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Licenciatura em Biologia Celular e Molecular

Preservation of Red Blood Cells using Natural Deep Eutectic Systems

Dissertação para obtenção do Grau de Mestre em
Bioquímica para a Saúde

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Nova School of Science and Tecnology – Universidade Nova de Lisboa

Março 2023

2023

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Dedico aos meus avós.

ACKNOWLEDGMENTS

First of all, I would like to express my deepest gratitude to my advisers, Professor Ana Rita Duarte and Dra Ana Rita Jesus, for teaching and guiding, me, and above all for showing patience and not giving up when I almost gave up. I am more grateful to you than you'll ever know and more than I could ever express. Thank you for my personal growth and for the opportunity to be part of this project.

I would also like to thank the DES Solve Group, a gathering of remarkable people full of energy and joy. Thank each of you for making this journey a joyful ride.

Thank you, FCT NOVA and Instituto Português de Sangue e Transfusão (IPST) for providing materials and space for personal research.

I also wanted to thank my parents for their unconditional support and love, without whom I wouldn't be here. Thank you, my brother, Dmytro, for all the help and for being such an amazing brother, couldn't wished any better, you did a wonderful job.

To all my friends, especially Tavaritos, I thank you for your support and patience, as well as a lot of positive and supportive talk during these years of our friendship. To Eduardo and Sophia, thank you for believing, and never miss the chance to say it. To Ana, my dear friend, for recording all the precious memories and making me feel confident about myself. Francisca, I also want to thank you for all the snacks, meals, and conversations, and for being such an awesome roommate. I also want to thank Odílio and Quitéria, for all the wisdom and laughter.

I would also like to thank, Professor Teresa Catarino, for reaching out to me and cheering for me. Thank you, Rodrigo, for being such a funny and selfless person and helping me whenever needed.

I also want to thank my "afilhados" for making my college years full of happy and positive memories, something to look forward to remembering.

Lastly, my volleyball team, for bringing me joy and excitement whenever I needed it, especially Carlos, for teaching me volleyball and giving me another passion in life.
Also to all my friends, that remained by my side and gave me support.

ABSTRACT

Blood transfusions are a life-saving procedure that is fundamental even nowadays. The appearance of SARS-CoV2 and the recent conflict between Ukraine and Russia showed that blood supplies can drop drastically from one day to another.[1,2] The importance of increasing the storage period as well as improving the quality of the blood became crucial.

Natural deep eutectic solvents (NADES) are a mixture of natural components in a certain molar ratio that experience a significant decrease in their melting point.

NADES components, being organic molecules, present different beneficial antioxidant properties that help minimize harmful secondary metabolites resulting from oxidative stress. To evaluate NADES' effect on red blood cells, outdated bags donated from IPST were used for preliminary tests to verify whether NADES systems can improve blood quality. Osmolarity and pH levels play an important role in red blood cells' morphological change, which is directly associated with oxygen transport and cell integrity; therefore, these parameters were evaluated over time. Our results showed that we were able to increase and maintain a satisfactory pH level between 6.8-7.3 for 5 more weeks. The erythrocyte shape shifted from initially biconcave to echinocyte and later to irreversible spherocyte, leading to haemolysis, however, there were still some normally shaped erythrocytes after 5 weeks of storage. During the first week, hemolytic activity in solutions of Pro:Glc:W and Pro:Gly:Sorb:W met the official requirements; however, after 5 weeks in NADES solution, haemolysis levels were significantly above the legal requirements for the USA (1%) and Europe (0.8%). These preliminary studies show that NADES represents a potential alternative as an additive solution to increase the shelf-life of blood products, especially red blood cells.

Keywords: Blood Transfusion, NADES, Natural compounds, Erythrocyte

RESUMO

As transfusões sanguíneas são procedimentos que salvam vidas e que são fundamentais atualmente. O aparecimento do SARS-CoV2 e o recente conflito entre a Ucrânia e a Rússia mostraram que a reserva nacional de sangue pode cair drasticamente de um dia para outro.[1,2] A importância de prolongar o período de armazenamento, bem como melhorar a qualidade do sangue, tornou-se fulcral.

Os NADES são uma mistura de componentes naturais, que numa certa proporção molar demostra uma diminuição significativa do ponto de fusão da mistura.

Os NADES, sendo moléculas orgânicas, apresentam diferentes propriedades antioxidantes benéficas que ajudam a minimizar os metabolitos secundários prejudiciais resultantes do stress oxidativo. Para avaliar o efeito NADES nos glóbulos vermelhos, os sacos caducados doados do IPST, foram usados nos testes preliminares para verificar se os sistemas NADES podem melhorar a qualidade do sangue.

Os níveis de osmolaridade e pH desempenham um papel importante na mudança morfológica dos glóbulos vermelhos, que está diretamente associada ao transporte de oxigénio e à integridade celular. Os nossos resultados mostram que conseguimos aumentar e manter um nível de pH satisfatório, entre 6.8-7.3 até mais 5 semanas. O eritrócito mudou de forma bicôncava para equinócito e posteriormente para esferócito irreversível, promovendo à hemólise, porém ainda havia alguns eritrócitos de formato normal mesmo após 5 semanas de armazenamento. Para a 1ª semana, a atividade hemolítica em Pro:Glc:W e Pro:Gly:Sorb:W estava conforme os requisitos oficiais, no entanto, após 5 semanas na solução NADES, os níveis de hemólise estavam além dos requisitos legais para os EUA (1%) e Europa (0,8 %).

Estes estudos preliminares mostram que o NADES representa uma alternativa promissora como solução aditiva para aumentar a prazo de validade de produtos sanguíneos, especialmente glóbulos vermelhos.

Palavras chave: Transfusão, NADES, Compostos Naturais, Eritrócitos

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ACRONYMS

2,3-DPG	2,3-Diphosphoglycerate
ATP	Adenosine Triphosphate
Bet	Betaine
CA	Citric Acid
CO_2	Carbon Dioxide
CPD	Citrate-Phosphate-Dextrose
EC	Erythrocyte Concentrate
Fe	Iron
Fru	Fructose
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
Glc	Glucose
Gly	Glycerol
GSH	Glutathione
Hb	Haemoglobin
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HTLV-I-II	Human T-Lymphotropic Viruses Type I and II
IPST	Instituto Português de Sangue e Transfusão
NAC	N-Acetylcysteine
NADES	Natural Deep Eutectic Systems
NADH	Nicotinamide Adenine Dinucleotide Reduced

O_2	Oxygen
PAGGSM	Phosphate–Adenine–Glucose-Guanosine–Saline–Mannitol
PC	Platelets Concentrate
Pro	Proline
RBC	Red Blood Cell
RCC	Red Blood Cell Suspension
ROS	Reactive Oxygen Species
SAGM	Sodium–Adenine–Glucose–Mannitol
SARS-CoV2	Severe acute respiratory syndrome coronavirus 2
SH	Sulfhydryl
Sorb	Sorbitol
Suc	Sucrose
Tre	Trehalose
U	Urea
W	Water
Wk	Week
WB	Whole Blood
WCC	White Cell Concentrate

1

Introduction

Even though we live in the 21st century, humanity does not possess all the answers to modern problems, and some issues, such as the shortage of blood, are still of global concern. In Portugal, during 2022, due to the Sars-Cov-2 pandemic, there was a lack of blood storage.[1] As a result, due to the need to support haemorrhagic events during childbirth, chronic patients, including those with sickle cell anaemia and cancer, among other diseases, continued to demand medical care as well as blood transfusions. Blood supply became a national concern during the COVID-19 pandemic since hospitalised patients had a higher need for blood transfusions than there were actual donations.[1]

The term blood transfusion first appeared in the 15th century, when Italian doctors suggested the consumption of young blood for rejuvenation.[3] However, since then this term has significantly changed its meaning and its administrative route. Nowadays blood transfusion is a life-saving procedure in which blood administration is done intravenously.

Since last November, Portugal has been struggling to restore satisfactory levels of blood units to meet the required needs[1] and there is a similar problem worldwide.[4] In addition to the anticipated blood costs, the number of patients who required immediate transfusions due to the COVID-19 rose drastically from day to night. Firstly, social distancing measures, lockdowns, and fear of getting infected resulted in cancellation of blood deliveries and voluntary blood donations. Furthermore, many severe COVID-19 cases required intensive medical care, such as surgeries and these patients often needed blood and blood products.

1.1 Composition of blood

The blood is a vital body fluid without which complex organisms cease to exist, because that blood nourishes and oxygenates every cell in the human body. However, that is not its only purpose, as it also removes waste from cells and provides nutrients for their normal activity. Blood controls body temperature by carrying heat from different parts of the body to the skin, where it can be released. Furthermore, it carries substances that control numerous bodily processes and fight infection.

Red blood cells (RBC) or erythrocytes, white blood cells (leukocytes), platelets, and plasma are the elements that give blood volume. Leukocytes, platelets, and erythrocytes make up around 40% of total blood volume, while plasma makes up about 60% of it.[5] The average volume of blood that circulates inside a person varies according to their size and weight, for instance an adult human has around 5 liters of blood in circulation. Compared to men, women usually have less blood volume. Men have a higher haemoglobin level and red blood cell count, in addition to a greater total blood volume.[6] These values of haemoglobin and RBC, are inversely proportional to total body fat, and females have a larger percentage of total body fat than males due to an adaptation that allows them to be better prepared for pregnancy and breastfeeding. Moreover, during their reproductive years, women experience periodic blood loss due to menstruation and are continually making up for this loss. Testosterone drives the synthesis of haemoglobin and the creation of red blood cells, and men have significantly higher testosterone levels than women.[6,7]

About 2 to 3 million red blood cells (RBCs) are generated every second in the bone marrow and dispersed into the bloodstream.[8] RBCs are the most prevalent kind of blood cell, they represent about 99% of the blood cells, corresponding to 4-6 million cells per cubic millimetre of blood. RBCs can fit through the smallest blood channels considering their diameter is only 6 μm.[9] They circulate throughout the body for up to 120 days before being eliminated from circulation by specialised cells called macrophages in the liver and spleen. In humans and other mammals, mature RBC lacks a nucleus as well as mitochondria, so the cell has more storage for the oxygen-binding proteins, haemoglobin, enabling the RBC to transport more oxygen in a more efficient way. As a result, RBCs rely almost exclusively on anaerobic metabolism for their energy needs. By relying on anaerobic metabolism and not consuming the oxygen they transport, RBCs efficiently deliver oxygen to tissues while maintaining their own energy needs.

This adaptation allows RBCs to execute their essential function in oxygen delivery and maintain the proper oxygenation of tissues throughout the body.[10]

From the top angle, erythrocytes appear to be a circular cell, however when looking more carefully, a profile shows that they are biconcave discs (Fig. 1.1).

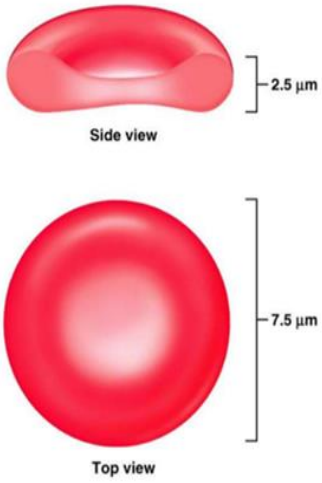


Figure 1.1 — Erythrocytes morphology from side and top view. Copyright © 2006 Pearson Education, Inc., publishing as Benjamin Cummings

The loss of the nucleus enables the RBC to include more oxygen-carrying haemoglobin, hence facilitating the transport of more oxygen in the blood and enhancing our metabolism. This shape was evolutionarily perfected over time and by increasing of the surface area-to-volume ratio of the cell, there is also an increase in the efficiency of diffusion of oxygen (O_2) and carbon dioxide (CO_2) into and out of the cell.[9] Erythrocytes, like many other human cells, also have a flexible plasma membrane. In non-mammalian vertebrates such as birds and fish, mature RBCs do have a nucleus, which provides an oval shape to the blood cell.[9] The most important function of erythrocytes is to transport oxygen throughout the body; nevertheless, it was just discovered that despite being nonimmune cells, bird erythrocytes are capable of taking part in some immune responses that are important for host defence. Also non-mammalian vertebrate erythrocytes have mitochondria and ribosomes responsible for synthesizing enzymes, these enzymes help the cell generate energy through Krebs cycle.[11,12]

All RBCs contain haemoglobin (Hb), which is the protein responsible for their red pigmentation. RCBs are loaded with oxygen in the lungs, where it is most abundant, and release it where it is needed throughout the body. Haemoglobin is organized into four globin proteins, each with a heme group, a ring containing iron, which constitute haemoglobin, and consisting of two

alpha and two beta chains. One iron atom (Fe), which is part of the heme group, has the ability to bind to one oxygen molecule.[9] Thus, haemoglobin can carry up to four oxygen molecules since each molecule has four globin. Not only can it carry oxygen but carbon dioxide as well, when it collects oxygen from lungs gas exchange happen, haemoglobin releases CO_2 and receives oxygen, following its purpose to oxidise the tissues.[10]

1.2 Types of blood and storage

From the initial phase of blood collection to the preparation of blood bags, the blood can be subjected to different procedures, resulting in distinct labile blood products such as whole blood (WB), red blood cell concentrates (RCC) or, more usually used, erythrocyte concentrate (EC), red blood cell suspensions, platelets concentrates (PC), plasma components and white cell components (WCC)[13]. Before the administration, the blood is submitted to several tests, including those for anti-HIV (Human Immunodeficiency Virus), anti-HCV (Hepatitis C Virus), Anti-HTLV-I-II (Human T-lymphotropic Viruses, type I and type II).[13,14] The storage condition and further indications depend on each blood product.[14]

The main purpose of RBC transfusion is to maintain tissue and organ oxygenation, so the procedure is designated for patients that suffer precisely from lack of oxygen delivery to organs and tissues. This condition may be the result of different circumstances: traumas, surgeries, hereditary disorders associated with normal RCB production or RCB destruction, and cancer.

EC results from the removal of plasma and leucocytes and once collected an additive solution, usually a hypertonic solution called SAGM (sodium–adenine–glucose–mannitol, 376 mOsm/L), or an isotonic solution designated by PAGGSM (phosphate–adenine–glucose-guanosine–saline–mannitol) is added. Then, ECs are stored between 2-6°C, to limit bacterial growth.[15] Their storage time depends on the additive solution used. For instance, EC in SAGM typically lasts up to 6 weeks (wk) and PAGGSM can hold up to 7 weeks.[16] This short storage period is considerably low for blood banking since, at least in densely populated locations, it has been verified that near-shortage situations are directly connected to seasonal festivities, occurring more frequently when donors are on vacation, usually in the middle of the summer and over the Christmas break.[17]

The scientific community has recently questioned whether transfusions of long-term preserved EC have any unfavourable effects.[18] Although recent clinical trials are encouraging best

practices, there is still no agreement on whether ECs held for a long time can be harmful to patients who are receiving transfusions.[16] However, it is evident that the characteristics, morphological and biochemical, of erythrocytes within ECs change throughout time.[16,19] Their oxygen perfusion efficiency and the length of time that they remain in circulation following a transfusion are particularly impacted.[20]

Overall, blood transfusions are very important but unfortunately the transfusion bags' lifespan is limited. Many studies are developing new alternatives for blood preservation, not only trying to extend its lifespan but also reducing the number of lesions on RBCs during the storage process.

In this study, our focus is on RBCs. By studying and understanding the morphological changes in the presence of natural components we hope to achieve longer preservation and fewer physicochemical changes.

RBCs used were organic material provided by IPST (Instituto Português de Sangue e Transfusão).

1.3 Effects of storage on RBC metabolism

The metabolism of the RBC undergoes a significant modification under non-physiological storage conditions. First, low temperatures change metabolic fluxes by slowing down biological rates,[21] then, in the bags containing RBCs and an additive solution nutrients are gradually used and possibly depleted while waste products build up inside the cells and in the supernatant.

Negative feedback for enzymatic activity is created by the low pH of additive solutions combined with the acidification of RCC supernatant following lactate build-up (glycolysis end-product).[22] The most important metabolites gradually disappear, including: after 2 weeks of storage, the O_2-Hb affinity regulator 2,3-diphosphoglycerate (2,3-DPG) decreases; an important redox cofactor - the reduced nicotinamide adenine dinucleotide (NADH), resulting from glyceraldehyde 3-phosphate dehydrogenase (GAPDH), lastly, adenosine triphosphate (ATP), the substance necessary to perform all metabolic pathways and protein activity.[23]

After suffering many chemical alterations, the RBC suffers haemolysis, which is a process that describes the loss of intracellular content and lesion of cell membrane, mainly expelling

haemoglobin into the surrounding. This occurs when the RCB membrane is damaged.[24] Osmolarity often contributes to this process.

A selectively permeable membrane allows water to travel down its osmotic gradient by osmosis. Despite different numbers of impermeable particles in solution on either side of the membrane, an osmotic pressure gradient, pressure needed to stop water from flowing along its gradient, is created.[25] Although water may pass straight through cell membranes, this process is slow since the membranes are made of lipid bilayers.

The total number of osmolyte particles, particles present in a solution, determines its osmolarity, affecting the physical characteristics of the solution, including its freezing point and osmotic pressure[26] (Fig 1.2). The nature of the molecules does not affect the osmolarity values.[27]

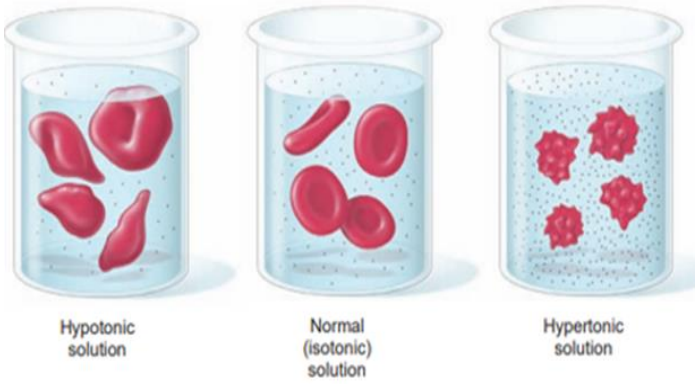


Figure 1.2 — Erythrocytes' morphology in hypotonic, isotonic, and hypertonic solutions. Adapted from M. Turgeon (2005).

1.3.1 RBC morphological changes

Some factors that contribute to a storage lesion, which is used to refer to a variety of biochemical and biomechanical abnormalities that are connected to these corpuscular changes, have been previously described.[28]

Erythrocytes that present short, scalloped or spike-like projections evenly spaced along the cell membrane are designated echinocytes (crenated erythrocytes) (Fig. 1.3B). Crenation is not a disease state, but an osmotic imbalance causes this cellular distortion, which is usually observed when intracellular water is physically lost. This membrane projection might present dif-

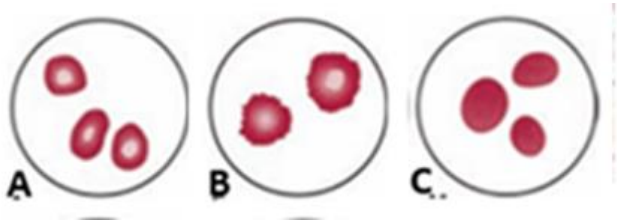


Figure 1.3 — Erythrocytes morphology. A – normal erythrocytes, B – Echinocytes, C – spheroechinocytes/ Spherocytes. Adapted from M. Tourgeon (2005).

ferent sizes and shapes. When the osmolarity is increased - hypotonic solution, the erythrocytes lose their biconcave shape by absorbing water and expanding, meanwhile, in a hypertonic solution, the water is loss and the cell presents shrinkage. (Fig 1.2)

Meanwhile, erythrocytes that have lost their typical biconcave shape are referred to as spherocytes (Fig. 1.3C). The morphology of these cells is highly rounded and compact. When stained, it has a strong orange red colour and is often less than 6 μm. These deformed cells develop due to the instability in the cell membrane leading to the loss of cell membrane. Premature cell death is caused by membrane instability and the diminished deformability of spherical cells.

Faced with these problems previously described, blood preservation solutions do not have a very long lifespan, and in more critical times when the demand is high, there is a lack of this resource, inciting difficult situations and sometimes even unnecessary loss. In order to try to avoid waste and extend as much as possible the possibility of using RBCs, this study resorts to Natural Deep Eutectic Solvents (NADES).

1.4 NADES

1.4.1 Natural Deep Eutectic Systems

NADES are a mixture of natural components, that in a certain molar ratio, experience a significant decrease in melting point (Fig. 1.4) when compared to either of their individual molecules.[29] The charge delocalization caused by hydrogen bonding between a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), as well as steric effects and ionic contributions from the anion and cation, may all be responsible for the decrease of melting point phenomena. (Fig. 1.4)

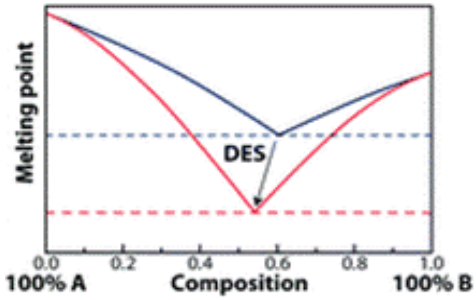


Figure 1.4 — Graphical representation of the behavior of a binary eutectic system. Adapted from Kollau et all (2018).

A new identity with different physical and chemical properties from the component elements is created as a result of the interactions between the HBA and the HBD during the preparation of the NADES. By the combination of natural molecules at different molar ratios, it is possible to obtain a stable viscous mixture with amazing properties and promising results. (Fig. 1.4) The blue line represents the melting point expected when mixing the individual compound, however, the red line is the real melting point observed.[30]

Generally, the use of these systems is more advantageous as the majority of mixtures are eco-friendly, and depending on the NADES application, its cytotoxicity may not necessarily be a drawback or limitation.[31] Some present low toxicity and low production cost, increasing their usage for diverse purposes, such as biomedical applications by increasing the permeability, absorption and solubility of certain drugs[29], extraction solvents, as an alternative to organic solvents[32] and stabilizing agent[33], such as the stabilization of the phenolic unstable compound carthamin[33].

One example of NADES can be obtained from menthol and lauric acid. Only by mixing and heating up two solid compounds at room temperature, in this case without adding water, does the interaction between these compounds result in a clear and viscous mixture. (Fig. 1.5)

Figure 1.5 — Preparation of NADES from menthol and lauric acid.

1.4.2 NADES selection and properties

Considering the additive solution SAGM, commonly used to preserve the RBCs, and understanding each substance's purpose, citrate operates as an anticoagulant by chelating calcium, dextrose is required for metabolism of stored red blood cells, adenine increases the vitality of red blood cells, and phosphate is utilized to reduce acidity and also promotes a higher concentration of 2,3-DPG and red cell phosphate.[16] Citrate-phosphate-dextrose (CPD) is the anticoagulant present in the solution to avoid cell coagulation.[16]

Molecules with identical properties were selected to prepare different NADES to study whether they have a positive effect on the storage of RBC.

1.4.2.1 Citric Acid and Ascorbic Acid

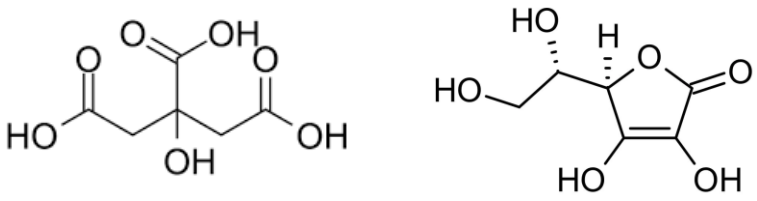


Figure 1.6 — Structure of citric acid (left) and ascorbic acid (right).

Antioxidants, such as Citric Acid (CA) (Fig. 1.6), may lessen oxidative damage and subsequently storage lesions when added to stored RBCs. Citric acid is a weak organic acid that can be found in the highest concentrations in citrus fruits, including lemon, grapefruit, tangerine, and orange. Other foods that contain citric acid include apples and grapes. In addition to its usage as a natural preservative, foods and soft drinks can be given an acidic, sour flavor with the addition

of this ingredient. Citric acid is present in all mammalian tissues because it functions as an intermediary molecule in the oxidative metabolic process[34]. It is a component of the tricarboxylic acid cycle or the Krebs cycle. Citric acid, according to certain studies, has been shown in studies to reduce harmful processes in the body, such as lipid peroxidation (cell damage caused by free radicals) and inflammation.[34]

The least harmful and likely most effective antioxidant found in mammalian systems is Ascorbic Acid (Fig. 1.6). Ascorbic Acid differs from Citric Acid by one oxygen molecule. The loss of oxygen makes ascorbic acid a water-soluble antioxidant that breaks chains and interacts directly with singlet oxygen, hydroxyl radicals, and superoxide.[35] Recent studies indicate a growing interest in administering pharmacologic doses of Ascorbic Acid to preserved RBCs.[36] Exposure to Ascorbic Acid dramatically decreased the mechanical fragility and haemolysis of stored RBCs.[37] These findings suggest that RBC preservation with the right amounts of Ascorbic Acid and Citric Acid may avert storage lesions.[36–38]

1.4.2.2 N-Acetylcysteine (NAC)

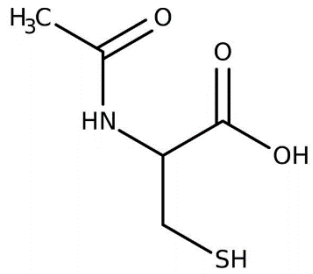


Figure 1.7 — Structure of N-Acetylcysteine.

N-Acetylcysteine (NAC) (Fig. 1.7) is a great source of sulfhydryl (SH) groups and is transformed by the body into metabolites that can boost the production of glutathione (GSH), aid in detoxification, and directly scavenge free radicals.[39] Some studies show the effectiveness of including NAC in the preservation solution, since oxidative stress has been found to be the primary cause of the accelerated ageing process that RBC experience while in storage.[40]

Besides, other studies demonstrated that NAC increases scavenger activity against reactive oxygen species (ROS) resulting from oxidative stress,[40] replenishes the intracellular reductive reserve[41] and reduces cell-free hemoglobin.[42]

1.4.2.3 Urea

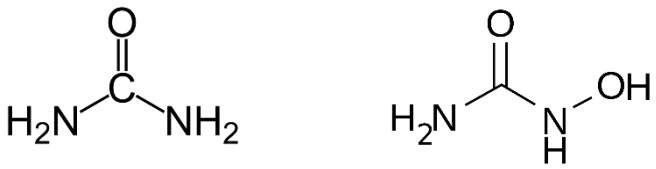


Figure 1.8 — Structure of urea (left) and hydroxyurea (right).

Rapid urea transport, a nitrogen-containing compound, due to the facilitated diffusion[43], helps maintain the osmotic stability and deformability of the cell and prevents the dissipation of extracellular osmotic gradients.[25] Urea (Fig. 1.8) that has had one of its hydrogens replaced with a hydroxy group is known as hydroxyurea (Fig. 1.8).[44] Hydroxyurea oral treatment drugs are prescribed for sickle cell disease patients, since they increases levels of fetal hemoglobin as well as levels of catalase and antioxidant enzymes at the RBC membrane.[45]

1.4.2.4 α-Tocopherol or Vitamin E

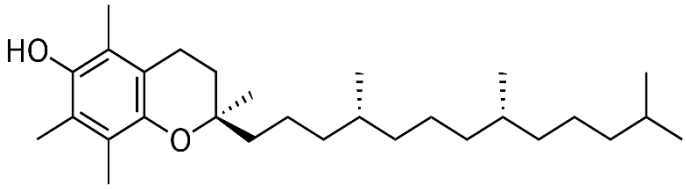


Figure 1.9 — Structure of α-Tocopherol.

Our organisms contain vitamin E (Fig. 1.9), which is a significant lipophilic antioxidant, in lipophilic compartments such as membranes or lipoproteins. It is thought to be the main molecule that prevents lipid peroxidation.[46] The antioxidant activity, which is thought to be the primary metabolic function of vitamin E, is also associated with a reduction of haemolysis in RBC[47], inhibition of oxidative damage caused by glucose (haemolysis, restoration of catalase, and GSH levels)[48], a decrease in the fraction of irreversible sickled RBCs[49] and decreased generation of ROS.[50]

1.4.2.5 Betaine

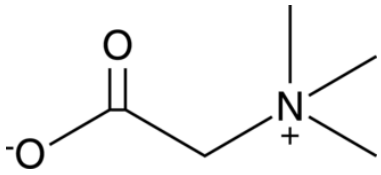


Figure 1.10 — Structure of betaine.

Betaine (Fig. 1.10) is a biocompatible osmolyte that replaces inorganic salts, enhances cellular water retention, and protects intracellular enzymes from being inactivated by osmotic pressure or temperature.[51] Its rapid absorption is also quite advantageous, since it also has the ability to stabilize cellular metabolic function when subjected to different kinds of stress.[51] Previous studies have used betaine in cryopreserving methodologies[52], however none was made just for the preservation of RBCs.

1.4.2.6 Glucose

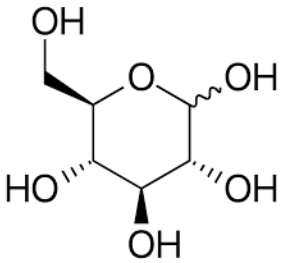


Figure 1.11 — Structure of glucose.

Glucose (Fig. 1.11) is the primary source of energy, providing power for normal cell function. It is verified that high glucose attenuated haemolysis, eryptosis, calcium accumulation, and glutathione loss seemingly as a result of maintaining the cells' energy supply. It is essentially solely through the breakdown of glucose that the body's metabolic functions obtain their source of energy.[53]

When compared to other cells in the body, an erythrocyte's capacity for metabolic activity is significantly lower. The cell is only capable of a limited amount of fatty acid and amino acid metabolism, as it lacks mitochondria, which are necessary for oxidative metabolism.[10] Glucose is responsible for the normal function of different pathways of erythrocyte glycolysis, which consist in ATP production, which is then used to maintain the energy level of the cell, the prevention of denaturation of the globin of the hemoglobin molecule as a result of oxidation, the

protection of heme iron from being oxidized and the regulation of the oxygen affinity of hemoglobin.[10]

1.4.2.7 Glycerol

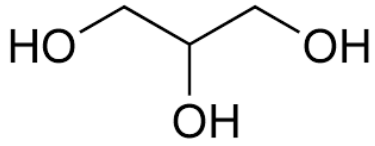


Figure 1.12 — Structure of glycerol.

By increasing blood plasma osmolality, glycerol (Fig. 1.12) facilitates the movement of water from tissues into the interstitial fluid and plasma. Additionally, glycerol functions as a carrier, and solvent in a variety of pharmacological formulations.[54] Hence, an increase in plasma glycerol concentrations has the potential to disrupt RBC homeostasis.[55]

1.4.2.8 Proline

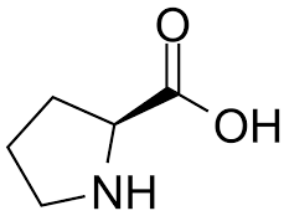


Figure 1.13 — Structure of proline.

Proline (Fig. 1.13) is one of the twenty amino acids utilized as vital protein components in living organisms. Proline is a ROS scavenger, by reacting directly with ROS and forming stable free radical adducts.[56] It is now known that the metabolism of proline regulates cellular signalling mechanisms that promote cellular survival or death.[56]

1.4.2.9 Trehalose

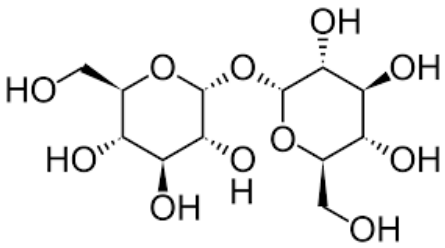


Figure 1.14 — Structure of trehalose.

Trehalose (Fig. 1.14) is a sugar that can prevent the protein denaturation and cellular membranes brought on by a range of stress-related circumstances.[57,58–60] Trehalose has generally been found to be more effective than other sugar molecules at protecting various biological components from stress. Many species that withstand osmotic stress, extreme dehydration, and low temperature duress include high quantities of a variety of these chemicals. In fact, it is currently believed that some agents, such as trehalose, betaine and proline, play a significant role in the molecular biology of osmoregulation and the ability of certain organisms to endure low intracellular water activity.[61]

1.4.2.10 Sorbitol

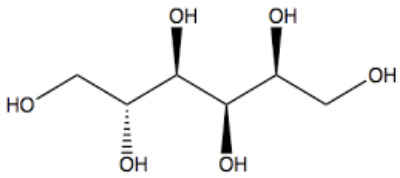


Figure 1.15 — Structure of sorbitol.

Since mannitol does not form NADES, sorbitol (Fig. 1.15) is used as its substitute, they both have identical structures and molecular weights.[60]

Sorbitol and mannitol have distinct properties, such as solubility, melting range, and stability.[62] For instance, mannitol has lower solubility than sorbitol. Sorbitol can slowly gain or lose moisture as the humidity levels of its surrounding environment changes, however there are no humectant characteristics found in mannitol.[62]

Mannitol is perhaps the most well-tolerated polyol, and it is possible to ingest, on average, no more than 20 g per day and no more than 10 g per serving before adverse effects are observed. With sorbitol, this number rises to approximately 40 g per day, with no more than 10 g per meal.[62]

1.4.2.11 Sucrose

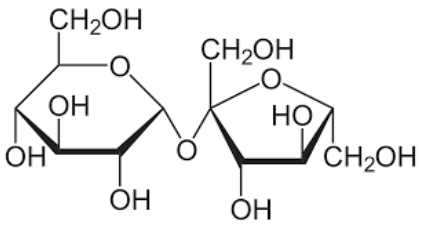


Figure 1.16 — Structure of sucrose.

Sucrose (Fig. 1.16) is a natural sugar that protects red blood cells from damage caused by freezing and thawing. Even though, trehalose has generally been found to be more effective than other sugar molecules at protecting various biological components from stress, sucrose shares similar features[63].

Glycerol, proline, trehalose, sorbitol, and sucrose are biologically significant osmolytes. In reaction to dryness, salt, and temperature extremes, these osmolytes build up. In addition, these molecules balance turgor pressure and lessen the effects of water stress.[56,64] Besides these properties, these osmolytes have an enormous cryoprotectant potential.[52,65,57]

2

AIM

The availability of sufficient RBC quantities, and the clinical safety and efficacy of long-term storage preparations are two ongoing concerns in transfusion therapy since blood transfusions allow the saving of numerous lives.

Nowadays, artificial blood substitutes are being investigated to eliminate biological variability and blood supply shortages. Nevertheless, the various clinical trials carried out up until 2008 were unsuccessful since it was established that these chemicals interacted with endogenous nitric oxide (NO), leading to undesirable side effects, and as well promoting oxidative stress. Once at the lab, blood components are typically separated mainly by centrifugation. Depending on the preservative administrated, RBCs can be kept in storage for up to 30–40 days[16], however this storage time is short and requires further studies in order to maximize the shelf-time.

The main goal of this project is to explore the use of NADES to increase the storage time of blood components, specially, red blood cells. Hence, this project comprises the following objectives:

- Preparation of NADES with suitable physico-chemical properties to be used as blood components preservatives
- Evaluation of the osmotic pressure of NADES solutions
- Evaluation of NADES toxicity in mammalian cells.
- Evaluation of the effect of NADES in red blood cells.

3

MATERIALS AND METHODS

3.1 NADES preparation

NADES were prepared using Citric Acid (CA, Panreac), Trehalose (Treh, Hayashibara Co., Okayama, Japan), Glucose (Glc, Merck), Sucrose (Suc, Sigma), Betaine (Bet, Sigma-Aldrich), Proline (Pro, Alfa Aesar), Glycerol (Gly, Sharlau), Sorbitol (Sorb, Sigma-Aldrich), Urea (U, Normax, Portugal). NADES were prepared using these compounds in different molar ratio. The mixtures were heated at 40–60 ºC under constant stirring, until clear mixture formation occurred. Afterwards, NADES were stored at room temperature (RT) for further use. After NADES preparation, NADES solutions were made. The samples used to further this study consist of different percentages of NADES dissolved in PBS (0.01 M phosphate buffer, pH=7.4, Sigma). The range varies from 1% to 25%.

3.2 Cell culture

Mouse adipose tissue cell line L929 (DSMZ - German Collection of Microorganisms and cell culture GmbH) was preserved in Eagle´s medium (MEM, Corning, USA) supplemented with 10% of fetal bovine serum (FBS, Corning, USA) and 1% of penicillin/streptomycin (PS, Corning, USA). L929 was kept at 37 °C under a 5% CO_2 atmosphere. When the cells reached about 80% of confluence, in 2-3 days, maintenance was performed, consisting in detaching the cells from the T-flask (T-75) by the addition of 3 ml trypsin (Corning, USA) for about 5 min, followed by the addition of MEM, for trypsin inactivation. The content was then centrifuged (centrifuge 601900066, Z 206, Hermle, Germany) 200 rcf, 10 min, the supernatant was discarded, and the

cells were resuspended in 1 ml of medium. L929 was then cultured in a new flask usually with 1:20 dilution.

3.3 Cytotoxicity assay

MTS (3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is a colorimetric reagent, soluble in water. This method consists of the conversion of MTS into a compound that is soluble in cell culture. In contact with the viable cells, reduction occurs allowing spectrophotometric quantification at 490 nm and the results are proportional to the number of living cells.[66]

Cells were seeded in 96-well plate (Falcon, Corning, USA) at $1x10^4$ cell/well for MTS assay and incubated for 24 h at 37 °C under a 5% CO_2. After medium removal, adherent cells were treated with NADES dissolved in culture medium with concentrations ranging from 1 to 25% (w/v). Negative control was the L929 in medium. The incubation period was 24 h, in the conditions previously described. Later, the medium was removed, the cells were washed with PBS and a 100 µL solution of medium + 16% of MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, PROMG3581, Promega, USA) was added to each well. The plates were incubated for 2 h in the same conditions, and the absorbance was measured at 490 nm in a microplate reader (HH35L2019044, Victor Nivo 3S, Perkin Elmer, USA). All measurements were performed with nine samples for each concentration analysed.

3.4 pH and Osmolarity

For pH measurements a 914 pH/Conductometer (Metrohm, Switzerland) was used. Concentration 1%, 2%, 2.5%, 5%, 10% and 25% of NADES dissolved in PBS were analysed.

For osmolarity measurements a Semi-Micro Osmometer (K-7400S, Knalier) was used. Firstly, the device was calibrated with 0, 400 and 850 mOsmol/kg solutions. After, 100 µL of NADES solutions were measured. All measurements were performed in triplicate for each concentration analysed.

3.5 RBC sample preparation

SAGM preserved, end-of-life leukoreduced RBC units were provided by the IPST (Instituto Português de Sangue e Transfusão). EC samples were taken from bags into sterile recipients and centrifuged at 1000 rcf for 10 minutes. After centrifugation, the serum was removed. RBCs were washed three times with PBS (0.01 M, pH 7.4) (1000 rcf, 10 min). After, RBC solutions were prepared by adding NADES solution to PBS previously prepared with a concentration ranging from 1 to 25% (w/v) in a 1:5 ratio (for 1 mL of EC, 4 mL of NADES solution) and stored between 1–6 ºC in a refrigerator for up to 4 weeks. Every week, the pH and cell morphology of these samples were assessed.

3.5.1 Haemolytic activity

Hemolytic assay is used to measure the degree of hemolysis, which is the rupture or destruction of red blood cells (RBCs).

The procedure for washing and preparing blood is described above.

After obtaining washed RBC, 200 µL of this erythrocyte suspension was added to 200 µL of NADES solutions with concentration ranging from 1 to 25% and incubated for 1 h at 37 °C. Then, the samples were centrifuged at 800 rcf for 10 min and 100 µL of the supernatant was collected into 96-well plate and absorbance at 570 and 630 nm was measured. For positive and negative controls instead of NADES, water and PBS were used, respectively. The values from Abs570 were normalized with the results from Abs630.[67] After obtaining the results, haemolytic activity was determined according to the formula:

$$Haemolytic\ Activity\ (\%) = (Abs\ S - Abs\ N)\ x\ 100/(Abs\ P - Abs\ N)$$

In which, Abs S – average absorbance of sample, Abs N – average absorbance of negative control in PBS (0% of haemolytic activity), Abs P – average absorbance of positive control in water (100% of haemolytic activity).

The haemolytic activity was measured at 1, 2, 3, 4 and 5 weeks.

3.5.2 Morphology

To observe the morphological RBC changes, one drop of RBC sample was visualized in inverted microscope (Zeiss, Axio Vert A1, Germany) using 63x/0.65 Ph 2 objective.

All samples examined are diluted in PBS and NADES a 1:5 ratio, since it was the minimum dilution that allow the clear visualization of the erythrocytes, during 1, 2, 3, 4 and 5 weeks.

4

RESULTS AND DISCUSSION

4.1 NADES characterization

NADES are mixtures of two or more components that have a lower melting point than their individual components when combined in a particular molar ratio. In this work, different NADES composed of different components and different molar ratios were investigated to determine which ones were liquid at room temperature, and when dissolved in PBS presented the pH and osmolarity close to the blood parameters. **Table 4.1** summarizes which NADES were made and studied. These NADES were previously studies by Solve.DES group and are registered in their database.

Table 4.1 — Different NADES prepared in different molar ratio.

NADES	Components				Molar ratio
	A	B	C	D	
CA:Glc:W	citric acid monohy-drate	glucose anhy-drous	water		1:2:4
Bet:CA:W	Betaine	Citric Acid	Water		2:1:2
Bet:Gly:Suc	Betaine	Glycerol	Sucrose		2:3:1
Bet:NAC:W	Betaine anhydrous	NAC (N-acetyl L-cysteine)	water		1:1:3
Bet:Sorb:W	Betaine	Sorbitol	water		1:1:3
Bet:Suc:Pro:W	Betaine anhydrous	Sucrose	proline	water	5:2:2:21
Bet:Treh:Gly:W	Betaine	Trehalose	Glycerol	water	2:1:3:5
Bet:Treh:NAC:W	Betaine	Trehalose	NAC (N-acetyl L-cysteine)	water	5:2:2:20
Bet:Treh:Pro:W	Betaine anhydrous	Trehalose	proline	water	5:2:2:21
Fru:Glc:Suc:W	Fructose	Glucose	Sucrose	Water	1:1:1:11
Glc:Pro:Gly:W	Glucose	Proline	Glycerol	water	3:5:3:20

Glc:Suc:W	Glucose anhydrous	Sucrose	water		1:1:6
Gly:Fru:Sorb:W	Glycerol	Fructose	Sorbitol	water	1:1:1:3
Gly:Glc:Sorb:W	Glycerol	Glucose	sorbitol	water	1:1:1:3
Gly:Treh:Sorb:W	Glycerol	Trehalose	Sorbitol	Water	2:1:2:10
Pro:Glc:W	Proline	Glucose anhydrous	Water		5:3:15
Pro:Gly:Sorb:W	Proline	Glycerol	sorbitol	water	1:1:1:3
Treh:Glc:Sorb:W	Trehalose	Glucose	sorbitol	water	1:2:1:13
Treh:Glc:W	Trehalose	Glucose anhydrous	water		1:2:13
U:Glc:Pro	Urea	Glucose	Proline		1:2:1
U:Glc:Pro:W	Urea	Glucose	Proline	Water	1:1:1:3

Some NADES formed with trehalose were not as stable as the ones made with sucrose, so as an alternative, sucrose was used instead when it was impossible to use trehalose.
To further characterize the samples, all NADES were later dissolved in PBS solutions in concentrations varying from 1% to 25%.

4.1.1 pH values

Table 4.2 — pH values for NADES prepared in different molar ratio dissolved in PBS.

NADES	**Components**				**Molar ratio**	**% in 5mL of PBS**	**pH**
	A	**B**	**C**	**D**			
Bet:CA:W	Betaine	Citric Acid	Water		2:1:2	1%	3.1
						2%	2.9
						2.5%	2.83
						5%	2.75
						10%	2.72
						25%	2.77
Bet:Gly:Suc	Betaine	Glycerol	Sucrose		2:3:1	1%	7.41
						2%	7.41
						2.5%	7.41
						5%	7.4
						10%	7.42
						25%	7.46

Bet:NAC:W	Betaine anidrate	NAC (*N*-acetyl L-cysteine)	water		1:1:3	0.05%	7.09
						0.08%	6.84
						0.10%	7.01
						1%	3.14
						2%	2.92
						2.5%	2.87
Bet:Sorb:W	Betaine	Sorbitol	water		1:1:3	1%	7.31
						2%	7.32
						2.5%	7.32
						5%	7.31
						10%	7.34
						25%	7.38
Bet:Suc: Pro:W	Betaine anidrate	sucrose	proline	water	5:2:2:21	1%	7.3
						2%	7.32
						2.5%	7.32
						5%	7.33
						10%	7.31
						25%	7.27
Bet:Treh: Gly:W	Betaine	trehalose	Glycerol	water	2:1:3:5	1%	7.46
						2%	7.46
						2.5%	7.47
						5%	7.47
						10%	7.46
						25%	7.5
Bet:Treh: NAC:W	Betaine	trehalose	NAC (*N*-acetyl L-cys-teine)	water	5:2:2:20	0.05%	7.16
						0.08%	7.1
						0.10%	7.02
						1%	4.89

						2%	4.04
						2.5%	3.84
Bet:Treh: Pro:W	Betaine anidrate	trehalose	proline	water	5:2:2:21	1%	7.32
						2%	7.33
						2.5%	7.34
						5%	7.33
						10%	7.35
						25%	7.36
CA:Glc:W	citric acid monohy-drate	glucose anidrate	water		1:2:4	1%	3.3
						2%	2.79
						2.5%	2.68
						5%	2.41
						10%	2.2
						25%	1.98
Fru:Glc:Suc :W	Frutose	Glucose	Sucrose	Water	1:1:1:11	1%	7.37
						2%	7.34
						2.5%	7.34
						5%	7.28
						10%	7.15
						25%	6.99
Glc:Pro:Gly :W	Glucose	Proline	Glycerol	water	3:5:3:20	1%	7.27
						2%	7.25
						2.5%	7.23
						5%	7.17
						10%	7.09
						25%	6.89
Glc:Suc:W	glucose anidrate	sucrose	water		1:1:6	1%	7.42
						2%	7.41
						2.5%	7.46

						5%	7.35
						10%	7.35
						25%	7.22
Gly:Fru: Sorb:W	Glycerol	Frutose	Sorbitol	water	1:1:1:3	1%	7.39
						2%	7.27
						2.5%	7.28
						5%	7.36
						10%	7.35
						25%	7.29
Gly:Glc: Sorb:W	Glycerol	Glucose	sorbitol	water	1:1:1:3	1%	7.24
						2%	7.23
						2.5%	7.2
						5%	7.18
						10%	7.13
						25%	6.96
Gly:Treh: Sorb:W	Glycerol	Trehalose	Sorbitol	Water	2:1:2:10	1%	7.38
						2%	7.36
						2.5%	7.34
						5%	7.34
						10%	7.32
						25%	7.22
Pro:Glc:W	Proline	Glucose anydrate	Water		5:3:15	1%	7.36
						2%	7.31
						2.5%	7.29
						5%	7.18
						10%	7.04
						25%	6.77
Pro:Gly: Sorb:W	Proline	Glycerol	sorbitol	water	1:1:1:3	1%	7.43
						2%	7.44
						2.5%	7.45

						5%	7.43
						10%	7.38
						25%	7.3
Treh:Glc: Sorb:W	trehalose	Glucose	sorbitol	water	1:2:1:13	1%	7.22
						2%	7.22
						2.5%	7.21
						5%	7.19
						10%	7.21
						25%	7.15
Treh:Glc:W	trehalose	glucose an-idrate	water		1:2:13	1%	7.37
						2%	7.36
						2.5%	7.36
						5%	7.26
						10%	7.32
						25%	7.27
U:Glc:Pro	Urea	Glucose	Proline		1:2:1	1%	7.32
						2%	7.33
						2.5%	7.32
						5%	7.31
						10%	7.27
						25%	7.25
U:Glc:Pro: W	Urea	Glucose	Proline	Water	1:1:1:3	1%	7.3
						2%	7.27
						2.5%	7.26
						5%	7.2
						10%	7.1
						25%	6.92

After studying the NADES pH in PBS, we selected for further studies those whose pH was above 6.5, because when RBCs were added the pH of solution (NADES-RBC) decreased.
NADES that presented very acidic behavior at 1%, 2% e 2.5% we decided to study their pH at lower concentration.

4.1.2 Osmolarity

The osmolarity of the NADES solution was also determined. Despite the fact that optimal range for RBC storage is 280-300 mOsm/kg, our NADES solutions showed higher values, especially at higher concentrations. (Fig. 4.1)

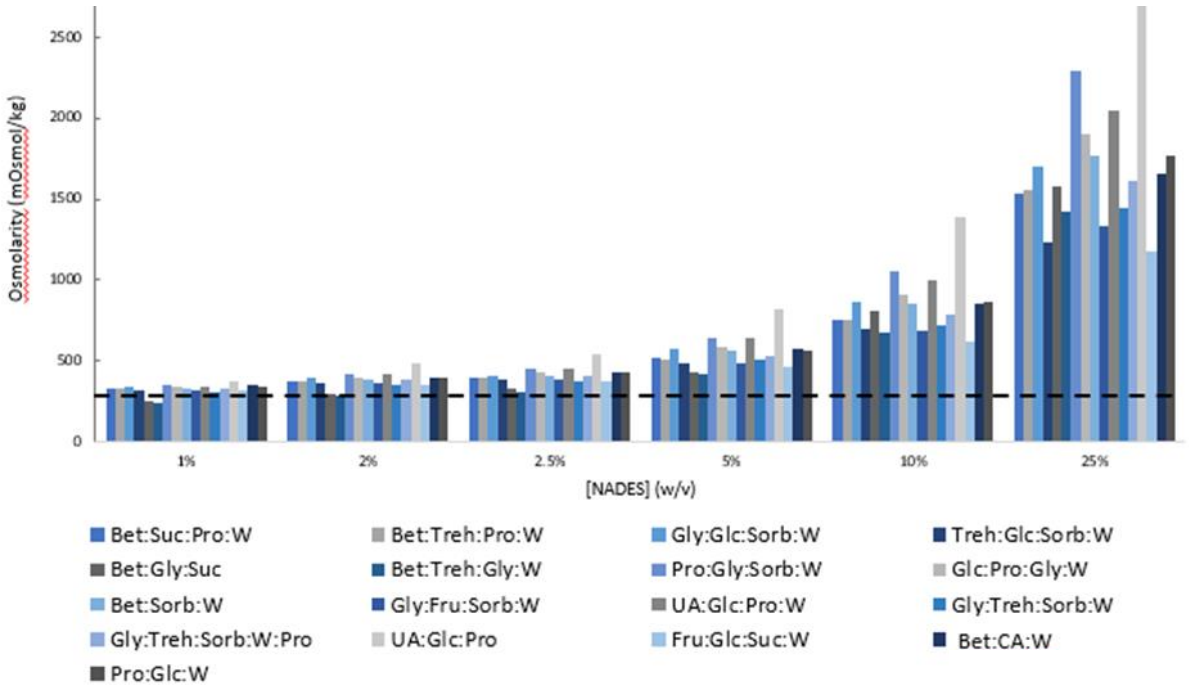


Figure 4.1—NADES osmolarity at 1%, 2%, 2.5%, 5%, 10% and 25%. PBS osmolarity is the reference as it has a similar osmolarity to RBC.

The osmolarity values of NADES solution for 1%, 2% and 2.5% did not change much, the values remained close to the reference value (PBS osmolarity = 280 mOsm/kg).

For NADES solution with concentrations of 5%, 10% and 25% there was an increase in osmolarity values, surpassing the reference osmolarity at least by at least twice as much.

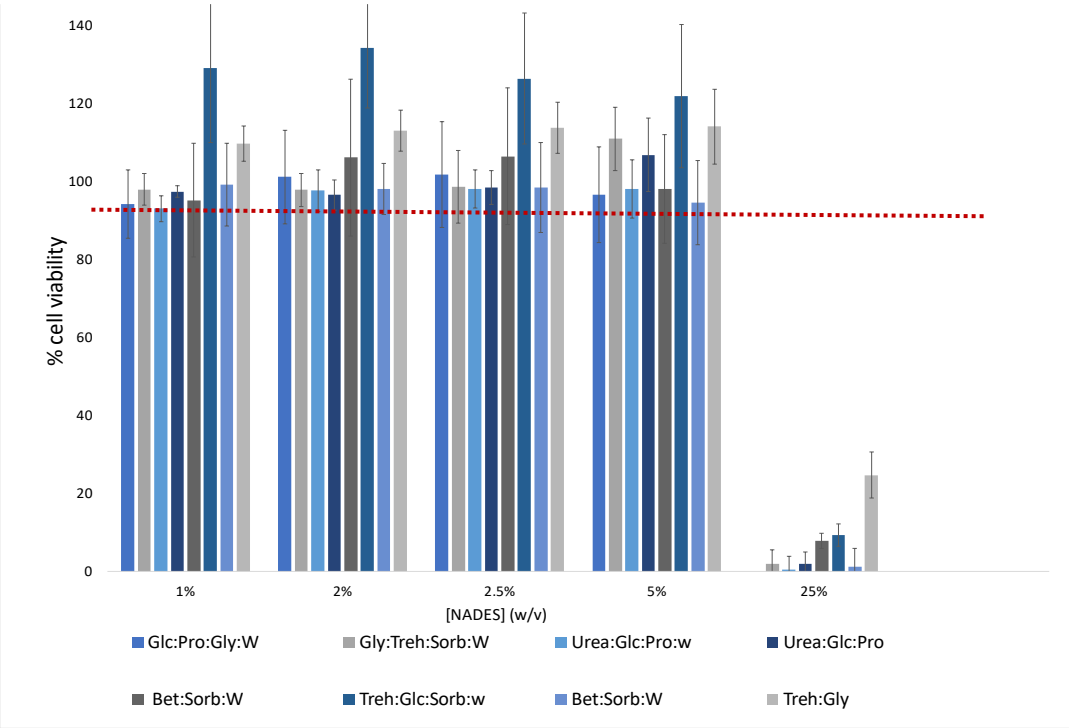
4.1.3 Cytotoxicity

In this work, cell lines L929 were used to determine the cell toxicity of NADES solutions previously described.

After testing NADES, we could observe that up to 5% stimulate cell proliferation, since the incubation of L929 cells with most NADES represented in Fig. 4.2 showed values of cell viability of 100% or higher. Only a few showed slight toxicity, such as the system Bet:CA:W, which

presented the highest toxicity despite concentrations. This result was expected, since the pH is very acidic, thus not providing suitable conditions for cell growth.

Above 25%, all NADES were toxic for this cell line.



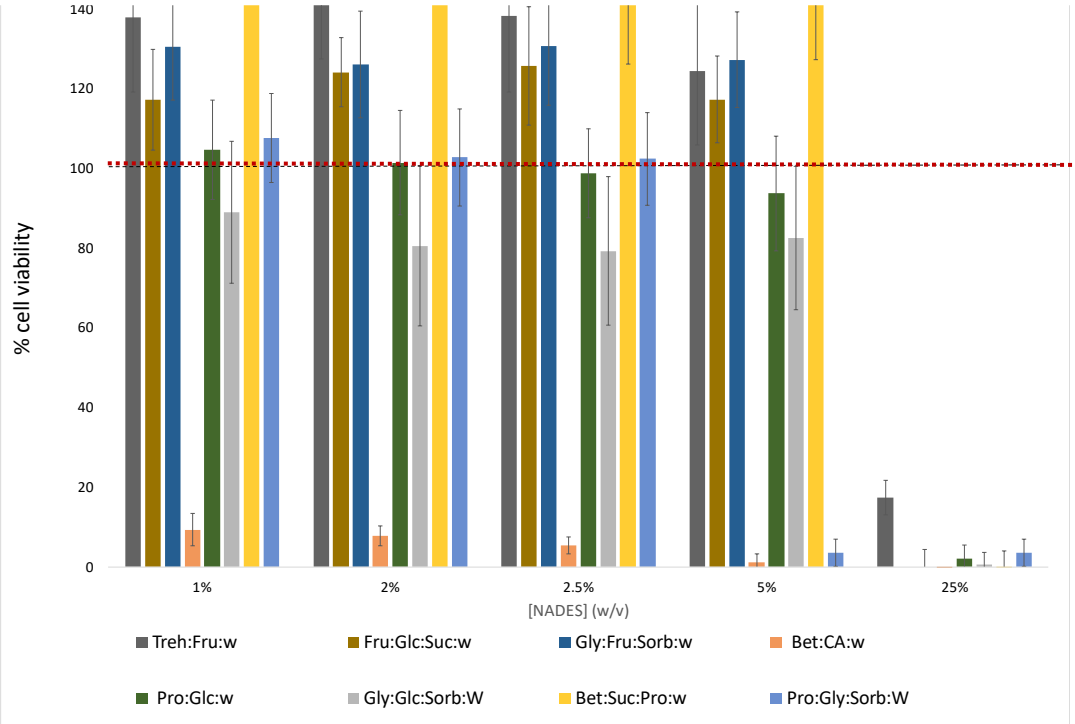


Figure 4.2 — NADES cytotoxicity results at 1%, 2%, 2.5%, 5%, 10% and 25% in cell line L929. Red Line represents the 100% of cell viability.

Some NADES had shown cell growth beyond 140%, even though the assay was performed in triplicates of three, these values may still be confirmed, specially Bet:Suc:Pro:w (Fig. 4.2 bottom chart). These elevated values admit that the NADES used are non-toxic for RBC at concentrations lower than 25%.

4.2 RBCs in NADES solutions

4.2.1 pH

The SAGM solution presents low level of acidity with a pH value of around 5.7[16]. However, when blood components are added, the pH decreases. From literature, it was verified that in additive solutions, like SAGM, at the beginning of the second week of storage and continuing throughout the storage period, the progression of acidosis occurred. The RBC sample that was stored for 6 weeks showed a change in pH from 7.05 to 6.4.[68]

The pH values for NADES-RBC used were overall above 6.5 (Table 4.2), except for the NADES-RBC containing citric acid and NAC (Annex I, Table A1) in the range of 1% to 25% where the pH values were less than 3. Despite having a lot of advantages and antioxidant properties[35–38,46], Citric Acid also presents acidic behaviour in concentrations from 1% to 25%. For example, at 1% Bet:CA:W in PBS the solution pH was 3.30 (Annex Table A1). Once in contact with RBCs, the suspension turned rusty brown, speculating that the iron (Fe) present in haemoglobin suffered immediate oxidation (data not shown), discarding this NADES immediately from further studies.

Fig. 4.3 represents plots for 5 weeks (Wk) for two selected assays, all the data about pH changes can be found in Annex Fig A1.

Comparing to control PBS (red line), in both systems Pro:Gly:Sorb:W and Bet:Suc:Pro:W there was an improvement in pH value at the 5Wk. We can observe at 0Wk, 1Wk and 2Wk there is a minor decrease in pH values and that this is more accentuated in PBS. The concentrations at which the pH value was the highest at 5Wk were 2.5% Pro:Gly:Sorb:W and 1% Bet:Suc:Pro:W. These two systems presented the best values for this assay among the rest.

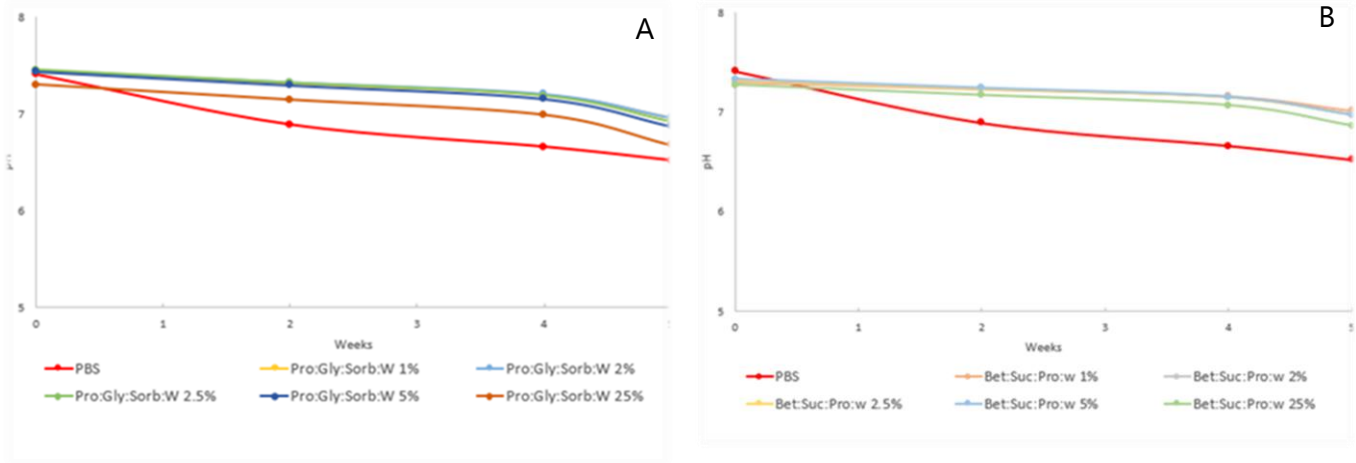


Figure 4.3 — Graphical representation of pH values changes from 0 to 5 weeks in different concentrations of NADES solutions with RBC (NADES-RBC) comparing to PBS (control). A - Pro:Gly:Sorb:W; B - Bet:Suc:Pro:W

The pH values of our solutions when compared to the values described in literature, beginning with pH=7.05 and by the end of the storage period pH=6.4[16] are promising results because our NADES solutions were able to stabilize the pH and maintain a constant over a period of 5 weeks. However, we must take into consideration that the RBC were already at their end-of-life point and the control used in this study is not 100% reliable, since in the 0Wk the hemolysis is already really high.

The NADES-RBC solutions used are abundant in fresh nutrients such as sugars, polyols, and aminoacids, which may promote RBC rejuvenation, for further resistance to haemolysis and prolonged life spam.

During the study PBS was used as a control, however, RBCs stored in PBS alone showed increased haemolysis. Therefore, for future studies, the control should be the solution in the bag or, recreate the SAGM solution[16] to treat and analyze all the NADES-RBC at the same time point, so the changes can be examined. Also, the samples should be performed in triplicate using different transfusion bags, if possible, at the same time point.

4.2.2 Osmolarity

The high osmolarity of the sample (NADES dissolved in PBS) and the cytotoxicity assay at 25% lead us to speculate that it was expected to cause a complete and immediate haemolysis when storing RBC in NADES solution at such a concentration, however it was not verified. As seen in Figure 4.4 after 1 week of storage, the medium is transparent (Fig. 4.4A) compared to the PBS (Fig. 4.4B) which means that the haemolysis process is not as straightforward as we imagined, suggesting the adaptability of RBC towards hostile environments.

During the 5th week of assay the osmolarity values within the NADES-RBC did not change, maintaining the same values as at the beginning of the study.

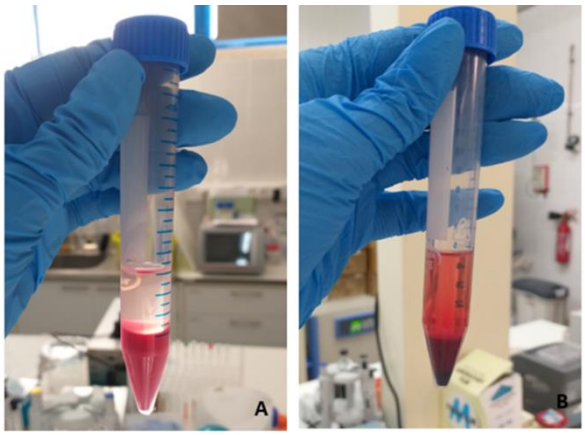


Figure 4.4 — NADES-RBC in Treh:Fru:W 25% after 1 week (A) NADES-RBC in PBS after 1 week (B)

4.2.3 Haemolysis and Morphology

According to literature research, significant RBC shape changes begin to occur during the second week of blood preservation and continue for the rest of the storage time.[20] Cell deformability has decreased as a result of these modifications to RBC shape. Similar to how blood clotting gradually gets worse after two weeks of storage, probably because procoagulant plasma factors run out.[69] RBCs experience a regular morphological change during storage, progressing from flexible biconcave disks (Fig. 4.5A) to even more deformed echinocytes (Fig. 4.5B) to irreversibly deformed spheroechinocytes (Fig. 4.5C).

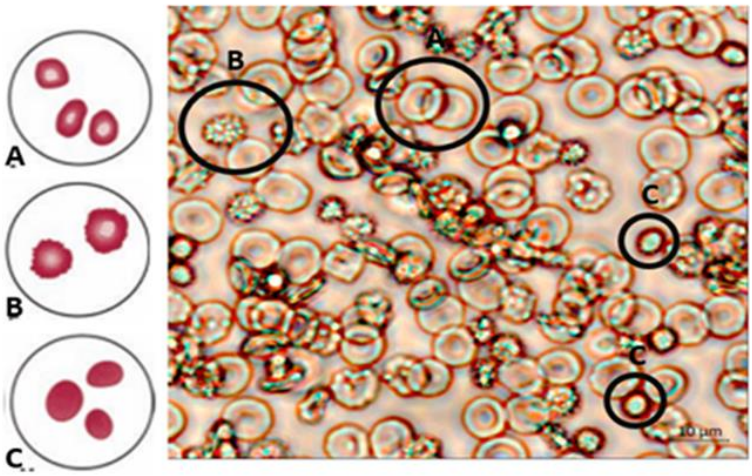


Figure 4.5 — Erythrocytes morphology in NADES after 24h (right). A – normal erythrocytes, B – Echinocytes, C – spheroechinocytes/ Spherocytes. Adapted from M. Turgeon (2005).

Although there is reported improvement in RBC deformability between the 35th and 42nd day of storage, the haemolysis reached 7.6% by the end of the storage period.[69] So the blood bags provided by the IPST used in this research project already present high levels of haemolysis and deformable RBC and are taken into consideration during the interpretation of the results. The conditions for storage, decrease in nutrients, acidosis, and an increase in oxidative stress, inside the blood bag are continuously depleting, which results in a rising rate of haemolysis with storage duration.[68,70] Current RBC storage systems meet the historical regulatory limits for the USA (1%) and Europe (0.8%) systems.[70] Nevertheless, since the shelf-life of RBC inside transfusion bags is limited, after 42 days the haemolysis percentage spikes, overcoming the regulatory limits and consequently the disposal occurs.

It would be expected that NADES at high concentration (25%) in contact with RBC would cause extremely high levels of haemolysis; however, during the haemolysis assay RBC presented flexibility and resistance to the concentration exposed. The cells were flexible enough to endure the high concentration, however the solution remains toxic, so keeping the RBC in this solution would require further dilutions before administering it intravenously.

Throughout this study we used PBS as a control method (Fig 4.6 red line), however, as seen in haemolysis chart, there is a high haemolysis level associated with the storage of RBC in PBS which does not fit into European nor USA regulatory values.

For the first week in Pro:Glc:W solution (Fig 4.6A) the haemolysis values are lower than in PBS and concentrations of 1%, 2%, 2.5%, 5% and 25% are within the Europe's and the USA's regulatory limits (<0.8% and <1.01%).

After the first week the haemolysis values in all concentrations increased, considerably exceeding both regulatory values. However, for lower concentrations (1%, 2%, 2.5% and 5%) this increase is less emphasized, only at 4Wk is the haemolysis increase sharp. While for a concentration of 25% the exponential increase is at 2Wk, as seen in Fig 4.6A (Annex III, Table A2).

For Pro:Gly:Sorb:W concentrations 1%, 2%, 2.5% and 5% are respecting Europe's and the USA's limit regulation standards at the beginning. After 2Wk the percentage also followed a gradual increase exceeding the regulatory standard. Haemolytic percentage for concentration 25% is above both USA and Europe's limit haemolysis (>1.01%) at the beginning of 0Wk, Fig 4.6B (Annex III, Table A2) reaching 100% of haemolysis within 1Wk.

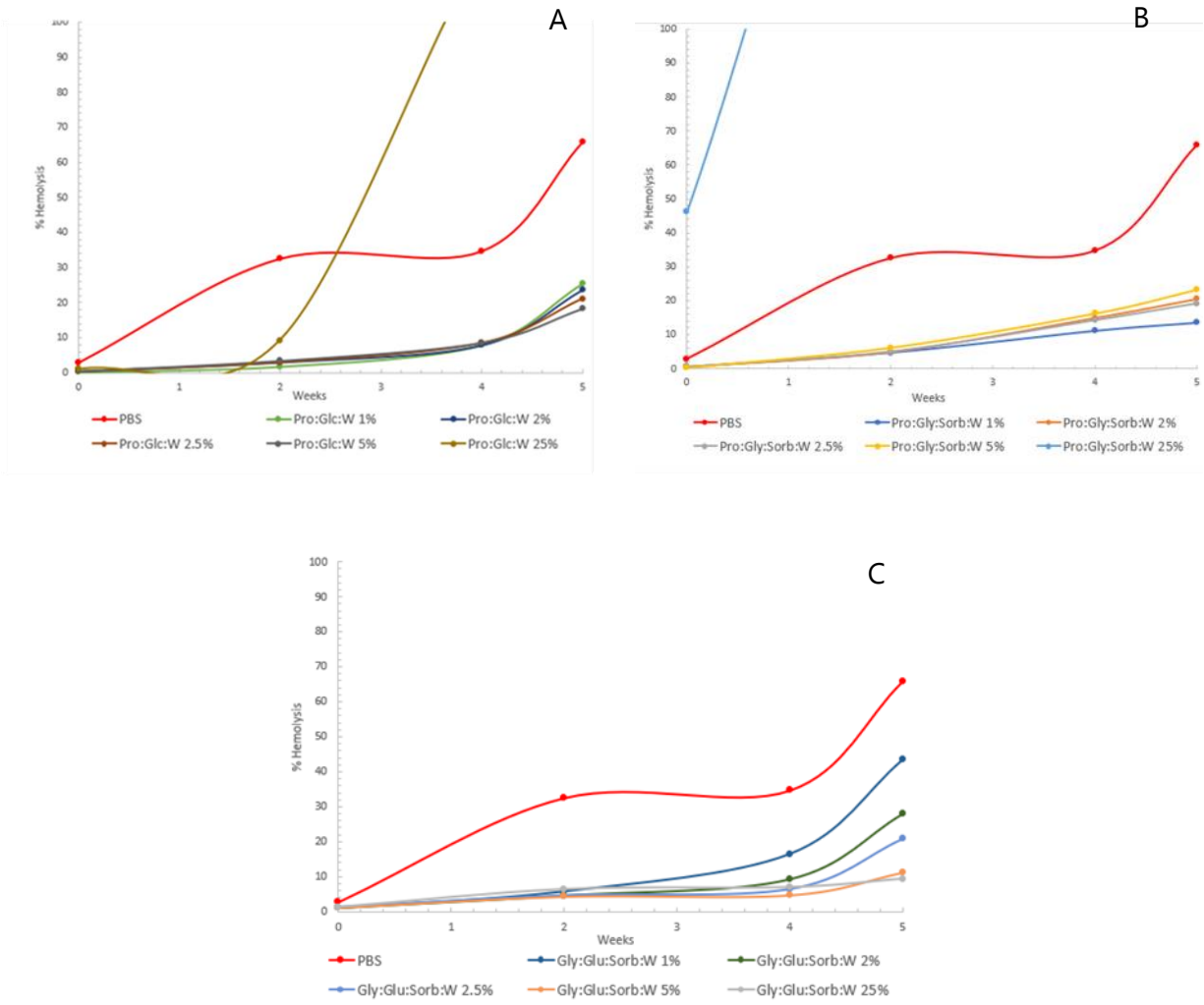


Figure 4.6 — A - Pro:Glc:W haemolysis in 1%, 2%, 2.5%, 5% and 25%, B - Pro:Gly:Sorb:W haemolysis in 1%, 2%, 2.5%, 5% and 25%, C - Gly:Glu:Sorb:W haemolysis in 1%, 2%, 2.5%, 5% and 25%. PBS (red line) control.

None of the Gly:Glu:Sorb:W concentrations meet the European and American regulatory limits (0.8% and 1.01%, respectively). Even though Gly:Glu:Sorb:W haemolytic percentage values are too elevated, they follow the same pattern as the other two systems, there is a slight increase in haemolytic activity followed by sharp boost (Fig 4.6C and Annex III, Table A2). Surprisingly, the results are inverted when compared to previous NADES, for higher concentrations the haemolytic activity is lower. It is possible that this inversion may result from glucose, glycerol, and sorbitol availability. Given their benefits in nature, and since there are more fresh nutrients available for the same quantity of RBC, and glucose is the primary source of energy, this helps preserve cells longer and in more adverse environments. Also, these compounds are associated with preserving properties.[65]

For morphological analysis, the samples were gently mixed, and a little drop was put in the middle of the lamina and covered with lamella.

When the Pro:Glc:W was added, in the beginning the RBC present some echinocytes but the majority were RBC normal cell for all concentrations, as seen in 0Wk (Fig. 4.7). After the 1Wk

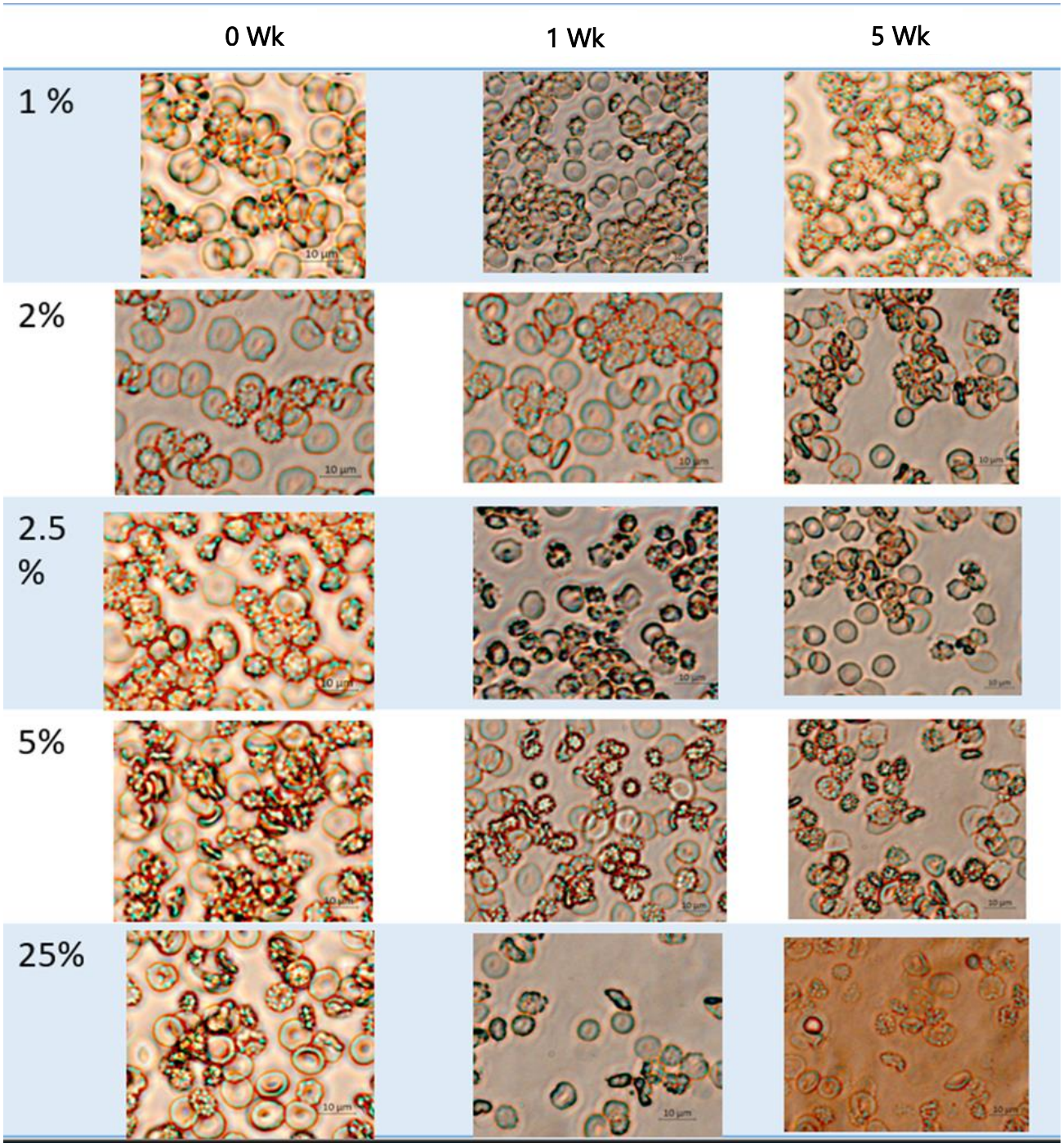


Figure 4.7 — Pro:Glc:W Cell morphology from 0 to 5 week (Wk). Each picture represents different Pro:Glc:W concentrations 1%, 2%, 2.5%, 5% and 25%, respectively during 0, 1 and 5 weeks.

there was an increase in deformed RBC followed by its shrinkage, especially in Pro:Glc:W at 25%.

In solutions at 25% we can observe fewer RBC, correlating these results with haemolytic activity, where 25% represents the higher values of haemolytic activity for this NADES at 1Wk. At the 5 week, the majority of RBC looks like it has almost completely lost its biconcave shape in the different concentrations. (Fig. 4.7)

A certain degree of coagulation was also observed, as seen in Pro:Glc:W 1% at 5W (Fig. 4.7), when cell lost their individual shape and merge into one form. Meanwhile, in Pro:Glc:W solution at 25% 5W an excessively red background, an indicator of high haemolytic activity, was observed with only few visible RBCs and a very high percentage of haemolysis (above 100%) (Fig. 4.7).

Regarding the morphology of RBC in Pro:Gly:Sorb:W (Fig 4.8) at the beginning of the study, all solutions except the 25%, presented nicely biconcave shaped RBC with little echinocytes. For the following week there was a significant deformity of RBC in all solutions, predominating the echinocytes. During the last week of the study, not only some echinocytes were visualized but also spherocytes, which are smaller, irreversible, and round cells.

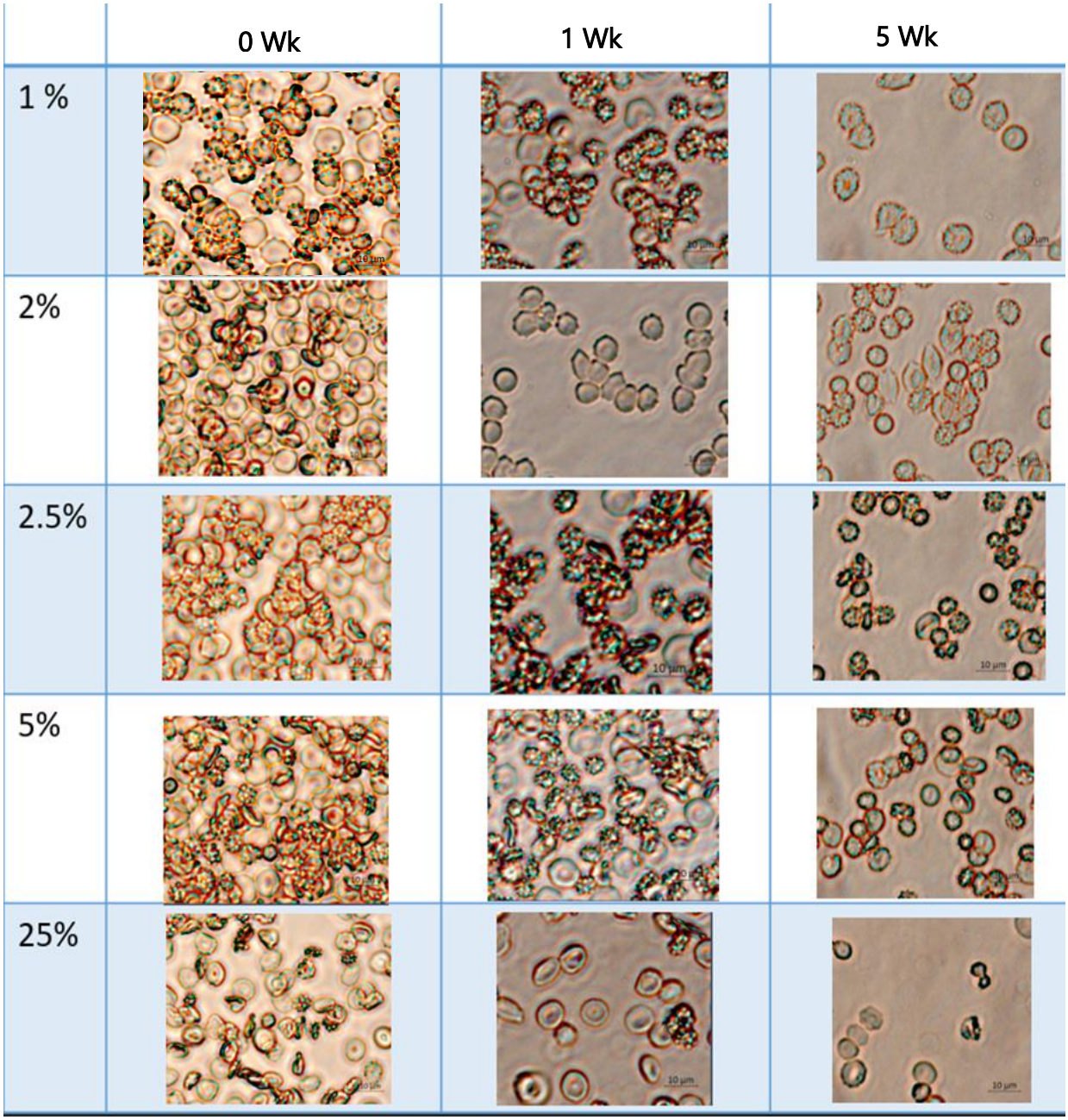


Figure 4.8 — Pro:Glc:Sorb:W Cell morphology from 0 to 5 weeks (Wk). Each picture represents different Pro:Glc:Sorb:W concentrations 1%, 2%, 2.5%, 5% and 25%, respectively during 0, 1 and 5 weeks.

Considering the morphology analysis for Gly:Glc:Sorb:W it is possible to observe that at the beginning 0Wk, the RBC present both normal and echinocyte form, but after 2 weeks, fewer echinocytes were detected and the cells appeared smaller in size, however the biconcave shape was still prevailing in 1%, 2%, 2.5%, 5% and 25%. (Fig 4.9)

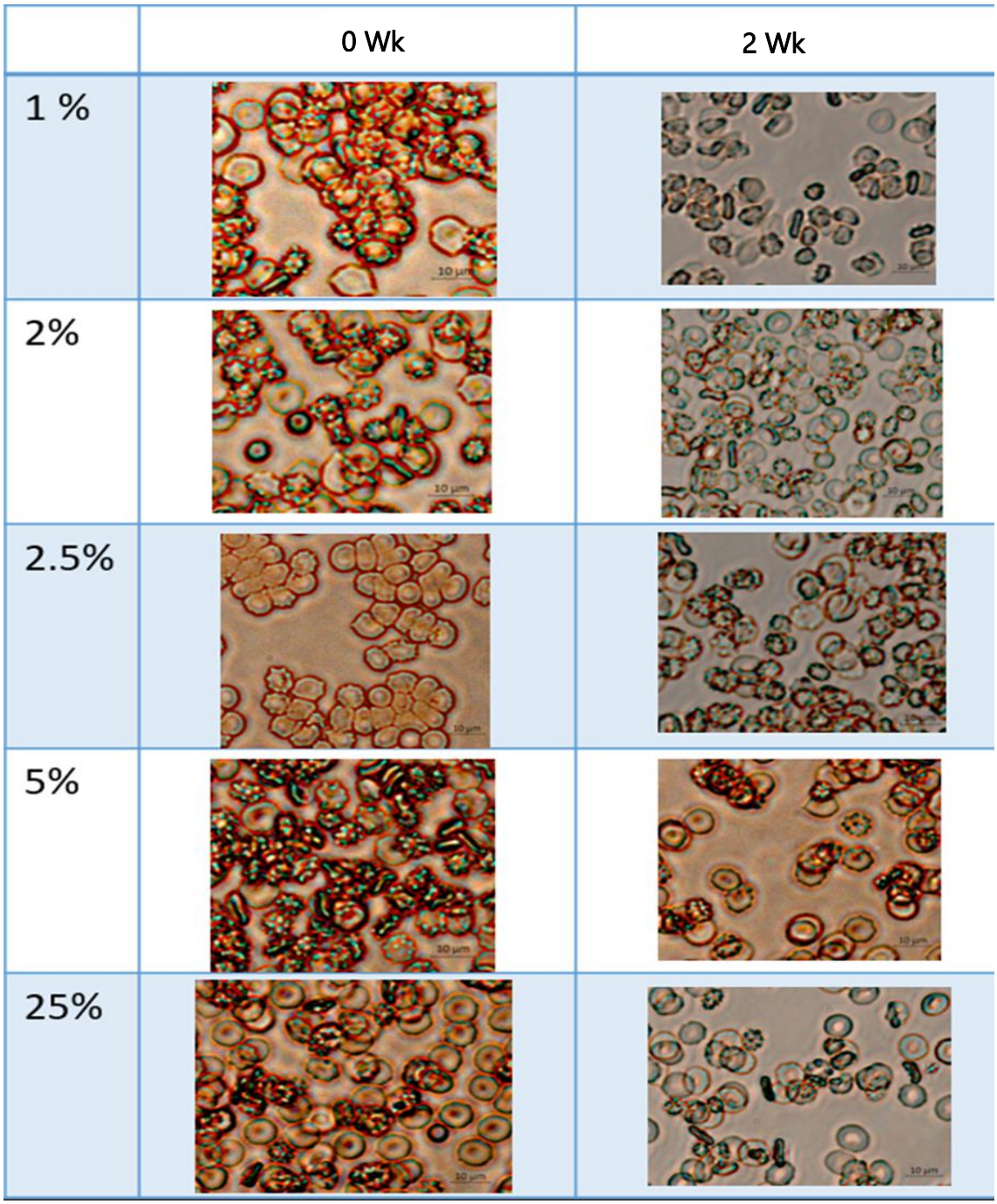


Figure 4.9 — Gly:Glc:Sorb:W Cell morphology from 0 to 2 week. Each picture represents different Gly:Glc:Sorb:W concentrations 1%, 2%, 2.5%, 5% and 25%, respectively during 0 and 2 weeks.

Even though there is a little agglomeration at 1% 0Wk, it may be due to the adaptation to the new solution, since in 2Wk RBC are separated and have a nice biconcave shape.
The presence of echinocytes in NADES-RBC could be due to the age of the cells, since the bags are outdated, and the osmotic imbalance could also affect the erythrocyte membrane.
It is also important to notice that there is no 'true' control because we are comparing to PBS and as mentioned there is complete haemolysis in PBS solutions. What we can conclude is that PBS is a terrible buffer to keep RBC in for long periods of time.
For further research, it would be more accurate to use freshly prepared solution of SAGM or the content of the bag as a control. In this work PBS was used since we have EC - erythrocyte concentrate, to perform morphology evaluation, it is important to dilute the sample from EC bag. It is also very important to have a control sample at the same time points as the tests, since the bags provided are already outdated, and the end time points have not been specified. In contrast to plasma and cells from the circulation (such as leucocytes, platelets, and endothelial cells), RBCs are surrounded by additive solution, plastic, other RBCs, and waste products as storage progresses in a blood bag, implying that the exchanges and equilibrium with the environment are completely upset. Furthermore, exposing the RBCs to hypothermia, or 4°C, has repercussions, particularly on the speed of metabolic fluxes. Consequently, we are not totally sure if the cell behaviour is due to the tests applied, the stress suffered from transportation and temperature shifting or their age.
The difference in haemolytic activity could be explained because different bags were used at different time points.

5

Conclusion and future perspectives

The best way to store blood products is a key part of the transfusion problem, since both RBCs get damaged when they are stored, which can affect their function and possibly their clinical outcome. Natural deep eutectic systems (NADES) have been made to solve problems in many different areas. They are made from established toxicity profiles, biocompatible, and biodegradable compounds. The preparation of NADES is quite simple, does not require a chemical reaction, and does not generate waste, making it a very appealing and promising alternative.

When adding RBC to NADES solutions it was possible to observe some improvements. The major improvements observed were relatively to pH levels, where we could maintain satisfactory levels of pH for 5 weeks, additionally helping reduce oxidation levels and consequently ROS formation.

RBC must meet quality controls and should display levels of haemolysis below 0.8% for European regulations and below 1% according to USA regulations by the end of the storage period. Despite the fact that in this work it was not possible to achieve a similar outcome there is a potential and promising alternative for the systems Pro:Gly:Sorb:W, Pro:Glc:W, and Gly:Glu:Sorb:W. Initially and up to the end of the first day of storage, the haemolytic activity is similar between the solutions of Pro:Gly:Sorb:W and Pro:Glc:W, with Gly:Glu:Sorb:W being a little higher.

The shape analysis showed that even after 2 weeks of RBC stored in Gly:Glu:Sorb:W solution a normal and intact appearance was observed. Even though we analysed NADES-RBC at 25% it should be taken into consideration that these percentages are toxic towards L929.

Even though osmolarity plays a vital role in our body, RBC are more resistant and flexible than initially expected, being able to outperform solution with osmolarities almost 10x higher.

Since this is the first report of using NADES for the preservation of blood cells and although the results were not remarkably good, they are very promising and there is a huge opportunity for improvement. Additional assays such as glucose and lactate quantification, antioxidant quantification of NADES solutions and levels of free haemoglobin present in solution should also be performed to fully understand the relevance of NADES in the preservation of blood cells.

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AN APPENDIX

A.1 Annex I

Table A.1— pH, osmolarity, average and deviation values for all NADES prepared in different molar ratio dissolved in PBS.

DES	Molar ratio	% in 5mL of PBS	pH	Osmolarity (mOsmol/kg)				Deviation
				Sample 1	Sample 2	Sample 3	Average	
CA:Glc:W	1:2:4	1%	3.3	234	228	227	230	3.79
		2%	2.79	237	234	232	234	2.52
		2.5%	2.68	246	251	247	248	2.65
		5%	2.41	328	326	321	325	3.61
		10%	2.2	455	459	458	457	2.08
		25%	1.98	906	906	905	906	0.58
Treh:Glc:W	1:2:13	1%	7.37	240	241	240	240	0.58
		2%	7.36	265	266	264	265	1.00
		2.5%	7.36	275	273	277	275	2.00
		5%	7.26	370	366	369	368	2.08
		10%	7.32	363	361	363	362	1.15
		25%	7.27	966	970	970	969	2.31
Glc:Suc:W	1:1:6	1%	7.42	192	191	192	192	0.58

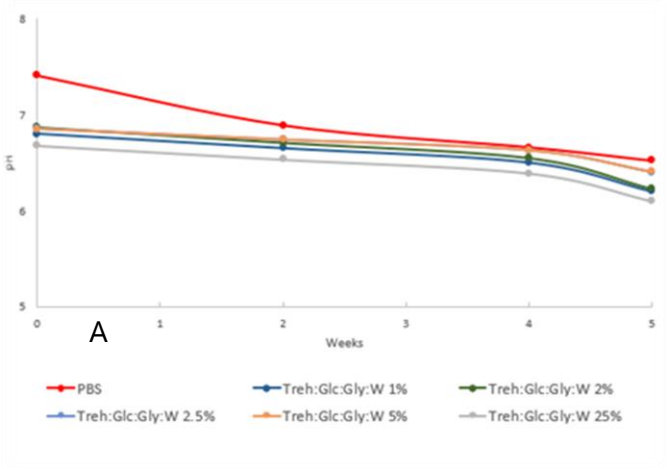
		2%	7.41	213	209	211	211	2.00
		2.5%	7.46	222	222	222	222	0.00
		5%	7.35	270	268	268	269	1.15
		10%	7.35	365	366	365	365	0.58
		25%	7.22	993	1007	992	997	8.39
Bet:NAC:W	1:1:3	0.05%	7.09	211	211	209	210	1.15
		0.08%	6.84	207	208	211	209	2.08
		0.10%	7.01	207	208	210	208	1.53
Bet:Suc:Pro:W	5:2:2:21	1%	7.3	330	329	330	330	0.58
		2%	7.32	372	375	371	373	2.08
		2.5%	7.32	397	397	399	398	1.15
		5%	7.33	520	519	514	518	3.21
		10%	7.31	757	755	762	758	3.61
		25%	7.27	1531	1538	1528	1532	5.13
Bet:Treh:Pro:W	5:2:2:21	1%	7.32	323	327	326	325	2.08
		2%	7.33	370	371	370	370	0.58
		2.5%	7.34	394	394	396	395	1.15
		5%	7.33	510	511	512	511	1.00
		10%	7.35	754	757	756	756	1.53
		25%	7.36	1546	1555	1550	1550	4.51
Gly:Glc:Sorb:W	1:1:1:3	1%	7.24	339	340	337	339	1.53
		2%	7.23	397	400	399	399	1.53
		2.5%	7.2	428	427	407	421	11.85
		5%	7.18	567	574	572	571	3.61
		10%	7.13	876	873	870	873	3.00
		25%	6.96	1720	1725	1714	1720	5.51
Treh:Glc:Sorb:W	1:2:1:13	1%	7.22	326	321	324	324	2.52
		2%	7.22	364	364	360	363	2.31
		2.5%	7.21	382	382	384	383	1.15
		5%	7.19	487	487	484	486	1.73
		10%	7.21	693	695	693	694	1.15
		25%	7.15	1232	1231	1238	1234	3.79
Bet:Gly:Suc	2:3:1	1%	7.41	256	254	256	255	1.15
		2%	7.41	302	298	298	299	2.31
		2.5%	7.41	323	329	323	325	3.46

		5%	7.4	432	434	431	432	1.53
		10%	7.42	810	810	819	813	5.20
		25%	7.46	1576	1584	1568	1576	8.00
Bet:Treh:Gly:W	2:1:3:5	1%	7.46	238	241	240	240	1.53
		2%	7.46	286	285	286	286	0.58
		2.5%	7.47	311	311	310	311	0.58
		5%	7.47	423	422	419	421	2.08
		10%	7.46	681	666	679	675	8.14
		25%	7.5	1432	1444	1451	1442	9.61
Pro:Gly:Sorb:W	1:1:1:3	1%	7.43	350	351	350	350	0.58
		2%	7.44	421	419	427	422	4.16
		2.5%	7.45	455	459	454	456	2.65
		5%	7.43	649	651	637	646	7.57
		10%	7.38	1057	1044	1049	1050	6.56
		25%	7.3	2293	2300	2297	2297	3.51
Bet:Treh:NAC:W	5:2:2:20	0.05%	7.16	285	288	284	286	2.08
		0.08%	7.1	286	285	287	286	1.00
		0.10%	7.02	287	290	290	289	1.73
		1%	4.89	331	333	333	332	1.15
		2%	4.04	377	372	376	375	2.65
		2.5%	3.84	401	404	401	402	1.73
Glc:Pro:Gly:W	3:5:3:20	1%	7.27	341	339	339	340	1.15
		2%	7.25	398	399	398	398	0.58
		2.5%	7.23	429	431	433	431	2.00
		5%	7.17	586	584	586	585	1.15
		10%	7.09	918	911	914	914	3.51
		25%	6.89	1892	1902	1897	1897	5.00
Bet:Sorb:W	1:1:3	1%	7.31	335	334	334	334	0.58
		2%	7.32	387	386	388	387	1.00
		2.5%	7.32	412	411	412	412	0.58
		5%	7.31	559	560	558	559	1.00
		10%	7.34	849	852	849	850	1.73
		25%	7.38	1767	1772	1769	1769	2.52
Gly:Fru:Sorb:W	1:1:1:3	1%	7.39	319	317	318	318	1.00
		2%	7.27	362	360	362	361	1.15

		2.5%	7.28	381	382	383	382	1.00
		5%	7.36	490	488	490	489	1.15
		10%	7.35	684	687	684	685	1.73
		25%	7.29	1332	1330	1328	1330	2.00
U:Glc:Pro:W	1:1:1:3	1%	7.3	347	346	345	346	1.00
		2%	7.27	422	424	423	423	1.00
		2.5%	7.26	456	456	457	456	0.58
		5%	7.2	638	638	638	638	0.00
		10%	7.1	997	991	995	994	3.06
		25%	6.92	2048	2048	2045	2047	1.73
Gly:Treh:Sorb:W	2:1:2:10	1%	7.38	312	311	312	312	0.58
		2%	7.36	356	356	354	355	1.15
		2.5%	7.34	377	380	377	378	1.73
		5%	7.34	506	504	504	505	1.15
		10%	7.32	720	719	721	720	1.00
		25%	7.22	1440	1456	1448	1448	8.00
U:Glc:Pro	1:2:1	1%	7.32	378	376	377	377	1.00
		2%	7.33	486	487	485	486	1.00
		2.5%	7.32	540	542	541	541	1.00
		5%	7.31	819	821	821	820	1.15
		10%	7.27	1393	1388	1393	1391	2.89
		25%	7.25	3225	3220	3219	3221	3.21
Fru:Glc:Suc:W	1:1:1:11	1%	7.37	322	323	322	322	0.58
		2%	7.34	357	357	357	357	0.00
		2.5%	7.34	373	373	370	372	1.73
		5%	7.28	461	458	460	460	1.53
		10%	7.15	624	622	625	624	1.53
		25%	6.99	1182	1181	1174	1179	4.36
Bet:CA:W	2:1:2	1%	3.1	350	349	349	349	0.58
		2%	2.9	402	401	403	402	1.00
		2.5%	2.83	435	435	433	434	1.15
		5%	2.75	576	577	576	576	0.58
		10%	2.72	864	860	850	858	7.21
		25%	2.77	1660	1661	1664	1662	2.08
Pro:Glc:W	5:3:15	1%	7.36	344	344	342	343	1.15

		2%	7.31	398	397	396	397	1.00
		2.5%	7.29	425	426	426	426	0.58
		5%	7.18	573	562	572	569	6.08
		10%	7.04	862	863	864	863	1.00
		25%	6.77	1770	1765	1768	1768	2.52
PBS		1%	7.4	281	280	281	281	0.58
		2%	7.4	282	280	281	281	1.00
		2.5%	7.4	280	282	282	281	1.15
		5%	7.4	280	280	280	280	0.00
		10%	7.4	280	280	280	280	0.00
		25%	7.4	281	281	280	281	0.58

A.2 Annex II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PBS
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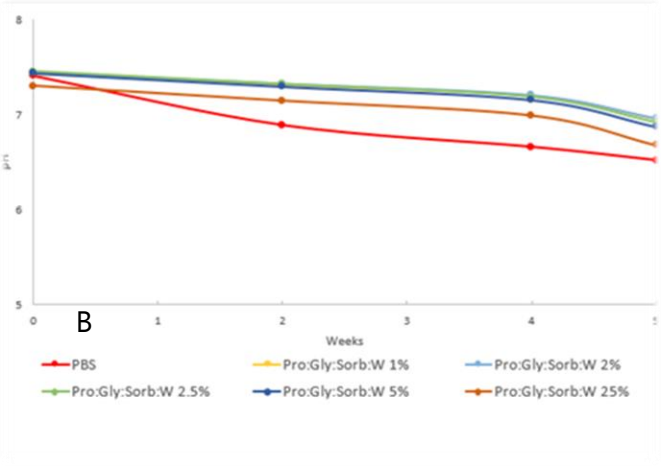
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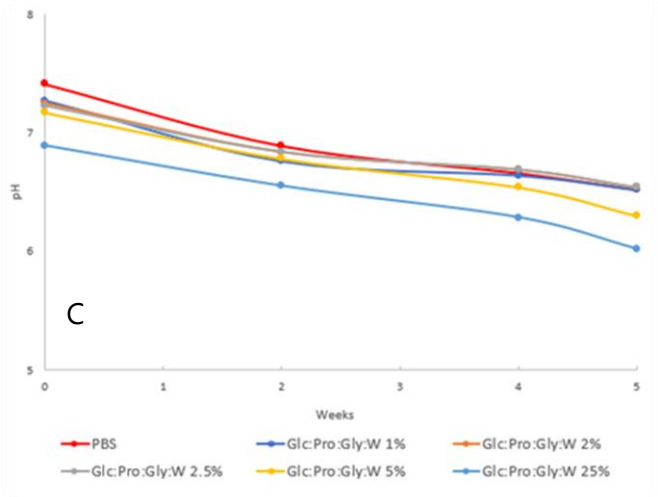
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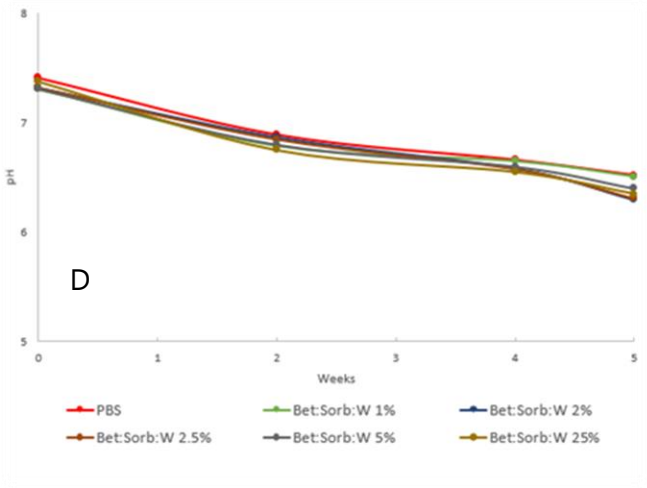
D
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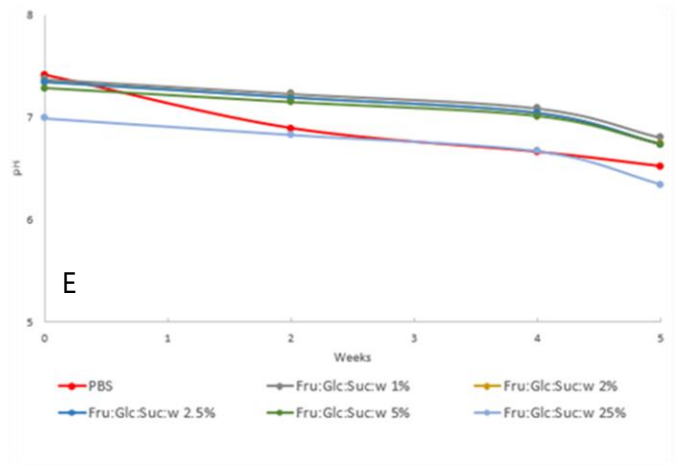
E
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Weeks
PBS
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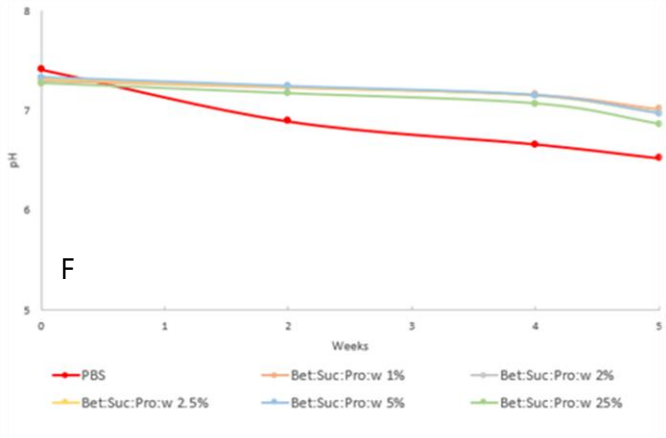
F
pH
Weeks
PBS
Bet:Suc:Pro:w 1%
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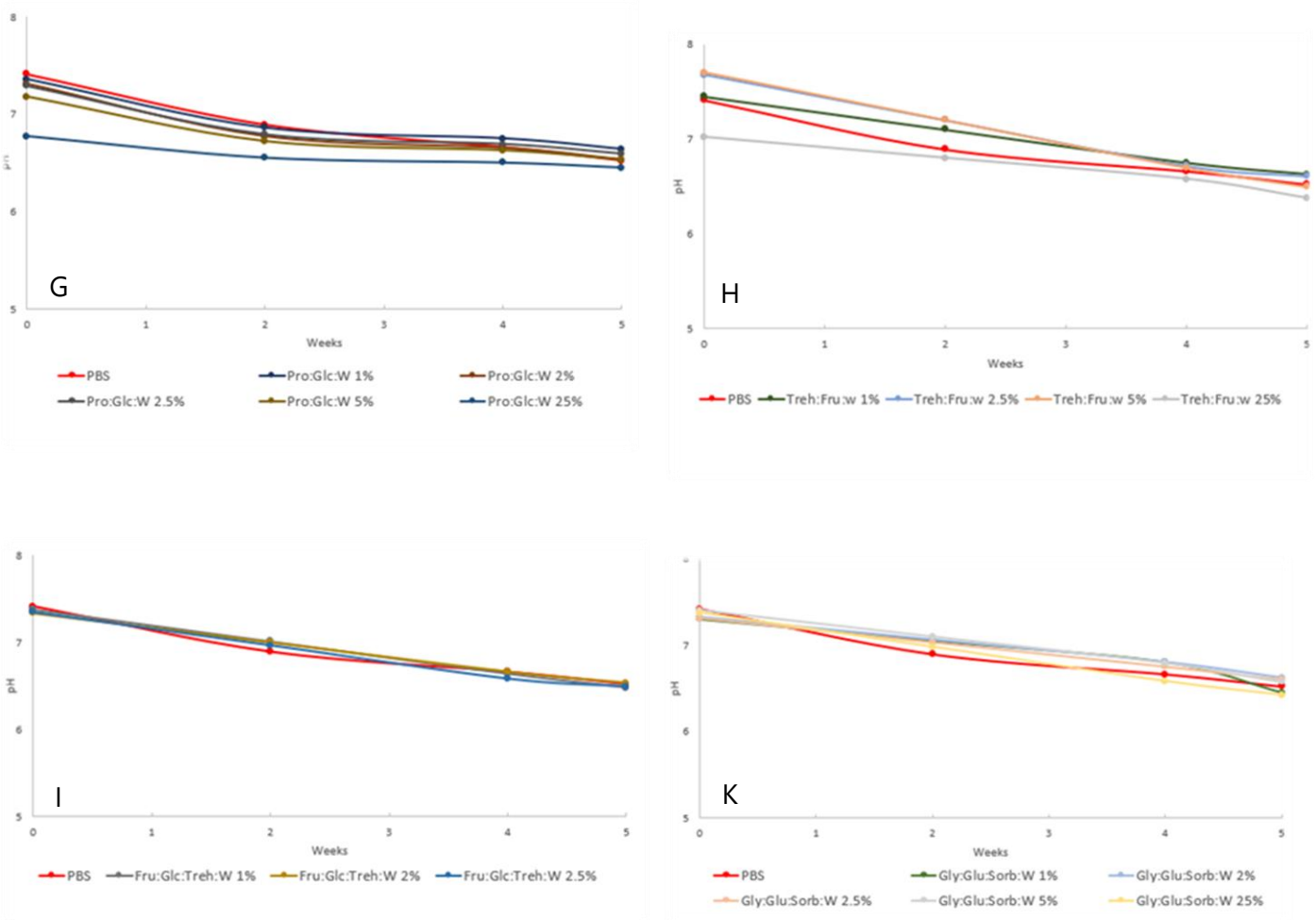

Figure A.2 — Graphical representation of pH values changes from 0 to 5 weeks in different concentrations of NADES solutions with RBC (NADES-RBC) comparing to PBS (control). A - Glc:Pro:Gly:W solution; B - Bet:Sorb:W; C - Treh:Glc:Gly:W; D - Pro:Gly:Sorb:W; E - Fru:Glc:Sucr:W; F - Bet:Suc:Pro:W; G - Pro:Glc:W; H - Treh:Fru:W; I - Fru:Glc:Treh:W; K - Gly:Glu:Sorb:W

A.3 Annex III

Table A.3 — Haemolytic activity values for three systems studied for 0, 2, 4 and 5 Weeks.

	0W	2W	4W	5W
Gly:Glu:Sorb:W 1%	1.1	5.9	16.6	43.4
Gly:Glu:Sorb:W 2%	1.1	4.6	9.3	28.0
Gly:Glu:Sorb:W 2.5%	1.2	4.6	6.4	20.8
Gly:Glu:Sorb:W 5%	1.1	4.3	4.7	11.1
Gly:Glu:Sorb:W 25%	1.3	6.4	7.0	9.4
Pro:Gly:Sorb:W 1%	0.5	4.7	11.1	13.5

Pro:Gly:Sorb:W 2%	0.4	4.8	14.9	20.5
Pro:Gly:Sorb:W 2.5%	0.4	4.9	14.2	19.2
Pro:Gly:Sorb:W 5%	0.2	6.2	16.2	23.2
Pro:Gly:Sorb:W 25%	46.2	216.7	220.4	230.9
Pro:Glc:W 1%	0.1	1.7	7.8	18.2
Pro:Glc:W 2%	0.4	2.9	7.8	21.1
Pro:Glc:W 2.5%	0.4	3.0	8.5	23.6
Pro:Glc:W 5%	0.4	3.3	8.5	25.3
Pro:Glc:W 25%	1.0	9.2	118.8	130.5