidase and nitric oxide are too deficient to sufficiently scavenge ROS (14). Therefore, ROS participates in SCD pathogenesis by accelerating hemolysis, causing hypercoagulability and endothelial dysfunction (15). In terms of hemolysis, ROS are the major cause of RBC membrane injury, targeting the membrane lipid bilayer via the lipid peroxidation mechanism (3, 15).

In addition, the auto-oxidation of Hb S elevates metHb, as described in the aforementioned formula. MetHb is the oxidized inactive form of Hb carrying iron atoms in ferric form (Fe^{3+}) instead of the typical ferrous form (Fe^{2+}). Normally, in healthy individuals, metHb comprises only 3% of the total Hb (3). In patients with SCD, the metHb formation of Hb S is 1.7 times faster than that of Hb A (15). MetHb is not considered a gas transporter, as it preferentially binds H_2O instead of O_2 or carbon dioxide (CO_2) (13). Therefore, the limited O_2 concentrations provided to the tissues causes hypoxia. In addition, binding Hb to H_2O within the RBC in deoxygenated conditions induces sickling (14). One-hour incubation of sickle cells with AC extracts from 42 different plants collected from Kisangani and Babogombe significantly decreased metHb concentrations (16). Delphinidin (Dp) ACs were also chosen for sickle cell reversal. The Dp-3-*O*-glucoside fraction in Taurasi red wine exhibited a strong antioxidant effect by decreasing ROS in normal RBCs after induction with 10 μM H_2O_2 after 5-minute incubation (10).

This chapter aims to examine the K^+ and metHb levels, which were measured to determine whether the hydration or antioxidation biomechanism, or both, reverses the sickle cell morphology. The 2 parameters were measured after 24 hours incubation at 37 ˚C. Normal and sickle RBCs were treated 1:1 with phosphate-buffered saline (PBS) as the control and 100

μg/mL of Delph-CL as the treated sample. The concentration of 100 μg/mL of Delph-CL was selected to study the sickle cell reversal biomechanisms. This concentration was used because it effected significant sickle cell reversal as compared to untreated sickle cells (P = 0.04), with a remarkable decrease in the sickle cell population, specifically, from 23.3 % to 9.7 % after 24 hours incubation in deoxygenated conditions, as seen in Chapter 2.

4.2 Materials and Methods

4.2.1 Biological Samples

Please refer to Section 2.2.1

4.2.2 Delphinidin Chloride Reagent Preparation

Please refer to Section 2.2.2.

4.2.3 Blood Sample Preparation

Please refer to Section 2.2.3

4.2.4 Hydration Biomechanism

To determine sickle cell hydration, the extracellular K^+ concentration in the sample supernatant was measured after 24 hours incubation with Delph-CL. After incubation, all samples were centrifuged at 2200 ×g at 4˚C for 10 minutes. Then, the clear supernatant was transferred to 1.5 mL microtubes. The obtained volume was around 1 mL per sample. The microtubes were labeled, and the samples were sent for analysis at the University of Alberta Hospital in the Chemistry lab. The K^+ concentration in the sample supernatant was measured by using a Beckman Coulter UniCel DxC 800 Synchron Clinical System. The value indicates the concentration of extracellular K^+ relative to the RBCs (6). As it is known that extracellular K^+ is very high in the dehydrated sickle cells and it is replaced with Ca^{2+}, therefore the desired result of Delph-CL moves K^+ into the cells and effects hydration.

4.2.5 Antioxidant Biomechanism

To compare the antioxidant effect of Delph-CL, vitamin C (ascorbic acid) was used as a natural anti-oxidant. Vitamin C was prepared by dissolving 20 mg, L (+) ascorbic acid powder (EMD

Chemicals Inc.), measured on a Mettler Toledo AX205 microbalance, in 100 mL PBS for a stock concentration of 200 µg/mL. This specific concentration was chosen because it is similar to that of Delph-CL. The stock solution was stored at 1–6˚C. The sickle cell suspension sample with 14–28 % Hct was divided into 2 treatment groups: Delph-CL and vitamin C. Based on the sickle cell suspension volume, an equal amount of RBC suspension and each treatment was pipetted in to separate microtubes. The reason for using equal volumes has been described in Section 4.2. The final Hct of the tested samples after treatment was 7–14 %. The final Delph-CL and vitamin C concentrations were also changed to 100 µg/mL. All samples were incubated at 37˚C for 24 hours.

After 24 hours, around 1 mL sample was obtained to measure hemolysis and Hb oxidation to determine the baseline levels before applying the oxidative stress agent, H_2O_2. Drabkin's method was used to measure hemolysis as described previously in 2.2.6. For Hb oxidation, 100 µL RBC suspension sample was lysed after incubation in 5 mL dH_2O. Each tube was mixed gently and then 200 µL was transferred into an appropriate well in a flat-bottom, medium-binding 96-well EIA/RIA plate (Corning Costar). At pH 7.2 (Mettler Toledo SevenMulti pH/conductivity meter), the absorbance of metHb, oxygenated Hb (OxyHb) and deoxygenated Hb (DeoxyHb) was read by the SpectraMax PLUS 384 spectrophotometer, (Molecular Devices, software 4.3) at 560, 576, 630 and 700 nm. The concentrations of each parameter were calculated as follows:

$$[\text{MetHb}] = 2.7\ (OD_{630} - OD_{700}) + 0.2\ (OD_{577} - OD_{700}) - 0.4\ (OD_{560} - OD_{700})$$

$$[\text{OxyHb}] = 0.6\ (OD_{630} - OD_{700}) + 1.4\ (OD_{560} - OD_{700}) - 1.9\ (OD_{576} - OD_{700})$$

$$[\text{DeoxyHb}] = 0.6\ (OD_{630} - OD_{700}) - 1.2\ (OD_{560} - OD_{700}) + 0.7\ (OD_{576} - OD_{700})$$

Thereafter, the percentage of metHb was calculated as follows:

$$\text{MetHb (\%)} = \text{MetHb concentration} \times 100/\text{Total Hb concentration (MetHb + OxyHb)}$$

Around 1 mL remaining blood sample from each treatment group was dispensed in to a clean tube. One mL of 4 mmol/L stock solution sodium azide (NaN_3), stored at 1–6˚C, was added to a final concentration of 1 mmol/L. The NaN_3 was used to inhibit the natural antioxidant catalytic enzyme activity. The NaN_3 was prepared by dissolving 13 mg NaN_3 powder, measured on a Mettler Toledo AX205 microbalance, in 50 mL PBS. Then, 1 mL of 2 mmol/L (30 %) H_2O_2 was

added to the sample for a final concentration of 0.5 mmol/L. The H_2O_2 was prepared by dispensing 11.3 µL H_2O_2 solution in 49.98 mL PBS. All treated samples were incubated at 37 ˚C for 1 hour to assess the effect of Delph-CL in decreasing or preventing Hb oxidation in the presence of H_2O_2 oxidative stress. After 1 hour, the percentages of hemolysis and Hb oxidation were measured as described earlier. At pH 7.4, the concentration of metHb, OxyHb and DeoxyHb was re-calculated as follows, based on the pH change after absorbance had been read at 560, 576, 630 and 700 nm:

$$[\text{MetHb}] = 2.9\,(OD_{630} - OD_{700}) + 0.4\,(OD_{560} - OD_{700}) - 0.2\,(OD_{576} - OD_{700})$$

$$[\text{OxyHb}] = 0.7\,(OD_{630} - OD_{700}) - 1.4\,(OD_{560} - OD_{700}) - 2\,(OD_{576} - OD_{700})$$

$$[\text{DeoxyHb}] = 0.7\,(OD_{630} - OD_{700}) - 0.7\,(OD_{576} - OD_{700}) + 1.3\,(OD_{560} - OD_{700})$$

The percentage of metHb was calculated as described earlier. The results were a comparison of the percentage of hemolysis and metHb between sickle cells untreated and treated with Delph-CL and vitamin C.

4.2.6 Statistical Analysis

The IBM SPSS Statistics 22 software package was used for data analysis. Nonparametric tests were chosen. The Wilcoxon signed rank test was used to compare sickle cells treated with Delph-CL or vitamin C and the untreated negative controls. The Mann–Whitney test was used for comparisons with the normal RBC positive control.

4.3 Results

4.3.1 Extracellular Potassium Electrolyte Concentration

The hydration biomechanism was tested by measuring K^+ concentrations in the sample supernatant after 24-hour incubation with 100 µg/mL Delph-CL at 37˚C. Delph-CL caused significant K^+ leakage into the extracellular environment in treated sickle cells as compared to untreated (0 µg/mL) sickle cells, $p = 0.04$. However, there were non-significant differences in the K^+ concentrations of normal RBCs and untreated sickle cells ($p = 0.1$), as seen in Figure 4.1.

4.3.2 Methemoglobin Concentration

The percentage of metHb was measured before and after H_2O_2 exposure, as seen in Figure 4.2. The Wilcoxon signed rank test revealed non-significant differences in the metHb percentages before and after H_2O_2 exposure in all groups ($p = 0.06$). Post-H_2O_2 exposure, all treatment groups had high levels of metHb formation that was not significantly different ($p > 0.05$) as compared to pre-treatment levels. Both vitamin C and Delph-CL caused Hb oxidation and elevated the metHb to 50 % and 48.2 %, respectively.

The percentage of hemolysis supported the Hb oxidation results. Overall, there was no significant difference in the percentage of hemolysis before and after H_2O_2 exposure in any group (Figure 4.3), $p > 0.05$. Delph-CL treatment before at the baseline, i.e., before H_2O_2 exposure, caused significantly more RBC lysis ($p = 0.008$) than that of the natural antioxidant vitamin C. Consequently, hemolysis increased more after exposure to H_2O_2.

4.4 Discussion

To determine sickle cell hydration through Delph-CL, concentrations of the cation K^+ were measured in the sample supernatant. Significant increases in extracellular K^+ levels were caused by 100 µg/mL Delph-CL as compared to the untreated sickle cells, which demonstrated dehydration as seen in Figure 4.1. In the study by Ballas (6), the intracellular sickle cell cations Na^+ and K^+ were measured using flame photometry. Sickle cells were incubated with 400 µg/mL P. *paniculata* for 5 hours at 37˚C. The results revealed increased intracellular Na^+ but decreased the K^+, indicating that extracellular K^+ was elevated, which were the same results obtained in the present study. Therefore, Delph-CL causing K^+ leakage could be the result of inactivation of the Gardos and/or K–Cl co-transport channels. The other explanation is that 100 µg/mL Delph-CL overhydrated the sickle cells by increasing the MCV to 101 fL ± 7 as compared to that of untreated sickle cells: 92.32 fL ± 6.2, as seen in Figure 2.7 ($p < 0.001$). Subsequently, 100 µg/mL Delph-CL caused hemolysis ($p < 0.05$), as depicted previously in Figure 2.3, via cell membrane damage. Overall, Delph-CL encouraged further dehydration of the sickle cells.

Methemoglobin was measured as a major outcome product in the Hb oxidation process. The catalytic enzyme activity was inhibited by NaN_3 in order to uncover the Delph-CL antioxidant properties. The results revealed non-significant differences in the metHb percentages across the treatment groups both before and after exposure to H_2O_2 ($p = 0.06$), as seen in Figure 4.2. This

indicates that the metHb levels were high before treatment with the oxidizing agent. This was also shown in another study, where a normal RBC aliquot was treated with Taurasi red wine containing Dp3-*O*-glucoside, peonidin 3-*O*-glucoside and malvidin 3-*O*-glucoside AC fractions. There was not much difference in the metHb before and after 3-hour exposure to H_2O_2 at room temperature (10). Vitamin C caused a significant induction in Hb oxidation. This finding was expected based on the reduction of Hb in Fe^{2+} form as a natural antioxidant protective effect. The antioxidant property of vitamin C first goes through a pro-oxidant step, rendering the vitamin C easy to oxidize and therefore produces dehydroascorbic acid. This occurs in the presence of O_2 and produces H_2O_2. Hydrogen peroxide causes DNA damage and cell death (17), and this may have occurred in the present study when sickle cells were incubated with vitamin C for long durations (i.e., 24 hours). Figure 4.2 also shows that the difference between the metHb concentrations in RBCs treated with PBS before and after H_2O_2 exposure is about 60%, whereas it decreased to 40% following the vitamin C and Delph-CL treatments. However, neither vitamin C nor Delph-CL could protect the cells from oxidation caused by H_2O_2. Exposure to H_2O_2 promoted Hb oxidation further, although not significantly ($p > 0.05$) in all treatment groups.

To sum up, Delph-CL enhanced sickle cell dehydration by discharging K^+ extracellularly. This was achieved after a hemolyzed toxic effect occurred following Delph-CL treatment. On the other hand, the antioxidant effect of Delph-CL did not decrease the Hb oxidation. On the contrary, Delph-CL caused Hb S oxidation by elevating metHb levels. Therefore, the biomechanism of reversal by 100 µg/mL Delph-CL ($p= 0.04$) achieved in this study is not defined yet. Accordingly, further study to define the sickle cell reversal biomechanism is required, but taking into account the limitations faced in this study. For example, in the hydration biomechanism, a short incubation period of 5 hours, as used by Ballas (6), could be used instead of 24 hours to measure the K^+ cation. Moreover, to assess the antioxidant effect of Delph-CL, it might be possible to add the natural antioxidant vitamin C to Delph-CL during preparation to increase the antioxidant efficacy as reported by Cesquini et al. (18). They found that, compared to quercetin alone, the combination of ascorbic acid and quercetin flavonoid decreased the Hb oxidation of sickle RBCs after oxidation had been induced by *tert*-butyl hydroperoxide (*t*-BOOH). Regarding the high level of Hb oxidation in sickle cells resulting from vitamin C treatment, a combination of two vitamins, such as vitamin C and E, may be used. Vitamin C functions as an antioxidant while vitamin E is an anti-hemolytic. Therefore, they might function

together to ultimately reduce Hb S oxidation, as reported by Atyabi et al. in their *in vitro* animal study, by reducing metHb significantly at 5 mmol/L (µg/mL) vitamin C and E (19).

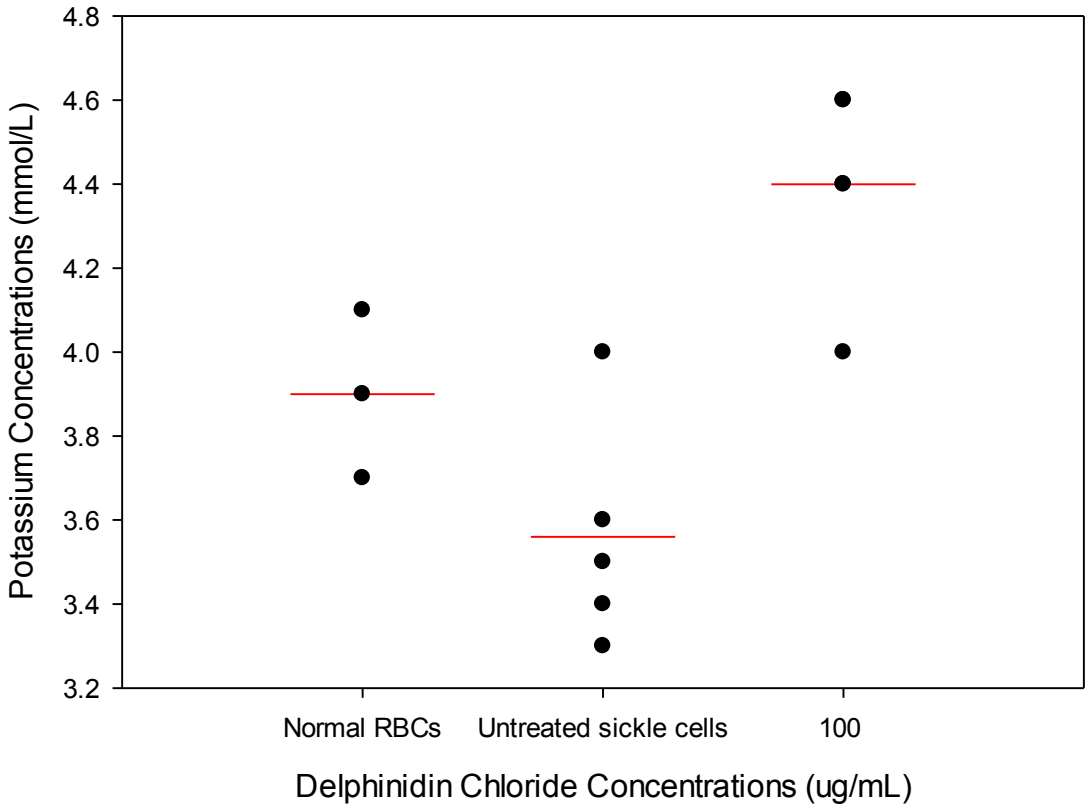

Figure 4.1: The K^+ concentrations in the sample supernatant after 24 hours incubation at 37 ˚C (n = 5).

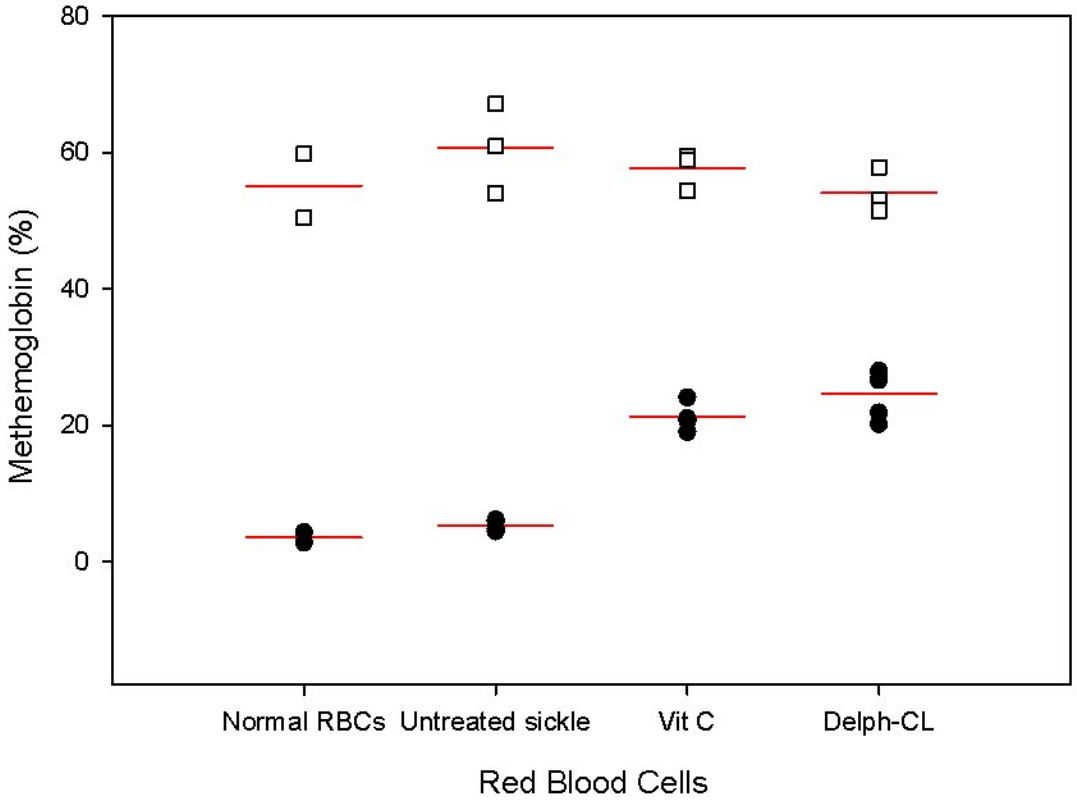

Figure 4.2: The percentage of metHb: (●) before; (□) after H_2O_2 exposure (n = 5).

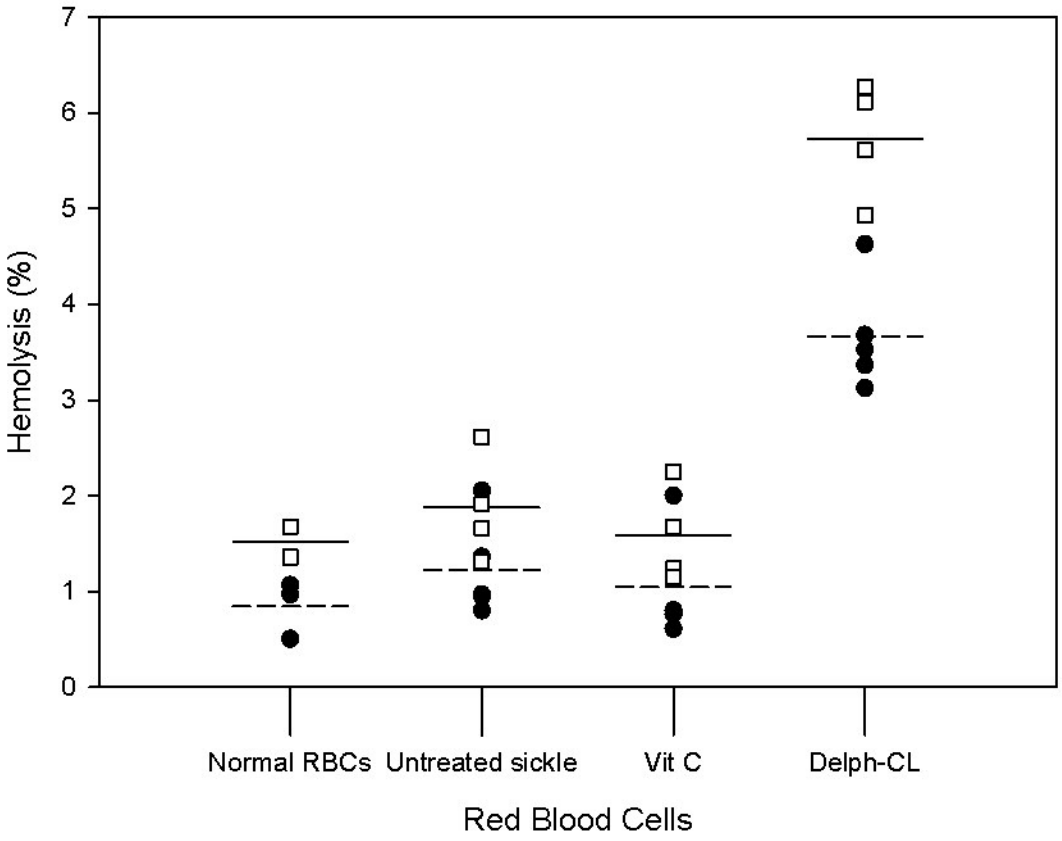

Figure 4.3: The percentage of hemolysis: (●, ---) before; (□,¬) after H_2O_2 exposure (n = 5).

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5 Discussion and Conclusion

This study hypothesized that delphinidin chloride (Delph-CL) can recover abnormal sickle cell morphology to a normal biconcave disc through inhibition of hemoglobin S (Hb S) polymerization. After applying 100 µg/mL Delph-CL for 24 hours in deoxygenated conditions, the sickle cell morphology was reversed by transformation from a sickle to an elliptical shape, as observed in Figure 2.6 (D). Delph-CL caused a remarkable decrease in the sickle cell population ($p = 0.04$) from 23.3 % to 9.7 %. At that concentration also, the mean cell volume (MCV) was increased significantly post incubation, $p = 0.03$, as seen in Figure 2.7, which indicated that Delph-CL had the ability to change the sickle cell morphology by increasing the cell volume. The MCV value obtained in this study was similar to Ballas' finding (111 ± 12 fL) when sickle cells were incubated with 400 µg/mL of *Pfaffia. paniculata* at 37 ˚C for 5 hours (1). This finding was referred to the *P. paniculata* hydration effect caused by increasing the intracellular sodium from 0.96 ± 0.31 to 1.6 ± 0.37 mEq/10 g Hb.

Unfortunately, a complete shape reversal was not achieved, as most of the cells were hemolyzed in a dose-dependent manner, as shown in Figure 2.3. At 250 µg/mL, the sickle cells were distorted and the shape was unrecognizable. This type of membrane distortion was also observed after incubating pig erythrocytes with 10 and 100 µg/mL of blackcurrant (*Ribes nigrum L.*) and blue honeysuckle (*Lonicera caerulea L. var. kamtschatica Sevast.*) extracts for 1 hour at 37 ˚C, after which the cells showed echinocytes, specifically disco-echinocytes (2, 3). Further study of blue honeysuckle found the projection formation of echinocytes was due to accumulation of polyphenolic compounds in the outer monolayer of the erythrocyte membrane. Membrane fluorescent examination revealed the extract was incorporated and caused disarrangement of the hydrophilic part of the lipid layer (3). As described in Chapter 2, the diffusibility of Delph-CL depends on cell membrane viscosity. Consequently, different RBC shapes were seen in the microscopic examination that varied from reversed to hemolysed cells. Accordingly, the hemolysis result in Figure 2.3 supports the increased red blood cell (RBC) fragility in hypotonic solutions of NaCl (9-0 g/L). Sickle cells treated with 100 µg/mL Delph-CL had increased fragility compared to 1 and 10 µg/mL, Figure 3.6. This assumes that the reversal of the sickle cell to normal biconcave disc impacts the membrane fragility. Overall, with increasing hemolysis, the RBCs had decreased deformability (4).

The hemolysis due to Delph-CL resulted in the release of the sickle cell cytoplasmic contents. The spectrophotometric absorbance of released Hb S was elevated as seen in Figure 3.1, which means that the Delph-CL agent interacted with Hb S and converted it from a polymer into a soluble form. Obtaining soluble Hb S enhanced the oxygen (O_2) binding affinity as very low P_{50} were achieved. Moreover, low P_{50} could be a result of Hb S auto-oxidation and elevation of metHb production. It is known that metHb is unable to bind O_2 (5). Therefore, increasing the production of metHb may boost the remaining Hb to be oxygenated by increasing the O_2 binding affinity and lowering P_{50} (6).

Nevertheless, the Delph-CL biomechanism in modest sickle cell reversal obtained at 100 µg/mL was investigated further. Two biomechanisms were hypothesized for sickle cell reversal in this study. First was a hydration biomechanism caused by increasing the intracellular potassium (K^+) electrolyte concentration after incubation with Delph-CL. However, Delph-CL seemed to have the reverse effect, where the K^+ was found to be mostly extracellular, Figure 4.1. A similar K^+ leakage was achieved also by *P. paniculata* extracts, where it was found that the K^+ intracellular concentration decreased from 1.52 ± 0.3 to 1.2 ± 0.39 mEq/10g Hb. In this study, the K^+ leakage is a consequence of Delph-CL toxicity where high hemolysis was reported in Figure 2.3. In addition, other work demonstrated that increased extracellular K^+ allowed the cells to dehydrate and become rigid (5, 7). These finding supports the low sickle cell deformability after treatment with Delph-CL as shown in Figures 3.3 and 3.4.

The second hypothesized biomechanism was anti-oxidation mediated by diminishing the metHb oxidation product. The free Hb and metHb levels were measured before and after oxidative stress, induced by the agent hydrogen peroxide (H_2O_2). Since there was a toxic effect due to Delph-CL before applying the H_2O_2 treatment, there was an insignificant difference in the metHb levels before and after H_2O_2 treatment, Figure 4.2. This was also shown when a normal RBC aliquot was treated with Taurasi red wine containing the Delphinidin 3-O-glucoside fraction and other compounds (8). There was not much of a difference in the metHb between pre- and post-100 µm of H_2O_2 treatment for 3 hours at room temperature. Moreover, the anthocyanin-rich black carrot extract showed insignificant protection of a colon cell line (HT29 clone 19 A) from the oxidative agent H_2O_2 at a concentration of 100 µmol/L (9). The genotoxicity occurred by inducing DNA damage and it persisted for 30 minutes. On the other

hand, the ROS are sometimes measured instead of metHb to detect an anti-oxidant effect property. In the do Nascimento et al. (10) study, intracellular ROS concentration was measured in normal and sickle erythrocytes after incubation with quercetin and rutin flavonoids compounds. The concentration of ROS revealed an insignificant difference between the two groups at H_2O_2 concentrations of 50 and 100 μmol/L, similar to what was detected here. Consequently, the ACs could have the susceptibility to be oxidized. A degradation of ACs extracted from blueberries (Vaccinium spp.) was attributed to ACs oxidations and condensation reactions with other phenolic compounds as Reque et al. mentioned (11).

Delph-CL is hydrophilic and is attracted to the hydrophilic phase of the sickle cell membrane, however the weakness between the membrane components enhance Delph-CL to reach hydrophobic alkyl chains region and cause hemolysis. The released Hb interacts with Delph-CL and decreases the hydrophobicity between valine and the nearby amino acids alanine, phenylalanine and leucine (12). Accordingly, the Hb S is converted from insoluble polymers into a soluble form as an outcome of Hb becoming oxygenated.

5.1 Limitations and Future Studies

This study faced many limitations; the major one was the source selection. Most studies of ACs use reagents extracted from a natural source. In this study, commercially manufactured Delph-CL powder was used for its purity and concentrated form. However, the natural extracts have additional compounds in addition to ACs that provide a supportive effect (13). The anti-sickling plant extract *P. nigrescens* contains flavonoids, alkaloids, glycosides, cardiac glycosides, tannins, saponins, and anthraquinones. In addition, protein, lipid, carbohydrate, crude fibre, ash, and moisture were also found after proximate analysis (14). Therefore, extraction of the ACs from a natural chosen source should be considered in future studies. On the other hand, as a potential therapeutic, it may be more appropriate to look at a chemically defined solution composed of two or more purified AC compounds (8).

The participants in this study were red cell exchange (RCE) patients. Their blood samples were taken before the exchange, which occurred on a monthly basis. As a result, many of the transfused RBCs from volunteer donors likely still remained in the patient's circulation. In future studies, both transfused and non-transfused patients should be included if possible to see the effect of AC treatment on a higher ratio of sickle cells. In addition, by examining these two

groups, it may help to identify the impact that the transfusion process has on sickle cell reversal agent therapy by involving or excluding the transfusion factor during the patient treatment. Non-transfused patients in this study were not included because most of the sickle patients are in the RCE program (18 out of 27 SCD patients who visit the University of Alberta Hospital). A larger sample size should be recruited to increase the study power. In addition, using patients with sickle cell trait as controls rather than normal healthy individuals, and comparing variables such as transfusion history, hydroxyurea (Hu) treatment, gender, and age should also be considered in further studies.

In the Emmel test, the difference in the cell volume between treated and untreated cells was not determined. Therefore, measuring the sickle cell radius, perimeter, and surface area through inverted microscope (Nikom TE 2000-U) may help. Simultaneously, involving a valid anti-sickling treatment such as Medox, Cikalvit®, or Niprisan as a control in a sickle cell reversal experiment should be proposed to determine the agent's relative efficacy (15). The 24 hours incubation period was chosen as a standard, although a negative outcome was obtained in some experiment such as hemolysis. Consequently, the incubation time needs to be justified for each experiment separately before testing. The reason for obtaining a contradicting result in reversal and hemolysis is not clear yet. Studying the ultrastructure of the sickle cell membrane is necessary to understand the mechanism of these two effects, such as in the Bonarska-Kujawa et al. study (3).

5.2 Summary

In summary, Delph-CL was able to transform the sickle cell morphology after increasing the Hb S solubility. This finding supports the hypothesis of reversing the sickle cell shape through the inhibition of Hb S polymerization. As Hb S solubility was increased, the Hb S-O_2 binding was enhanced. However, Delph-CL treatment decreased the deformability and increased hemolysis, Hb S oxidation and potassium leakage. In this study, Delph-CL did not appear to work through either of the proposed biomechanisms.

Delphinidin chloride could be a potential therapy for sickle cell disease management based on the shape transformation and increased Hb solubility demonstrated in this thesis. However, Delph-CL treatment resulted in an increase in sickle cell rigidity and increased hemolysis. Further work into establishing the mechanism of sickle cell reversal would help improve upon

the potential use of this compound in treating patients with sickle cell disease. In addition, further research is needed to further explore the *in vitro* and *in vivo* toxicity profile of this compound. As herbal extracts, like Delph-CL have shown to have an anti-sickling effect, further studies on the mechanism by which these compounds function at the molecular level may open new opportunities for SCD treatment.

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Appendix A

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INFORMATION LETTER

Title of Project: Investigating the Effect of Anthocyanins on Sickle Red Blood Cell

Principal Investigator(s): Ms. Kristi Lew
Co-Investigator(s): Ms. Asmaa Basonbul, Dr. Jason Acker

Purpose: This study will investigate the effect of a herbal extract on sickle cell shape. We are looking for blood samples from patients with sickle cell disease and normal healthy controls.

Background: Sickle cell anemia is a blood disease caused by an abnormal hemoglobin. In this disease, red blood cells take on a crescent shape instead of a normal biconcave disc shape. The abnormal shape of these red blood cells can cause problems such as decreased red blood cell survival, leg ulcers and blood vessel blockage. Previous research has shown that a herbal extract (anthocyanins) may correct the sickle cell shape. The goal of this project is to study the mechanism of anthocyanins for reversing the sickle cell shape.

Procedures: If you are agree to participate in the study, one small tube of blood (4 mL) will be collected from you.

Benefits: There are no direct benefits to you, but your participation will be very beneficial to our study. You are being asked to be in this study because your blood sample will help us to further understand the effect of anthocyanins on recovering the abnormal sickle cell shape.

Risks: The risks of participating in the study are minimal and are limited to having your blood drawn. These include slight discomfort during insertion of the needle and the possibility of minor bruising. Your blood will be collected by a trained professional who will use sterile collection supplies.

Confidentiality: Your identity in the study will remain confidential to only the study investigators. All of your personal data will be kept private. Once your sample has been collected, its identity and the data collected from it will not be directly linked to your personal information. Your samples will be kept in a restricted access lab for up to one week for analysis, after which the samples will be disposed following standard laboratory practice. Original data from the sample analysis will be kept in a locked drawer for five years according to standard research practice.

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Freedom to Withdraw: You are not required to take part in this study. Your treatment will not be affected by whether you take part, and you are free to withdraw at any time. If you leave the study after the experimental testing of your sample, we will stop with any further testing and collection of information, but we will need to keep the data we have already collected.

Additional Contacts: If you have any concerns about any aspect of this study, you may contact any one of the following:

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If you have any concerns about your rights as a research participant, you can contact the Research Ethics Office at 780-492-2615. This office has no affiliation with the researchers.

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CONSENT FORM

Title of Project: Investigating the Effect of Anthocyanins on Sickle Red Blood Cell

Principle Investigators:

Asmaa Basonbul E-mail: basonbula@hotmail.com
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Dr. Jason Acker E-mail: jacker@ualberta.ca

Part 2 (to be completed by the research subject):

	Yes	No
Do you understand that you have been asked to participate in a research study?	☐	☐
Have you read and received a copy of the attached Information Sheet?	☐	☐
Do you understand the benefits and risks involved in taking part in this research study?	☐	☐
Have you had an opportunity to ask questions and discuss this study?	☐	☐
Do you understand that you are free to withdraw from the study at any time, without having to give a reason?	☐	☐
Do you understand who will have access to your records, including personally identifiable information?	☐	☐

This study was explained to me by:_____

I _____ agree to take part in this study.

Signature of Participant _____Date _____

Signature of Investigator or Designee _____ Date _____

THE INFORMATION SHEET MUST BE ATTACHED TO THIS CONSENT FORM AND A COPY GIVEN TO THE RESEARCH SUBJECT

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