Aus dem Institut für Physiologie der Universität Tübingen

Abteilung Physiologie I

Direktor: Professor Dr. F. Lang

Mechanisms of Apoptotic Death of Red Blood Cells Induced by Environmental Contaminants and Natural Products

Inaugural Dissertation

zur Erlangung des Doktorgrades

der Humanwissenschaften

der Medizinischen Fakultät

der Eberhard Karls Universität

zu Tübingen

vorgelegt von

Mohanad Zbidah

Aus

Aleppo, Syrien

2014

Dekan: **Professor. Dr. I. B. Autenrieth**

1. Berichterstatter: **Professor. Dr. F. Lang**

2. Berichterstatter: **Professor. Dr. A. Nordheim**

Contents

ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CNS	Central nervous system
COX	Cyclooxyginase
Ctr	Control
EDTA	Ethylenediaminetetraacetic acid
EGTA	glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EPO	Erythropoietin
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothyocyanate
FL	Fluorescence channel
FSC	Forward scatter
g	gram
GLUT	Glucose transporter
GSH	Glutathion
h	hour
Hb	Hemogblobin
HCT	Hematocrit
HEPES	32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

IL	Interleukin
IONO	Ionomycin
JAK	Janus kinase
K^+	Potassium
KCC	K^+/Cl^- cotransporter
mM	Milimolar
Na^+	Sodium
NF	Nuclear factor
NHE	Na^+/H^+ exchanger
NO	Nitric oxide
PAF	Platelate activating factor
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2
PGN	Peptidoglycan
PLA	Phospholipase A
PPAR	Peroxisome proliferators activated receptor
PS	Phosphatidylserine
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SM	Sphingomyelin
SSC	Side scatter
t-BHP	Tert-butylhydroperoxid
(VI)	Hexavalent

Chapter 1

Introduction

Chapter 1

1. INTRODUCTION

1.1. Premise

Blood is considered a circulating tissue comprising cells (red blood cells, white blood cells, platelets) with fluid matrix plasma. Blood is also classified as a connective tissue, depending on its origin root in the bones as well as to its role. In a healthy body various functions are performed by blood, some of them is to deliver oxygen and nutrients to the body's cells, also to spread warmth to those tissues that need it the most, filter injurious waste products such as carbon dioxide out of the body, defend against diseases, also maintain the optimum pH of the intracellular fluid in order to work the cells' enzyme systems efficiently.

The formation of red blood cells (RBC) in the myeloid tissue is called erythropoiesis and is stimulated by the hormone-erythropoietin (EPO) synthesized by the kidneys. The hormone erythropoietin stimulates erythrocyte formations from undifferentiated pluripotent stem cells which continuously divide and give rise to various blood cells[1]. They lose nuclei upon maturation, and take on a biconcave, dimpled shape with an average diameter of 6-8 µm, and a thickness of 1.5-1.8 µm. Humans have roughly about 4-6 million erythrocytes per cubic millimetre of blood.

Due to the absence of nucleus and mitochondria in Erythrocytes, the total intracellular space is available for O_2 transport, bearing in mind that these cells do not use any of the oxygen they carry[2].

RBC's live about 120 days and do not self-repair[3]. At the end of their life, they are retained, e.g. - by the spleen, where they undergo physiological phagocytosis.

1.2. Membrane Composition of Red Blood Cells

Erythrocytes have a coating around them is so- called the cell membrane. This membrane includes the entire cell material within it and plays many principal roles.

In order to meet with the demands of function, the red cell membrane should have specific properties, cellular flexibility, cell recognition, immune response and adhesion properties. The composition of the cell membrane determines its properties. The membrane is freely permeable to water, oxygen, carbon dioxide, glucose, urea, and certain other substances, but it is impermeable to haemoglobin.

The membrane of erythrocytes is usually composed of a lipid bilayer, integral membrane proteins and a membrane skeleton. Phospholipids and cholesterol form the lipid bilayer, where phospholipids are asymmetrically dispersed in the bilayer[4]. The outer monolayer contains mainly phophatidylcholine and sphingomyelin, where the inner one include phosphatidylethanolamine, phosphatidylserine (PS) and to a lesser extent, phosphoinositide constituents. Dependent or independent of energy, various phospholipids transport proteins such as scramblases, flippases and floppases have proven involvement in the phospholipids movement [5].

A large number of membrane proteins come from diversified blood group antigens. The most important membrane proteins could be mentioned as: band 3, Glut 1, Kidd antigen protein, aquaporin 1, Na^+-K^+-ATPase, Ca^{2+}-ATPase, $Na^+/K^+/2Cl^-$co-transporter, Na^+-Cl^- cotransporter, Na^+-K^+ cotransporter, K^+/Cl^- co-transport, and Gardos channel. ICAM-4 and laminin binding protein function as adhesion proteins.

The red cell membrane skeleton is a group of protein complexes constructed by structural proteins encompassing α and β spectrin, ankyrin, protein 4.1, actin, adducin, dermatin, tropomyosin and tropomodulin. Key regulators of membrane mechanical stability are the spectrin dimer-dimer interaction and the spectrin actin - protein 4.1R junctional complex. Moreover,

they play a crucial role in withstanding shear stresses in the circulation[6, 7, 8].

1.3. Membrane Ion transport in Erythrocyte

The unceasing traffic of ions in erythrocyte is undergoing to various regulating transporters such as : (1) the Gardos channel, (2) Ca^{2+}-ATPase, (3) Na^{+}-K^{+}-ATPase, (4) K^{+}/Cl^{-} co-transport, (5) $Na^{+}/K^{+}/2Cl^{-}$ co-transporter, (6) Band 3 anion exchanger, (7) glucose transporter [9] (figure. 1).

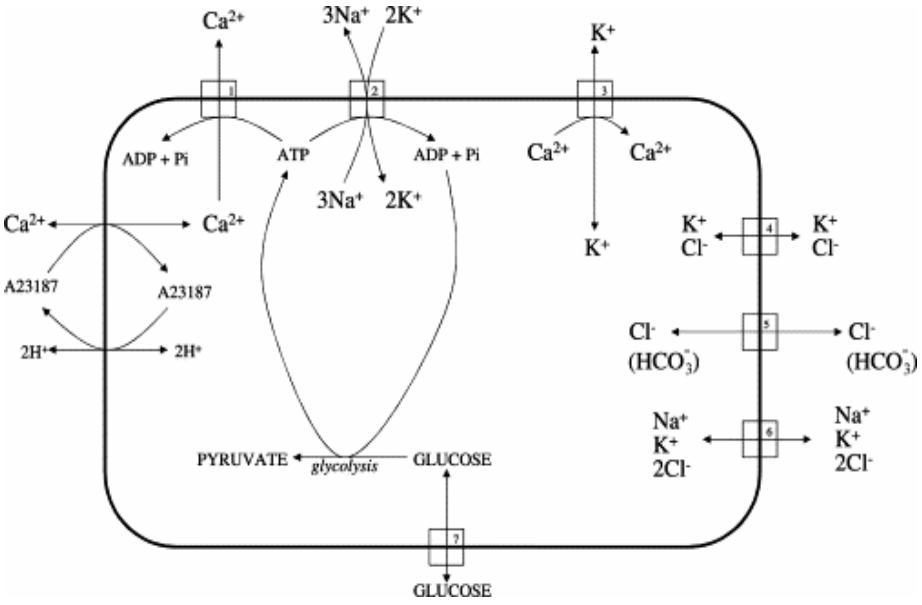

Figure 1: Ionic transport pathways in the erythrocyte membrane (Maher and Kuchel 2003).

The plasma membrane Ca^{2+} ATPase (PMCA) is a transporting protein embedded in the membrane of erythrocytes, which serves as vital regulator of Ca^{2+} amount within cells by removing the cytosolic calcium (Ca^{2+}). The entry of the ion into cells is driven by a very large transmembrane electrochemical gradient of Ca^{2+}, therefore it is very important for cells to maintain low

concentrations of Ca^{2+} for proper cell signalling, thus cells use ATP molecules as a power supply for Ca^{2+}- pump, where they are introduced as a power after suffering hydrolysis. Ultimately, calcium ions are ejected outside the cell.

In principle, the blocking of Ca^{2+} ATPase protein by one of ATPases blockers such as vanadate (VO_4^{3-}) [10] will keep cytosolic (Ca^{2+}) at high levels, which in turn will induce the apoptosis of erythrocytes[11].

The Na^+/K^+-ATPase with energy-dependent system is an intrinsic membrane protein (Figure 2). It is responsible for pumping Na^+ and K^+ against their membrane gradient[12]. It mediates the transport of three Na^+ ions out of the cell and two K^+ ions into the cell per ATP molecule, where it catalyzes the hydrolysis of ATP to active transport of Na^+/K^+ across the cell [13].

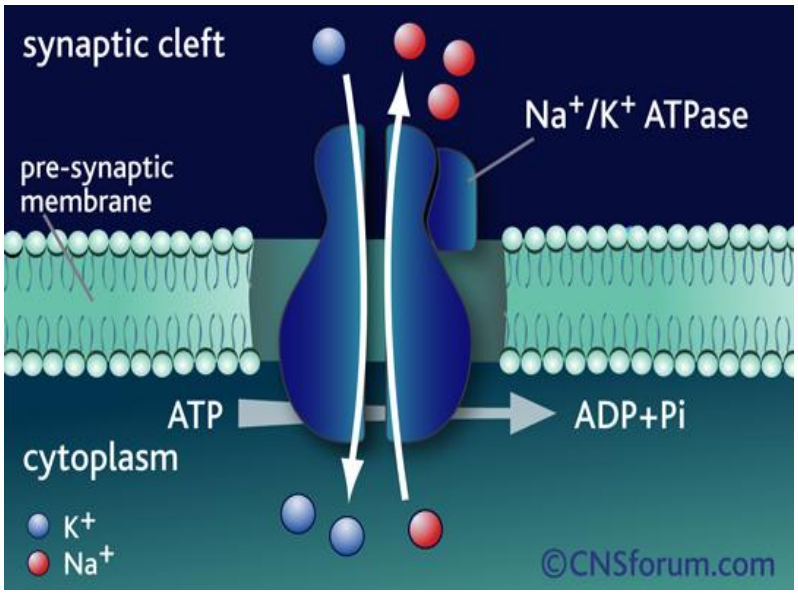

Figure 2: Na+/K+-ATPase pumb (Molecular Biology of the Cell, 4th edition. Alberts. B et al. New York, USA).

A Ca^{2+}-sensitive K^+ channel (Gardos channel, hIK1, or hSK4) is the major route, by which the cellular loss of KCl takes place, this results in an obliged

cellular water loss and consequently cell shrinkage [3]. Approximately 100-200 Gardos channels are found in the erythrocyte membrane[14, 15, 16]. Gardos channel activation is stimulated by high relative levels of intracellular Ca^{2+} concentrations, which in turn increases the mean K^+ flux through the plasma membrane,[17, 18, 19]. Dehydrated erythrocytes is the hallmark of sickle cell disease, it is followed by the loss of K^+, Cl^- and water from the cell[9].

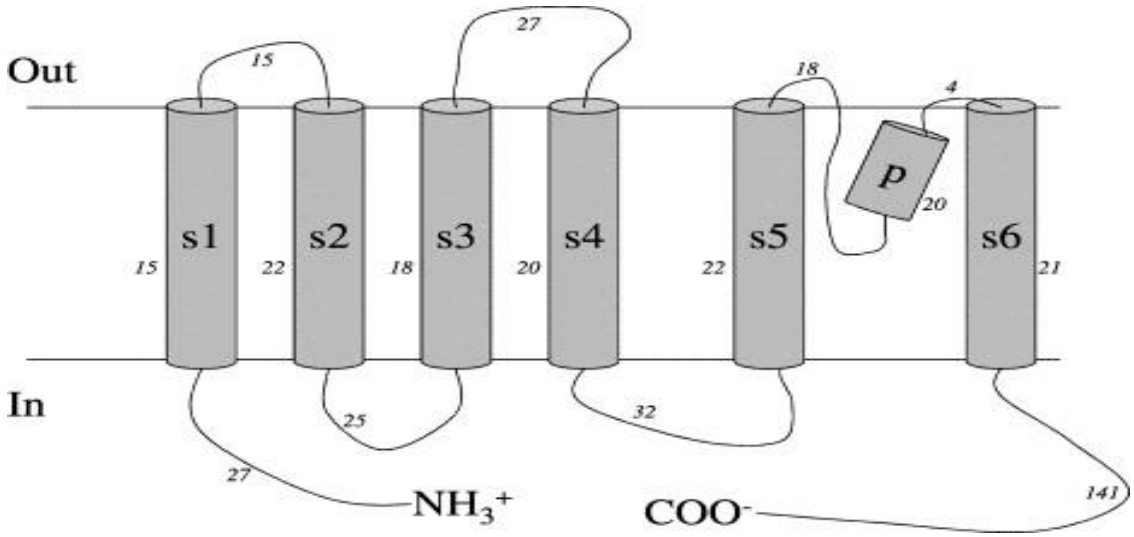

Figure 3: schematic representation of the monomeric subunit of the Gárdos channel in a cell membrane as predicted by Vandorpe et al. (1998).

Tram34 (1-((2-chlorophenyl)diphenylmethyl)-1H-pyrazole), clotrimazole, Charybdotoxin, are common pharmacological inhibitors of the Gardos channel [9, 20].

The KCl cotransporter is a protein involved in the active transport of potassium and chloride traffic across the cell membrane. It is abundant in erythroid precursors[21, 22] but also have a reduced activity during the erythrocytes maturation [3]. Independently of the membrane potential, the KCl

cotransporter mediates mainly a strictly coupled electroneutral transport of K^+ and Cl^- [23].

1.4. Apoptosis

Multicellular organisms do not become infinitely large according to one fact, that the proliferation of cells is balanced by cell death. Cells die through two different ways, one is accidental, often referred to as necrosis, it is a result of mechanical trauma or exposure to some kind of toxic agent. In injured cells, the concentrations of ATP can decrease abruptly, where Na^+/K^+ ATPase can no longer operate, therefore, this stop results in ionic imbalances, cell swelling and subsequent release of cytoslic contents to the exterior.

The other type of death is deliberate, where cells die by suicide mechanism known as apoptosis, they shrink bundling their contents into small membrane-bound packets named blebs. The nuclear DNA is broken into small fragments, each one is enclosed in a small part of the nuclear envelope [24]. The modified plasma membrane of the dying cell signal to macrophages, parenchymal cells, or neoplastic cells, which in turn engulf the cell debris and degrade them within phagolysosomes, so that inhibition of the inflammation is achieved by secreting special cytokines. Apoptosis is used basically as a defence mechanism in response to immune reactions or to damaged cells by disease or detrimental agents [25]. Inappropriate apoptosis either too little or too much is a hall mark in many human pathological conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders, cardiovascular diseases, acquired immunodeficiency syndrome (AIDS) and many types of cancer [25].

A part of the cellular constitution in addition to diverse factors outside the cellular environment can play a substantial role in apoptosis induction[26]. Two main pathways have been described in apoptosis, the extrinsic (death receptor-mediated) and the intrinsic (mitochondria-dependent) pathways. The intrinsic pathway launches within the cell triggered by variety of stimuli such as DNA

damage, stress, or the deprivation of a growth factor. It is depicted as mitochondria-dependent pathway where the mitochondria play a central role in the apoptotic procedure.

Mitochondria-dependent pathway activating brings on permeabilization of the outer membrane of the mitochondria, cytochrome C realeas and caspases 9 activation, which in turn stimulates the downstream caspase 3. Caspase 3 takes it upon himself to break down the main cell structures, to this end, the actual degrading enzymes are regulated indirectly by mitochondrial permeability (figure 4).

The extrinsic pathway is considered the more dynamic, where the extracellular environment events control the spark of its beginning and thus the cell death. It is expressed as constant interaction between the cell and stimuli derived from the external environment, where viruses, radiation, and drugs may all act as stimuli. Cells are known to possess death receptors on thier surface, this death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signalling pathways. The binding of certain ligands to these death receptors on the cell surface such as FAS will be followed by the formation of a death-inducing signalling complex (DISC) which activates death caspases [27, 28]. In a similar way the TNF inducing ligand (TRAIL) operates likewise as the FAS pathway. Processed caspase-8 directly activates other members of the downstream caspases such as caspase 3 (figure 4), which in turn triggers the execution of cell apoptosis by inducing the cell's chromosomal DNA degradation which leads to fragmentation and subsequently the apoptotic demise of the cell [29, 30, 31, 32, 33].

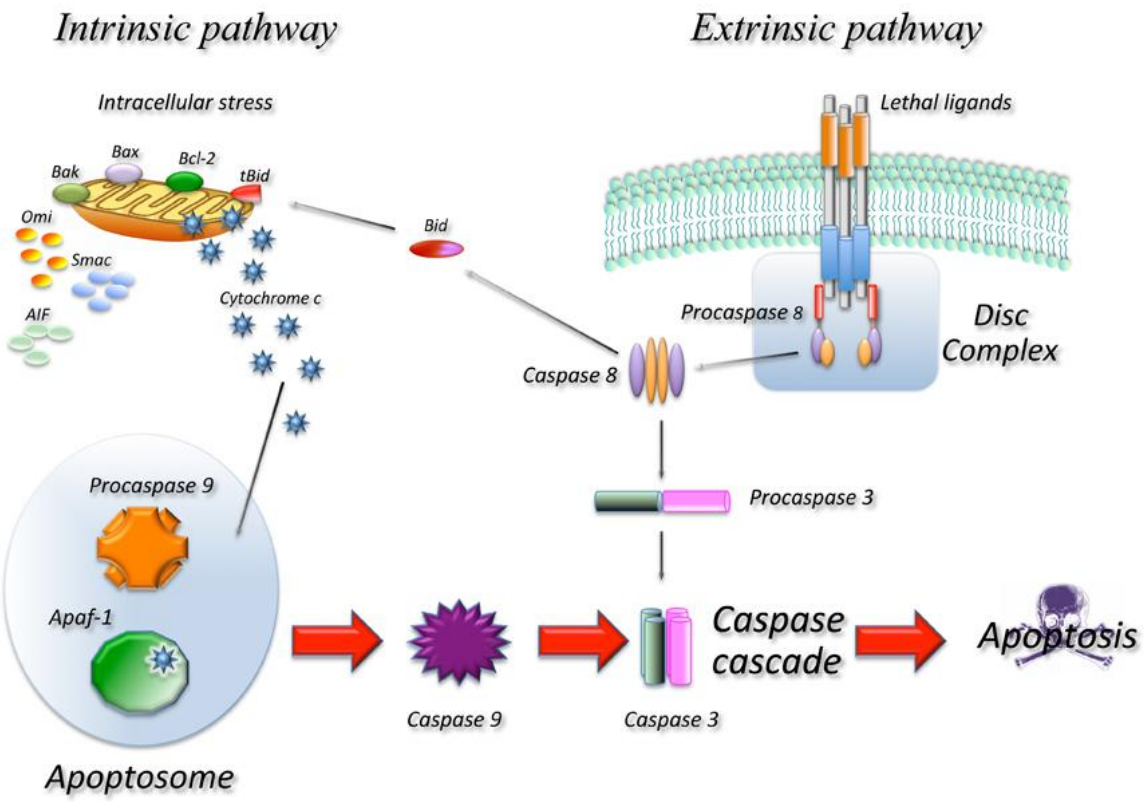

Figure 4: Schematic representation of the intrinsic, extrinsic cellular pathways of apoptosis (B. Favaloro, 2012).

1.5. Programmed erythrocyte death "Eryptosis"

Post a 120-day life span or in response to environmental signals mimic mature erythrocytes nucleated cells in self-destruction, therefore this procedure was coined "Eryptosis" to signify the physiological condition of apoptosis or the suicidal death of erythrocytes.

Eryptosis is characterized by cell shrinkage, cell membrane blebbing and membrane phospholipid scrambling with phosphatidylserine exposure at the cell surface. Eryptotic cells are removed by macrophages which have special receptors, these receptors can identify the exposed phosphatedyl serine on the outer leaflet of the apoptotic cell. Subsequently, cells will be engulfed,

degraded, and removed from the circulation system and thus prevented from undergoing hemolysis.

1.5.1. Eryptosis Inducers

Various clinical conditions in addition to a wide range of endogenous mediators and xenobiotics have proven their involvement in suicidal erythrocyte death. Oxidative stress [34, 35, 36], osmotic shock [37], energy depletion [35], activation of the death receptor CD95/Fas [38], Some of the known xenobiotics could be presented as eryptosis stimulators such as the following : aluminium [39], cadmium [40], lead [41], lithium [42], arsenic [43], selenium [44], silver [45], bismuth [46], tin (Nguyen, Foller et al. 2009), zinc [47], amantadine [48], vanadium [49], amiodarone [50], amphotericin b [51], chlorpromazine [52], ciglitazone [53], curcumin [54], cyclosporine [55], gadolinium [56], hemin [57], hemolysin [58], listeriolysin [59], methyldopa [60] and valinomycin [61].

Most xenobiotics set off eryptosis by augmenting cytosolic calcium activity, but also on the other side eryptosis could be enhanced by stimulating ceramide formation as reported by methyldopa and menadione [60, 62]. We may also encounter definite types of triggering eryptosis like phytic acid that decreases only the cellular ATP content [63]. Thymoquinone induced suicidal erythrocyte death employing the induction of protein kinase C pathway without being depend on the appreciable stimulation of ceramide or intracellular calcium activity[64].

The hindering of PGE2 formation and antioxidant activity as a mechanism proved its ability and efficacy to inhibit Ca^{2+} permeable cation channels partially or completely and thus attenuates suicidal erythrocyte death. However, Eryptosis is inhibited by nitric oxide, catecholamines and a variety of further small molecules [65, 66]. Erythropoietin plays a dual role, on one hand erythropoietin partially inhibits the Ca^{+2} permeable cation channels which counteract eryptosis. On the other hand, it contrarily may foster the formation of erythrocytes by prolonging the life span of circulating erythrocytes via inhibition

of the apoptosis of erythrocyte progenitor cells and slowing the clearance of mature erythrocytes. This effect explains the tow mechanisms which erythobiotin depends on [67]. Eryptosis is also inhibited by resveratrol [68], flufenamic acid [69], caffeine [70], zidovudine [71], vitamin C [72], thymol [73]. Adenosine is also an inhibitor of eryptosis [74]. Staurosporine can play as Pharmacological inhibition of eryptosis by inhibiting protein kinase C[75]. Blunting suicidal erythrocyte can be achieved by Urea, which abrogates ceramide formation [76].

1.5.2. Ion channels in eryptosis

The altered activity of ion channels is the turning point of apoptosis machinery. Different channels in erythrocytes like Cl^- channels, Ca^{2+}-permeable cation channels and Ca^{2+} -sensitive K^+ channels etc regulate the death machinery. The channels modify intracellular ion composition and contribute to apoptotic cell shrinkage. Cl^- channels is responsible for cell shrinkage and cytosolic acidification by permitting the exit of $HCO3^-$. K^+ exit through Gardus channel contributes to cell shrinkage and minimize intracellular K^+ concentration, which in turn fosters apoptotic cell death. Cell membrane potential is determined by K^+ channel activity, which has the upper hand at Ca^{2+} entry through Ca^{2+} channels. Ca^{2+} may enter intercellular environment through nonselective cation channels and activates several enzymes that carry out apoptosis in response to the cytosolic Ca^{2+} signal augmentation. Suicidal cell death could be promoted or impaired by Specific ion channel blockers.

1.5.3. Ca^{2+} permeable cation channels

The elevation of cytosolic Ca^{2+} activity in epoptosis may result from activation of monovalent and divalent cation-permeable channels known as nonselective cation channels that mediate Ca^{2+} entry[77]. The molecular identity of the cation channels is so far not completely understood, but supposedly involves the transient receptor potential channl TRPC6 [77]. A wide variety of

challenges can activate cation channels comprising osmotic shock, oxidative stress, and Cl^- removal [78], they affect at least partially by way of stimulation of cyclooxygenase with the generation of prostaglandin E_2, which in turn activate the cation channel[66]. This augment of cytosolic Ca^{2+} promotes Gardos channels activation which mediate K^+ loss together with Cl^- via parallel anion pathways resulting in osmotic water loss and subsequently cell shrinkage[79]. Simultaneously, high intracellular Ca^{2+} level activates a membrane scramblase that increases the abundance of phosphatidylserine in the outer leaflet of the erythrocyte membrane [79].

1.5.4. Cl^- channels

In general, Cl^- channels play a crucial role in controlling the ionic composition of the cytoplasm and the volume of cells. It is one of the most important players in the eryptosis events, where its activation leads to depolarize the cell membrane by the exit of the Cl^- ion, this efflux is usually accompanied with K^+ exit. The loss of the somatically active KCl is paralleled by the loss of osmotically obliged water and subsequent cell shrinkage, one of the decisive Manifestations of eryptosis [80].

$HCO3^-$ exit is also considered as Cl^- channels activation outcome resulting in cytosolic acidification. The inhibition of the Na^+/H^+ -exchanger during apoptosis may have the upper hand in the acidification process. This inhibition is due to at least partially caspase-dependent degradation of the carrier protein NHE1 [81, 82, 83, 84].

1.5.5. Oxidative stress and eryptosis

Oxidative stress is one of the most frequent triggers of eryptosis [36]. Defects of the antioxidative defence trigger eryptosis in part by stimulating Ca^{2+} entry via activation of the Ca^{2+} permeable cation channels [85, 86, 87], Oxidative stress also go further to activate erythrocyte Cl^- channels, which play a basic

role in erythrocyte shrinkage so that it participates in eryptosis triggering [88, 89, 90]. The stimulation of eryptosis by oxidative stress is proven to be paralleled by aspartyl and cysteinyl proteases activation[91]. Caspases have been shown to be expressed in erythrocytes[92, 93] and activated in response to oxidative stress [34, 91], they play an interesting role via cleaving the anion exchanger band 3 [94] and stimulating phosphatidylserine exposure [93]. However, Caspases activation is not an irreplaceable requirement for ionomycin or hyperosmotic shock induced eryptosis [76, 95, 96].

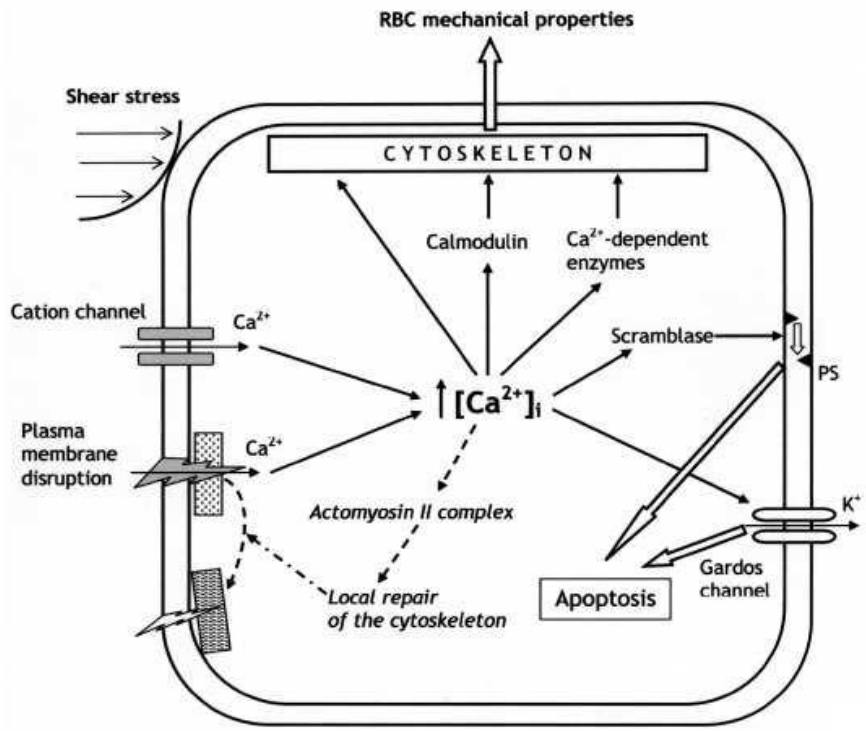

Figure 5: Scheme shows shear stress-induced Ca^{2+}-dependent mechanisms in RBCs (Barvitenko, Adragna et al. 2005).

1.5.6. Cycloxygenase and Prostaglandin E2 pathway

Hyperosmotic shock and Cl^- removal trigger the release of prostaglandin E2 (PGE2)[66, 78]. Where cation channels are activated as a result of PGE2 formation[66], this in turn will lead to increase of the

cytosolic Ca^{2+} concentration[66, 97], and thus stimulates phosphatidylserine exposure at the erythrocyte surface[66], subsequently, cell membrane blebbing. Cyclooxygenase inhibitors such as diclophenac and acetylsalicylic acid in addition to phospholipase-A2 inhibitors such as quinacrine and palmitoyltrifluoromethyl-ketone could abolish pharmacologically the activation of the cation channels by Cl^- removal [66], PGE2 proved its role via activating the Ca^{2+} dependent cysteine endopeptidase calpain, but apparently was not required for stimulation of phosphatidylserine exposure [66].

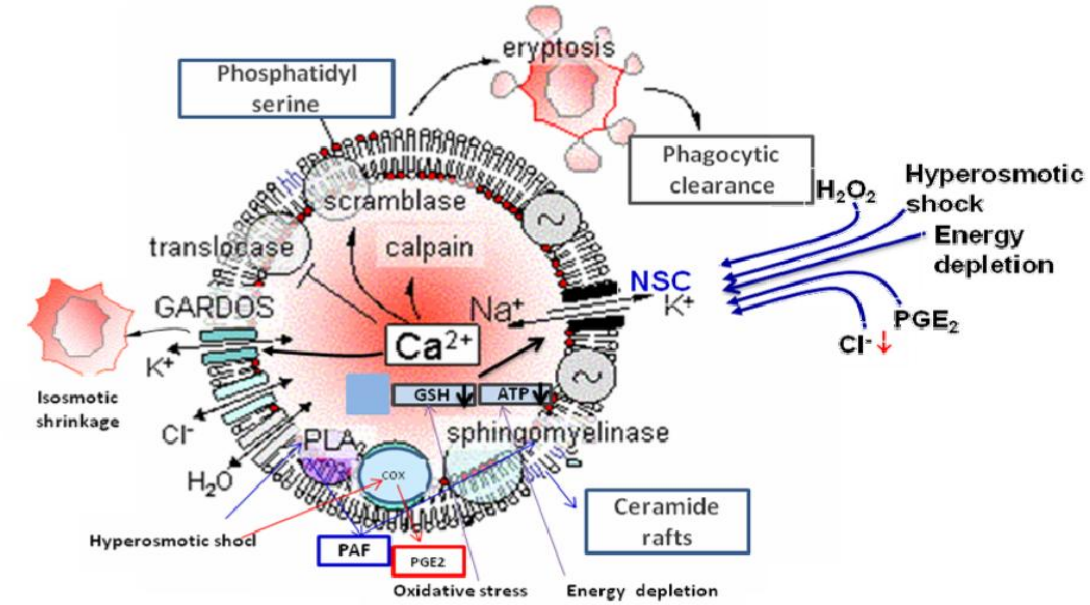

Figure 6: Synopsis of the eryptosis mechanism (Lang, Lang et al. 2006).COX, cyclooxygenase; PAF, platelet activating factor; PGE2, prostaglandin E2; PLA2, phospholipase A2; GSH, glutathione and NSC, nonselective cation channel.

1.5.7. Ceramide is formed by a sphingomyelinase following several stressors

The absence of calcium could not fully block the effect of osmotic shock on phosphatidylserine exposure[98]. this drew attention to other involved mechanisms and excluded the entirely role of nonselective

cation channel. Osmotic shock resulted in the occurrence of ceramide at the erythrocyte surface[98], C6-ceramide revealed in further experiments its ability to trigger eryptosis upon treatment with bacterial sphingomyelinase[98]. However, induced eryptosis by osmotic shock can be inhibited by the sphingomyelinase inhibitor 3,4-dichloroisocoumarin[11]. Moreover, the nominal absence of Ca^{2+} in hypertonic medium blunted the induction of eryptosis but not total abolished[98]. Accordingly, the presence of C6 ceramide accelerated eryptosis post osmotic shock exposure. Due to cell shrinkage, the phospholipid mediator platelet activating factor (PAF) is released, which in turn stimulates the breakdown of sphingomyelin and the formation of ceramide in erythrocytes[66], thus PAF participates in the stimulation of the sphingomyelinase activation and subsequent "eryptosis"[11]. PAF further triggers cell shrinkage and phosphatidylserine exposure of erythrocytes[99]. The stimulation of phosphatidylserine exposure is blunted by the genetic knockout of PAF receptors (PAF receptor knockout mice), and by the PAF receptor antagonist ABT491[66].

1.5.8. The physiological importance of eryptosis and its role in diseases

Eryptosis is an extra magnificent process, by which the circulating blood gets rid of defective erythrocytes avoiding the negative side injuries of hemolysis. Phagocytes are equipped with specific receptors sensing phosphatidylserine, which are exposed by apoptotic erythrocytes as a detected signal by phagocytes's navigator. Engulfed erythrocytes are degraded and thus cleared from blood stream [100]. Eryptosis has shown disclosed in various clinical dysfunctions and normal physiological processes its ability to limit erythrocyte survival. The cytosolic calcium activity remarkably increased at ageing erythrocytes [101]. The Life span of erythrocytes was significantly shortened in many haematological disorders, in sickle cell disease and glucose-6-phosphate dehydrogenase deficiency erythrocytes were more sensitive to stressors

impacts as osmotic shock, energy depletion and oxidative stress and jogged to eryptosis faster than healthier erythrocytes[35]. Energy depletion attenuates the Na^+/K^+ –ATPase function, cell swelling is resulted from the leakiness of the cell membrane which enhances the accumulation of Na^+, Cl^- and osmotically obliged water [102]. In general Na^+ entry is followed by the cellular loss of K^+ which brings on decreasing the equilibrium potential of K^+, gradual depolarization and entry of Cl^-. Erythocytes rupture could be the result of swelling releasing the intracellular haemoglobin into the blood circulation. Haemoglobin filtration procedure take place in the glomeruli, thus the precipitation of free haemoglobin in renal tubules could cause acute renal failure[11]. To this result, eryptosis pop up as protective technique works against excessive hemolysis [11, 103].

Eryptosis can play a crucial role in limiting the aggressive invasion of malaria Plasmodium falciparum by influencing the course of the pathogen [104]. The parasite induces novel permeability pathways (NPP) in the intact cell membrane facilitating the exchange of nutrients and disposal of waste products [105]. The oxidation of the cell membrane is the way used by parasites to activate host cell channels and these channels constitute the NPP. Cation channels which increase cytosolic Ca^{2+} and Na^+ are playing here dual function, it is indispensable for parasite survival, in addition to its activation which leads to eryptosis .

1.6. Environmental contaminants

Environmental contaminants are substances that have the potency to harm people by loading accidentally or deliberately into environment, wildlife and plants. A Hazardous substance, as defined in s. 292.01, Wis. Stats., means “any substance or combination of substances, including any waste of a solid, semisolid, liquid or gaseous form which may cause or significantly contribute to an increase in the mortality or an increase in serious irreversible or incapacitating reversible illness, or which may pose a substantial present or

potential hazard to human health or the environment because of its quality, concentration or physical, chemical or infectious characteristics. This term includes, but is not limited to, substances that are toxic, corrosive, flammable, irritants, strong sensitizers or explosives”[106]. People's exposure to chemicals or toxins in the environment requested a special expression known as biomonitoring, it is the direct measurement that indicates the amount of a chemical that actually gets into people from all environmental sources, containing substances measuring, or detecting the products in human specimens such as blood and urine after their breaking down. With such bio monitoring measurements, the most health-relevant assessment of exposure will be available.

1.6.1. Hexavalent Chromium

Potassium dichromate is an orange-red colour powder with the molecular weight of 294 g/mol,

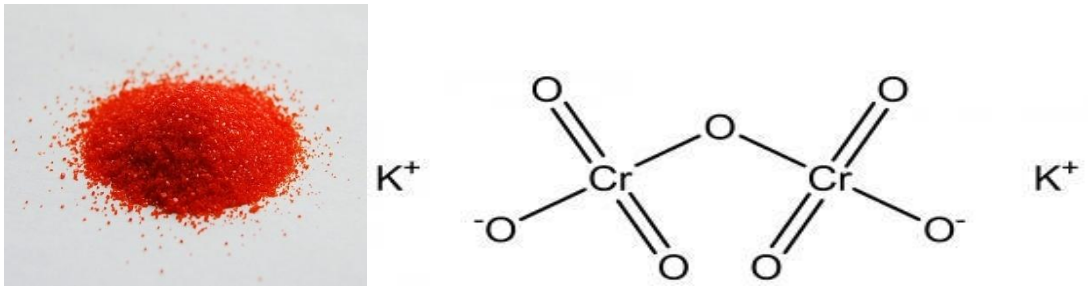

Figure 7: Potassium dichromate, the chemical structure and its crystal normal form (wikimedia).

it was awarded a wide spectrum of names as potassium bichromate, dichromic acid, dipotassium salt, chromic acid, bichromate of potash, dipotassium salt, and dipotassium dichromate. It has a chemical formula: $K_2Cr_2O_7$.

Potassium dichromate could exist as result of the chemical reaction of chromium trioxide and potassium hydroxide in a reactor creating mother liquor. The reaction is as follows:

$$2\ CrO_3 + 2\ KOH = K_2Cr_2O_7 + H_2O$$

The widespread industrial application of this heavy metal resulted in the global occurrence of soluble hexavalent (VI) chromium, which pushed it to enter the ranking as a critical environmental pollutant [107, 108]. Chromium as a pollutant is found in such components of wood preservatives, pigments, anticorrosive primers, metal plating, fossil fuel, etc[109], and in nature could be encountered in two main species, the relative innocuous form trivalent Cr(III), and the hexavalent form Cr(VI) rated more toxic species [107, 108]. Aqueous Cr(VI) could reach serious levels in surface and ground water, where the contaminant Cr(III) undergoes natural processing through oxidation converting it into the dissolved form [110]. Releasing chromium from orthodontic appliances can do damage the DNA in oral mucosa cells of children[111]. Chromium may further be released from metal on stainless steel implants [112, 113, 114]. Elevated concentration of hexavalent chromium pollution leads to health hazards of mining in India[115]. Oxidative stress, inflammation, cell proliferation, malignant transformation, growth arrest, cytotoxicity, and apoptosis are triggered by Chromium (VI) which is absorbed in the intestines [116, 117, 118, 119, 120]. DNA damage including base modification, mitochondrial damage as well as single-strand breaks, double-strand breaks, Cr–DNA adducts, DNA–Cr–DNA adducts, protein–Cr–DNA adducts, and mutagenesis are mostly the implicated mechanisms in chromium (VI) toxicity [116, 121, 122].

1.6.2. Rotenone

Rotenone, an odorless chemical of botanical origin, is obtained from extracts of Derris root and Lonchocarpus plants [123].

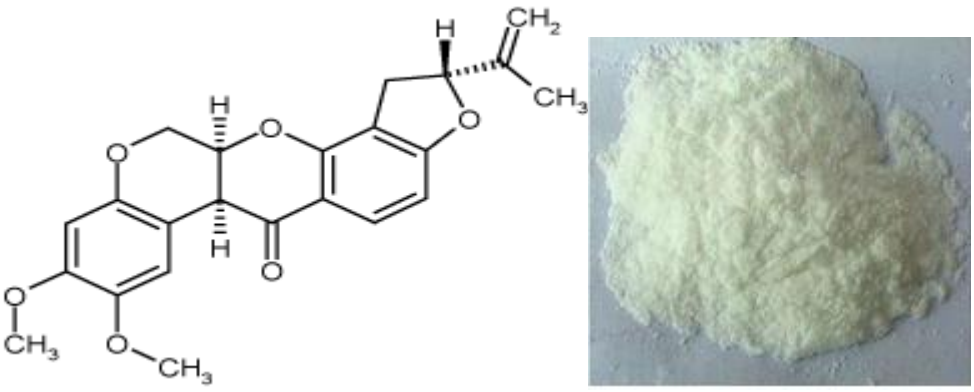

Figure 8: Structural formula of Rotenone and its crystal normal form (Alibaba Hebei Chinally International Trade Co., Ltd.).

It has been utilized extensively for a long time as a broad-spectrum insecticide and pesticide, In California, 200 kg of rotenone per year is used as insecticide for organically grown lettuce and tomatoes[124]. It is a flavonoid, naturally formed in stems of several plants and in various seeds as well, such as the jicama vine plant [125, 126, 127]. Rotenone is considered an environmental factor and has been implicated in neuronal cell death of Parkinson's disease (PD)[125, 128]. It is a lipophilic compound that can easily access cytoplasm and mitochondria by crossing cell membranes. Due to Rotenone`s involvement in the production of many features of PD in rats, its application in PD research has enlarged exponentially over the last few years largely, This is including development of α-synuclein-positive cytoplasmic inclusions similar to LBs in human PD in the remaining nigral neurons[129]. Neurons themselves showed also toxic sensitivity to rotenone[130, 131, 132, 133]. Animals treatment with Rotenone reproduce a neurochemical and neuropathological feature of Parkinson's disease [127, 134, 135, 136]. Rotenone has a capacity to induce neoplastic, paraneoplastic and preneoplastic lesions so that it is strongly suggested to be carcinogenic [126]. However, It is published that, effective modulators could penetrate multidrug resistance (MDR) proteins that belong to the ATP-binding cassette superfamily in tumor cells such as rotenone[137]. The mechanism which rotenone depends on is at least in part

effective by interference with mitochondrial function in turn induced the suicidal cell death [138, 139, 140, 141, 142, 143].

1.7. Natural products

Compounds, organic molecules, naturally occurring as end products of secondary metabolism. They are often isolated from animals, plants, or microbes, and are considered unique compounds for particular organisms or classes of organisms; Natural products are the cornerstone of modern therapeutics. They fulfil an important role in the development of therapeutic agents. Many of the natural products of medicinal value offer us new sources of drugs which have been used effectively for centuries in traditional medicine.

Plants are sources of hallucinogens, poisons, and addictive drugs. Wide range of people has preference to use natural products from plants for preventive medicine, but potential harmful effects of such compounds should put in account.

1.7.1. Apigenin

Flavonoids have been considered recently as being potential chemotherapeutic agents depending on their biological activities, which include involvement in cell cycle arrest in addition to stimulation of Apoptosis[144].

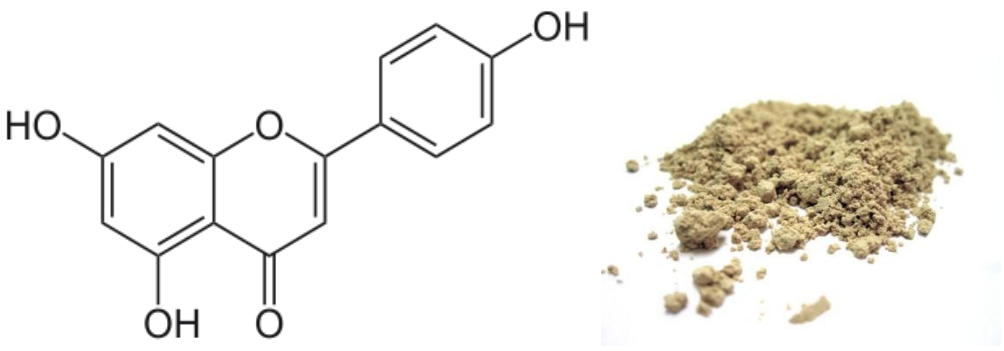

Figure 9: structural formula of Apigenin and its powder form (skinactives.com).

Apigenin is a yellow crystalline solid natural product, classified as a flavone subclass of Flavonoids, and can be encountered in vegetables and fruits and further plant materials [145, 146]. It is believed to have a number of biological function including anti-inflammatory, anticancer, and free-radical scavenging[147, 148, 149]. Malignant cancer cell line researches indicated to the inhibiting role of Apigenine in cancer cell activity by enhancing cell cycle arrest and apoptosis [150, 151]. Apigenin exhibits antiproliferative activity in human breast cancer cells[152]. This flavones also triggers autophagia which reflects its chemopreventive properties, concurrently it induces resistance against chemotherapy[153]. Moreover, Apigenin reportedly showed nonmutagenic and low toxicity compared to related flavonoides[154]. This flavon has shown ability to counteract inflammation, oxidative stress and the development of cancer [146, 155]. The protective effect of apigenin against tumor growth is at least partially due to the induction of apoptosis [156]. On the other hand, apigenin may protect against apoptosis [157].

The apoptosis stimulating effect is at least partially due to the influence of apigenin on mitochondria [158] and on gene expression [159]. Apigenin is partially effective through the targeting of the JAK/STAT pathway [153].

1.7.2. Honokiol

The polyphenol honokiol [(3,5-di-(2-propenyl)-1,1-biphenyl-2,2-diol], present in the cones, bark, and leaves of Magnolia officinalis [160].

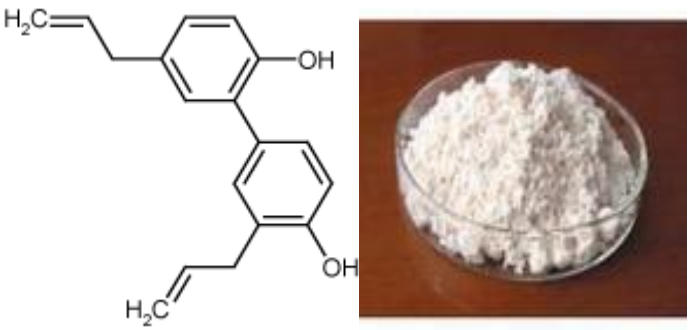

Figure 10: Honokiol, the chemical structure and its crystal normal form (Alibaba Hebei Chinally International Trade Co., Ltd.).

Honokiol is a small molecule that has shown its ability to act as antiangiogenic and antitumor in various tumors, including sarcomas, multiple myeloma, and chronic lymphocytic leukemia[161]. Honokiol, has been used for almost three decades as a muscle relaxant[162] also extensive research has shown that honokiol inhibits skin tumor promotion [163]. The two phenolic groups of the honokiol molecule awarded it antioxidant properties similar to vitamin E[164] or polyphenols such as flavonoids[165], inhibits nitric oxide synthesis and tumor necrosis factor (TNF) expression[166] [167], inhibits invasion [168], down-regulates antiapoptotic protein Bcl-xL[169]. Moreover, it has been reported that honokiol could inhibit free radical induced lipid peroxidation[170] and prevented oxidative modification of LDL [171]

It has previously been shown to counteract inflammation, infection, anxiety, depression, stress, angiogenesis, oxidation and malignancy [160, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184], The anti-tumor activity of honokiol has been attributed to its ability to stimulate apoptosis [160, 185, 186, 187, 188, 189, 190, 191, 192]. On the other hand, honokiol has been shown to inhibit apoptosis [193, 194, 195]. Mechanisms mediating effects of honokiol include death receptor pathways [160], mitochondria [160, 190, 196], caspases [160], apoptosis-related proteins [172], nuclear factor kappa B (NF-κB) [172], signal transducers and activator of transcription 3 (STAT3) [172, 197, 198], epidermal growth factor receptor (EGFR) [172, 199], mammalian target of rapamycin (m-TOR) [172], phospholipase D [196], calpain [200], adhesion molecules [189],15-lipoxygenase-1 [201].

Chapter 2

Materials and Methods

Chapter 2

2. MATERIALS AND METHODS

2.1. Modulation of erythrocyte survival by Hexavalent Chromium

2.1.1. Erythrocytes, solutions, and chemicals

The blood bank of Tübingen University supplied perpetually the Leukocyte-depleted erythrocytes friendly. Before donation, Hepatitis A, B, and C , HIV, and syphilis, were tested in the volunteers (age range 18–68 years) providing blood. The ethics committee of the University of Tübingen (184/2003V) approved this study, the volunteers provided informed consent. At a hematocrit 0.4%, pH 7.4, at 37 °C for 48 h, blood samples were incubated in vitro in Ringer solution contained (in mM): 125 NaCl, 5 KCl, 1 MgSO4, 32 N-2-hydroxyethylpiperazine- N-2- thanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; or in serum as indicated. 1 mM glycol-bis(2-aminoethylether)-N,N,N0,N0-tetraacetic acid (EGTA) was used to substitute 1 mM $CaCl_2$ in Ca^{+2}-free Ringer solution preparation. This Ca^{2+} free Ringer solution was used to study the role of calcium entry in phsophatidylserine exposure on erythrocytes treated with chromium (VI). $CaCl_2$ was removed in another experiment without addition any chelating agent to the solution. Erythrocytes were subjected to potassium dichromate (K2Cr2O7; Sigma, Freiburg, Germany), where concentrations represent chromium (VI) ions. Ringer solution with 5 mM $CaCl_2$ was used to wash the erythrocytes following pre-treatment with both Ringer solutions with / without Ca^{2+} for the indicated time periods, the same solution loaded with FITC-conjugated Annexin V was used to resuspend the erythrocyte for 20 min which is considered too short to trigger eryptosis.

2.1.2. FACS analysis of annexin V-binding and forward scatter

The protocol Andree, Reutelingsperger [202] was used to perform FACS analysis. The pre-treated cells under the respective experimental condition were washed with Ringer solution containing 5 mM $CaCl_2$. FITC-conjugated Annexin-V (Immuno- Tools, Friesoythe, Germany) was used to stain Erythrocytes at a 1:200 dilution. By flow cytometric analysis (FACS-Calibur from Becton Dickinson; Heidelberg, Germany) samples were properly measured after 20 min of incubating with light protection at 37C°. Cells volume was estimated by forward scatter (FSC) determination, where annexin V fluorescence intensity was measured in fluorescence channel FL-1 applying an excitation wavelength of 488 nm and an emission wavelength of 530 nm. In the different flow cytometric measurements ranged the total events between 10,000 to15,000. The standard software CellQuest Pro (BD biosciences) was used to perform the data acquisition and analysis.

2.1.3. Measurement of intracellular Ca^{2+}

Pre-incubated suspension of erythrocytes with volume 50 µl was washed in Ringer solution and followed later with Fluo-3/AM (Biotium, Hayward, USA) loading in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo- 3/AM. The cells were reincubated at 37°C for 30 min and washed tow times in Ringer solution with 5 mM $CaCl_2$. 200 µl Ringer was used to resuspend Fluo-3/AM-loaded erythrocytes. Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1.

2.1.4. Determination of intracellular ATP concentration

Intracellular erythrocyte ATP was measured by incubating 90 µl of erythrocytes for 48 hours at respective temperature conditions in Ringer solution with or without chromium (VI) (final hematocrit 5%). As a positive control, glucose-depleted Ringer solution was used instead of

Ringer solution by erythrocytes incubation. After Cells lysing in distilled water, proteins were precipitated using HClO4 (5%). Following cells centrifuging, an aliquot of the supernatant (400 µl) was adjusted to pH 7.7 by the addition of saturated KHCO3 solution. By employing the luciferin- luciferase assay kit (Roche diagnostics) in accordance to the manufacturer's protocol on luminometer (Berthold Biolumat LB9500, ad Wildbad, Germany) ATP concentrations of the aliquots were determined in mmol/l cytosol of erythrocytes. To avoid ATP degradation, all the manipulations in this experiment were performed at 4°C.

2.1.5. Determination of ceramide formation

A monoclonal antibody-based assay was used to determine Ceramide abundance in erythrocytes. Incubated erythrocytes with and without chromium (VI) were stained for 1 hour at 37°C with 1 µg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) with a dilution of 1:5. Following tow times of washing with PBS-BSA, cells were stained with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody for 30 minutes (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Frequent washing with PBS-BSA ensures removing unbound secondary antibody. On a FACS-Calibur in FL-1samples were then analysed by flow cytometric analysis.

2.1.6. Measurement of hemolysis

To determine the subsequent hemolysis of the treated erythrocytes, the samples were undergone to a centrifugation (3 min at 400 x g, room temperature), which is followed by supernatants collecting. The hemoglobin (Hb) concentration of the supernatant was measured by a photometer at 405 nm. lysed erythrocytes in distilled water was

specified as 100% hemolysis and determined by reading the supernatant absorption.

2.1.7. Confocal microscopy and immunofluorescence

4 µl of treated erythrocytes in respective experimental conditions was incubated to display their eryptotic condition, the cells were stained with FITC-conjugated annexin-V (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 µl Ringer solution with 5 mM $CaCl_2$. Following tow times of erythrocytes washing, cells were resuspended in 50 µl of Ringer solution with 5 mM $CaCl_2$. Prolong Gold antifade reagent (Invitrogen, Darmstadt, Germany) was added to 20 µl sample to mount up a glass slide covered with a coverslip. Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) was used to take images for the eryptotic erythrocyte with a water immersion Plan-Neofluar 63/1.3 NA DIC.

2.1.8. Statistics

Paired ANOVA with Tukey's test as post-test were used for statistical analysis as indicated in the Figure legends. The arithmetic mean ± the standard error of the mean (SEM) has been calculated from the averaged values obtained from the different erythrocyte preparations. In all graphs, data were expressed as arithmetic means ± SEM. n indicates the number of various erythrocyte specimens studied. In Both control and experimental conditions the same erythrocyte specimens have been used. Specimens are differently susceptible to eryptotic effects where different erythrocyte specimens were tested in diverse experiments.

2.2. Involvement of Rotenone in the erythrocyte cell membrane scrambling

2.2.1. Erythrocytes, solutions and chemicals

Experiments were performed at 37°C with isolated erythrocytes drawn from healthy volunteers and provided by the blood bank of the University of Tübingen. The study is provided with approval from the ethics committee of the University of Tübingen (184/2003 V). At a hematocrit 0.4%, pH 7.4, at 37 °C for 48 h, blood samples were incubated in vitro in Ringer solution contained (in mM): 5 KCl, 1 $MgSO_4$, 125 NaCl, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4 at 37°C for 48 h. Erythrocytes were subjected to rotenone (Sigma, Freiburg, Germany) at the donated concentrations. To prepare Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was replaced with 1 mM glycol-bis(2-aminoethylether)- N,N,N_,N_-tetraacetic acid (EGTA).

2.2.2. FACS analysis of annexin V-binding and forward scatter

FACS analysis was performed as described in protocol Andree, Reutelingsperger [202]. Cells were washed post incubation in Ringer solution with 5 mM $CaCl_2$ and followed by Annexin-V-FITC staining (1:200 dilution; ImmunoTools, Friesoythe, Germany), where incubated at 37°C for 20 min with light protection. In FL-1 the forward scatter (FSC) of the cells was determined, and annexin V fluorescence intensity was measured using an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany). To perform annexin V fluorescence analysing a distinct marker was placed at the end of the annexin V negative population, where the batch analysis was launched acquiring the percentage of annexin V positive erythrocytes using the CellQuest Pro software. On

the other side, the batch analysing of forward scatter was accomplished by recording the geometric mean of the cell population

2.2.3. Measurement of intracellular Ca^{2+}

Pre-incubated suspension of erythrocytes with volume 50 µl was washed in Ringer solution and followed later with Fluo-3/AM loading (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo- 3/AM. For 30 min at 37°C the cells were incubated and washed afterwards tow times in Ringer solution with 5 mM $CaCl_2$. 200 µl Ringer was used to resuspend Fluo-3/AM-loaded erythrocytes. Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1.

2.2.4. Determination of ceramide formation

A monoclonal antibody-based assay was used to determine Ceramide abundance in treated erythrocytes with Rotenon. Incubated erythrocytes with and without Rotenon were stained for 1 hour at 37°C with 1 µg/ml anti- ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) with a dilution of 1:5. Following tow times of washing with PBS-BSA, cells were stained with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody for 30 minutes (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Frequent washing with PBS-BSA ensures removing unbound secondary antibody. On a FACS-Calibur in FL-1 samples were then analysed by flow cytometric analysis

2.2.5. Measurement of hemolysis

To determine the subsequent hemolysis of the treated

erythrocytes, the samples were undergone to a centrifugation (3 min at 400 x g, room temperature), which is followed by supernatants collecting. The hemoglobin (Hb) concentration of the supernatant were measured by a photometer at 405 nm. lysed erythrocytes in distilled water was specified as 100% hemolysis and determined by reading the supernatant absorption.

2.2.6. Statistics

Paired ANOVA with Tukey's test as post-test was used for statistical analysis as indicated in the Figure legends. The arithmetic mean ± the standard error of the mean (SEM) has been calculated from the averaged values obtained from the different erythrocyte preparations. In all graphs, data were expressed as arithmetic means ± SEM. n indicates the number of various erythrocyte specimens studied. In Both control and experimental conditions the same erythrocyte specimens have been used. Specimens are differently susceptible to eryptotic effects where different erythrocyte specimens were tested in diverse experiments.

2.3. Participation of Apigenin in eryptosis

2.3.1. Erythrocytes, Solutions and Chemicals

Experiments were performed at 37°C with isolated erythrocytes drawn from healthy volunteers and provided by the blood bank of the University of Tübingen. The study is provided with approval from the ethics committee of the University of Tübingen (184/2003 V). At a hematocrit 0.4%, pH 7.4, at 37 °C for 48 h, blood samples were incubated in vitro in Ringer solution contained (in mM): 5 KCl, 1 $MgSO_4$, 125 NaCl, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4 at 37°C for 48 h. Erythrocytes

were subjected to apigenin (Tocris, Bristol, U.K.) at the donated concentrations. To prepare Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was replaced with 1 mM glycol-bis(2-aminoethylether)- N,N,N_,N_-tetraacetic acid (EGTA).

2.3.2. FACS Analysis of Annexin V Binding and Forward Scatter.

FACS analysis was performed as described in protocol Andree, Reutelingsperger [202]. Cells were washed post incubation in Ringer solution with 5 mM $CaCl_2$ and followed by Annexin-V-FITC staining (1:200 dilution; ImmunoTools, Friesoythe, Germany) where incubated at 37°C for 20 min with light protection. In FL-1 the forward scatter (FSC) of the cells was determined, and annexin V fluorescence intensity was measured using an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

2.3.3. Measurement of Intracellular Ca^{2+}

Pre-incubated suspension of erythrocytes with volume 50 µl was washed in Ringer solution and followed later with Fluo-3/AM loading (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo- 3/AM. For 30 min at 37°C the cells were incubated and washed afterwards tow times in Ringer solution with 5 mM $CaCl_2$. 200 µl Ringer was used to resuspend Fluo-3/AM-loaded erythrocytes. Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1.

2.3.4. Determination of Intracellular ATP Concentration

Intracellular erythrocyte ATP was measured by incubating 90 µl of

erythrocytes for 48 hours in the respective temperature conditions in Ringer solution with or without chromium (VI) (final hematocrit 5%). As a positive control, glucose-depleted Ringer solution was used instead of Ringer solution for erythrocytes incubation. After Cells lysing in distilled water, proteins were precipitated using HClO4 (5%). Following cells centrifuging, an aliquot of the supernatant (400 µl) was adjusted to pH 7.7 by the addition of saturated KHCO3 solution. By employing the luciferin- luciferase assay kit (Roche diagnostics) in accordance to the manufacturer's protocol on luminometer (Berthold Biolumat LB9500, ad Wildbad, Germany) ATP concentrations of the aliquots were determined in mmol/l cytosol of erythrocytes. To avoid ATP degradation, all the manipulations in this experiment were performed at 4°C.

2.3.5. Determination of Ceramide Formation

A monoclonal antibody-based assay was used to determine Ceramide abundance in treated erythrocytes with Apigenin. Incubated erythrocytes with and without Apigenin were stained for 1 hour at 37°C with 1 µg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) with a dilution of 1:5. Following tow times of washing with PBS-BSA, cells were stained with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody for 30 minutes (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Frequent washing with PBS-BSA ensures removing unbound secondary antibody. On a FACS-Calibur in FL-1samples were then analysed by flow cytometric analysis

2.3.6. Measurement of Hemolysis

To determine the subsequent hemolysis of the treated

erythrocytes, the samples were undergone to a centrifugation (3 min at 400 x g, room temperature), which is followed by supernatants collecting. The hemoglobin (Hb) concentration of the supernatant were measured by a photometer at 405 nm. lysed erythrocytes in distilled water was specified as 100% hemolysis and determined by reading the supernatant absorption.

2.3.7. Statistics

Paired ANOVA with Tukey's test as post-test was used for statistical analysis as indicated in the Figure legends. The arithmetic mean ± the standard error of the mean (SEM) has been calculated from the averaged values obtained from the different erythrocyte preparations. In all graphs, data were expressed as arithmetic means ± SEM. n indicates the number of various erythrocyte specimens studied. In Both control and experimental conditions the same erythrocyte specimens have been used. Specimens are differently susceptible to eryptotic effects where different erythrocyte specimens were tested in diverse experiments.

2.4. Honokiol in the triggering of erythrocyte cell membrane scrambling

2.4.1 Erythrocytes, solutions and chemicals

Experiments were performed at 37°C with isolated erythrocytes drawn from healthy volunteers and provided by the blood bank of the University of Tübingen. The study is provided with approval from the ethics committee of the University of Tübingen (184/2003 V). At a hematocrit 0.4%, pH 7.4, at 37 °C for 48 h, blood samples were incubated in vitro in Ringer solution contained (in mM): 5 KCl, 1 $MgSO_4$, 125 NaCl, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

(HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4 at 37°C for 48 h. Erythrocytes were subjected to Honokiol (Enzo, Lörrach, Germany) at the donated concentrations. DMSO was used to dissolve Honokiol and the final concentration of DMSO in the samples did not exceed 0.15%. To prepare Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was replaced with 1 mM glycol-bis(2-aminoethylether)- N,N,N_,N_-tetraacetic acid (EGTA). Substitution of 1 mM EGTA for 1 mM $CaCl_2$ allow the deletion of calcium traces derived from extracellular Ca^{2+} present in the erythrocyte concentrates .

2.4.2 FACS analysis of annexin-V-binding and forward scatter

FACS analysis was performed as described in protocol Andree, Reutelingsperger [202]. Cells were washed post incubation in Ringer solution with 5 mM $CaCl_2$ and followed by Annexin-V-FITC staining (1:200 dilution; ImmunoTools, Friesoythe, Germany) where incubated at 37°C for 20 min with light protection. In FL-1 the forward scatter (FSC) of the cells was determined, and annexin V fluorescence intensity was measured using an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD, Heidelberg, Germany).

In the different measurements by flow cytometry the total events of 10,000-15,000 were counted for their analyzing. The standard software CellQuest Pro (BD biosciences) was used to perform the data acquisition and also analysis.

To perform annexin V fluorescence analysing a distinct marker was placed at the end of the annexin V negative population, where the batch analysis was launched acquiring the percentage of annexin V positive erythrocytes using the CellQuest Pro software. On the other side, the batch analysing of forward scatter was accomplished by recording the geometric mean of the cell population.

2.4.3 Determination of erythrocyte diameter

Flow Cytometer size calibration Kit (F13838) from Molecular Probes was purchased to determine the accurate erythrocyte diameter. Short vortex mixing and sonicating pervious to sampling any of the kit components were used to suspend the microspheres uniform, where the larger microspheres will settle out within minutes. Addition one drop of each six standard microspheres to approximately 2 mL of sheath fluid or buffered saline was performed to prepaere a mixed suspension of six standard microspheres. Nonetheless, a subset of six separated suspensions with different diameter is prepared in the given experiment depending on the needed size markers. In FL-1well suspended microspheres and blood samples were measured.

2.4.4 Measurement of intercellular Ca^{2+}

Pre-incubated suspension of erythrocytes with volume 50 µl was washed in Ringer solution and followed later with Fluo-3/AM loading (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 2 µM Fluo- 3/AM. For 30 min at 37°C the cells were incubated and washed afterwards tow times in Ringer solution with 5 mM $CaCl_2$. 200 µl Ringer was used to resuspend Fluo-3/AM-loaded erythrocytes. Ca^{2+-} dependent fluorescence intensity was measured in fluorescence channel FL-1.

2.4.5 Measurement of hemolysis

To determine the subsequent hemolysis of the treated erythrocytes, the samples were undergone to a centrifugation (3 min at 400 x g, room temperature), which is followed by supernatants collecting. The hemoglobin (Hb) concentration of the supernatant were measured by a photometer at 405 nm. lysed erythrocytes in distilled

water was specified as 100% hemolysis and determined by reading the supernatant absorption.

2.4.6 Confocal microscopy and immunofluorescence

4 µl of treated erythrocytes in respective experimental conditions was incubated to display their eryptotic condition, the cells were stained with FITC-conjugated annexin-V (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 µl Ringer solution with 5 mM $CaCl_2$. Following tow times of erythrocytes washing, cells were resuspended in 50 µl of Ringer solution with 5 mM $CaCl_2$. Prolong Gold antifade reagent (Invitrogen, Darmstadt, Germany) were added to 20 µl sample to mount up a glass slide which was covered with a coverslip. Zeiss LSM 5 EXCITER confocal laser scanning microscope (CarlZeiss MicroImaging, Oberkochen, Germany) was used to take images for the eryptotic erythrocyte with a water immersion Plan-Neofluar 63/1.3 NA DIC.

2.4.7 Determination of ceramide formation

A monoclonal antibody-based assay was used to determine Ceramide abundance in treated erythrocytes with Honokiol. Incubated erythrocytes with and without Honokiol were stained for 1 hour at 37°C with 1 µg/ml anti-ceramide antibody (clone MID 15B4; Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) with a dilution of 1:5. Following tow times of washing with PBS-BSA, cells were stained with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody for 30 minutes (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Frequent washing with PBS-BSA ensures removing unbound secondary antibody. On a FACS-Calibur in FL-1samples were then analysed by flow cytometric analysis

2.4.8 Statistics

Paired ANOVA with Tukey's test as post-test was used for statistical analysis as indicated in the Figure legends. The arithmetic mean ± the standard error of the mean (SEM) has been calculated from the averaged values obtained from the different erythrocyte preparations. In all graphs, data were expressed as arithmetic means ± SEM. n indicates the number of various erythrocyte specimens studied. In Both control and experimental conditions the same erythrocyte specimens have been used. Specimens are differently susceptible to eryptotic effects where different erythrocyte specimens were tested in diverse experiments.

Chapter 3

Results

Chapter 3

3. RESULTS

In a non-nucleated cells acquires programmed death special interest, where the regulation of cell survival is still relative enigmatic at least by talking at non-genomic level. Hence, four different agonists were applied on erythrocyte to reveal apoptotic pathways and their involved associated signalling.

3.1. Modulation of erythrocyte survival by Hexavalent Chromium

The present study addressed the effect of Hexavalent Chromium on eryptosis. Cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$) was determined utilizing Fluo3 fluorescence in an attempt to elucidate the mechanisms that govern the triggering of erythrocyte shrinkage and cell membrane scrambling following Hexavalent Chromium exposure. To this end, erythrocytes were loaded with Fluo3-AM and Fluo3 fluorescence determined in FACS analysis following incubation in Ringer solution without or with Hexavalent Chromium (1-20 µM). As illustrated in Figure 11. statistically significant increase of Fluo3 fluorescence as a result of human erythrocytes treatment with chromium (VI) at a concentration of ≥10 µM, an observations pointing to an increase of cytosolic Ca^{2+} concentration. Ca^{2+} sensitive K^+ channels is activated as a result of an increase of cytosolic Ca^{2+} concentration which in turn followed by exit of KCl with osmotically obliged water leading to cell shrinkage [203].

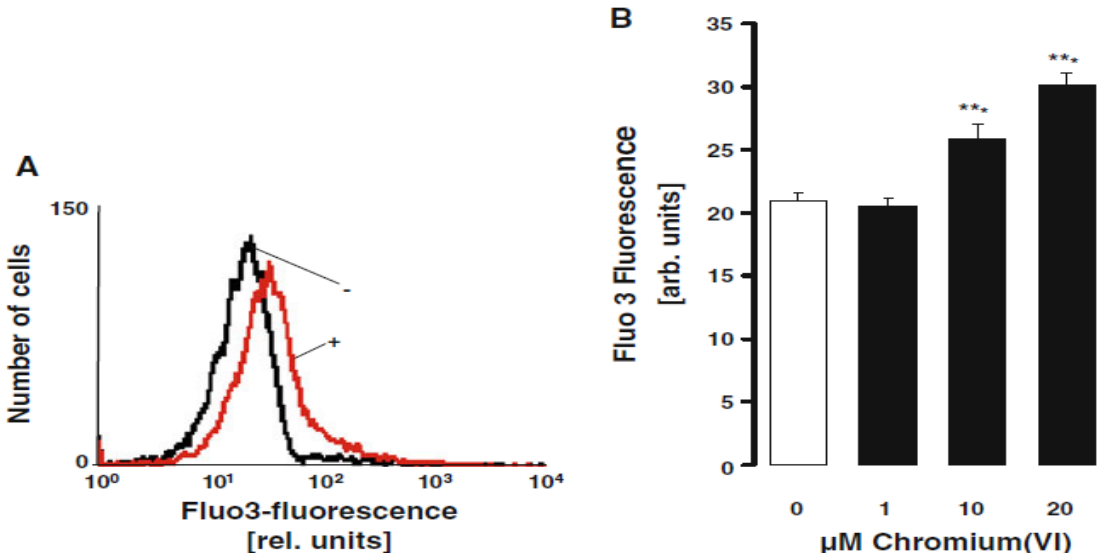

Figure 11: Effect of chromium (VI) on Fluo3 fluorescence in erythrocytes.

a) Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 20 µM chromium (VI) (indicated as, +)

b) Dose dependence of the effect of chromium (VI) on Fluo3 fluorescence. Arithmetic means ± SEM (n = 15) of the normalized Fluo3 fluorescence of erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to chromium (VI) (black bars) at the indicated concentrations. *** ($P < 0.001$) indicate significant difference from values in control Ringer solution (ANOVA).

Cell shrinkage is a hallmark of eryptosis, therefore cell volume was estimated utilizing forward scatter. The forward scatter was determined using flow cytometry. As shown in Figure 12, at concentrations ≥10 µM chromium (VI) treatment was substantially followed by a decrease of forward scatter .

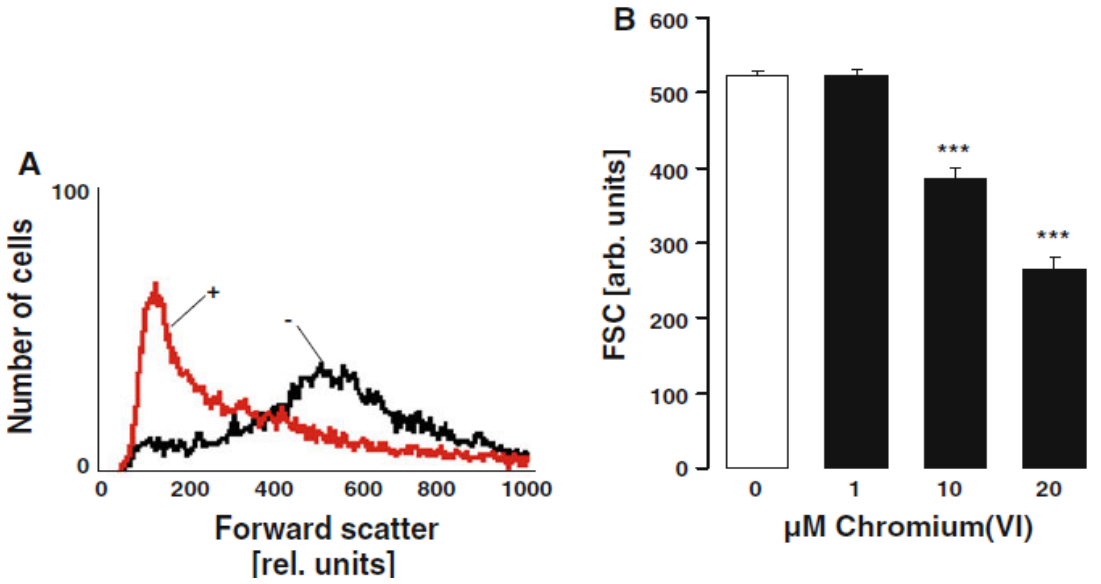

Figure 12: Effect of chromium (VI) on erythrocyte forward scatter.

a) Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 20 µM chromium (VI) (indicated as, +).

b) Effect of chromium (VI) on forward scatter. Arithmetic means ± SEM (n = 15) of the normalized forward scatter of erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to chromium (VI) (black bars). ***P<0.001 indicate significant difference from values in control Ringer solution (ANOVA).

Cell membrane scrambling with subsequent increase of phosphatidylserine abundance at the cell surface is considered the second hallmark of eryptosis. Accordingly, Confocal imaging was performed to visualize eryptosis in the absence and presence of 20 µM chromium (VI) as a first step (Fig. 13). The number of annexin V positive erythrocytes in chromium (VI) treated cells was indeed higher than in untreated erythrocytes incubated for 48 hours in Ringer solution.

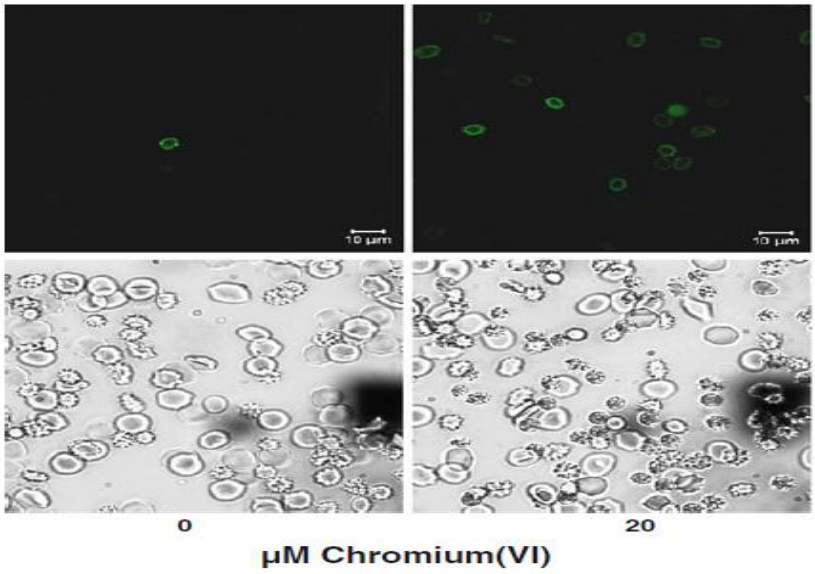

Figure 13: Confocal images of phosphatidylserine-exposing erythrocytes with or without chromium (VI) treatment.

Confocal microscopy technique was used in tow forms, light microscopy (lower panels) and FITC-dependent fluorescence (upper panels) to visualise stained human erythrocytes with FITC-conjugated annexin-V post 48h incubation in Ringer solution without (left panels) and with (right panels) 20 µM chromium (VI).

Phosphatidylserine exposing erythrocytes identification was achieved as a second step by exploiting annexin-V-binding in flow cytometry. As illustrated in Fig. 14a and b, the percentage of annexin-V-binding erythrocytes post a 48 hours exposure to chromium (VI) increased, an effect reaching statistical significance at 10 µM chromium (VI) concentration.

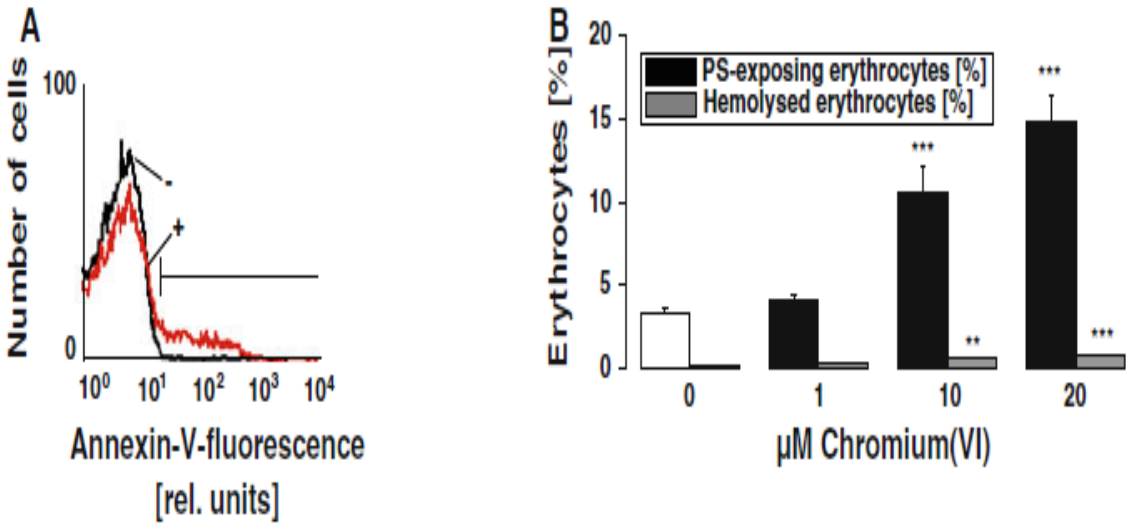

Figure 14: Effect of chromium (VI) on phosphatidylserine exposure.

a) Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 20 µM chromium (VI) (indicated as ,+).

b) Effect of chromium (VI) on phosphatidylserine exposure. Arithmetic means ± SEM (n = 15) of the percentage of annexin V-binding erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to chromium (VI) (black bars). For a purpose of comparison, arithmetic means ± SEM (n = 4) were used to express the percentage of hemolysis is shown as grey bars. **P<0.01, *** (P < 0.001) indicates significant difference from values in control Ringer solution (ANOVA).

The fluorescent annexin V access to phosphatidylserine could result from permeabilization of erythropcytes under hemolysis, in order to elucidate, whether chromium (VI) exposure leads to hemolysis, the estimated percentage of hemolysed erythrocytes was quantified from the released haemoglobin in the supernatant. As shown in Fig. 14b, the exposure to chromium (VI) for 48 hours increased the hemoglobin concentration in the supernatant, an effect reaching statistical significance at 10µM chromium (VI) concentration. Hemolytic erythrocytes percentage is notably still almost one order of magnitude

smaller than the percentage of phosphatidylserine exposing erythrocytes (Fig. 14 b).

To test whether chromium (VI)-induced increasing in the cytosolic Ca^{2+} concentration is indeed in charge of cell membrane scrambling and phosphatidylserine exposure harnessing Ca^{+2} entry, erythrocytes were exposed to 20 µM chromium (VI) in the nominal absence of extracellular $Ca^{2+.}$ As shown in Fig. 14c, the impact of chromium (VI) -induced phosphatidylserine exposure was significantly blunted but not abolished in the nominal absence of extracellular Ca^{2+}.

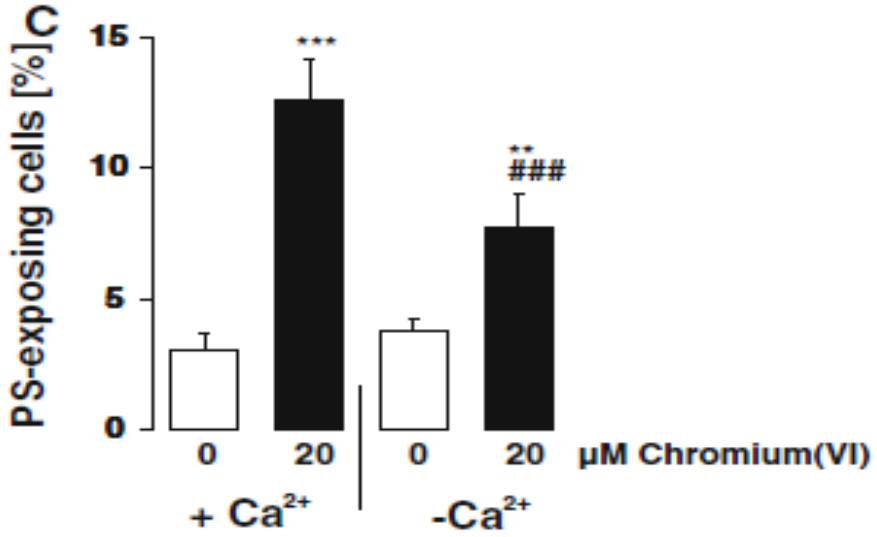

Figure 14c: Effect of chromium (VI) on phosphatidylserine exposure in the presence and absence of calcium.

C) Annexin V-binding erythrocytes percentage is expressed with arithmetic means ± SEM (n = 8) post exposure for 48 hours to plain Ringer solution (white bars) or to 20 µM chromium (VI) (black bars) in the presence (left bars) and absence (right bars) of Ca^{2+} in extracellular fluid. **P<0.01, ***P<0.001 indicates significant difference from values

in control Ringer solution (ANOVA). ### (P < 0.001) indicates significant difference from respective values in the presence of Ca^{2+} (ANOVA).

The cell membrane scrambling post chromium (VI) exposure can be traced largely to an increase of intracellular Ca^{+2} activity.

To investigate further whether phosphatidylserine exposure on the cell surface in erythrocytes incubated in serum would likewise be induced by chromium (VI). Exposure to 20µM chromium (VI) for 48h as shown in Figure 14d, annexin V binding increased in serum to a similar extent in erythrocytes incubated in Ringer solution.

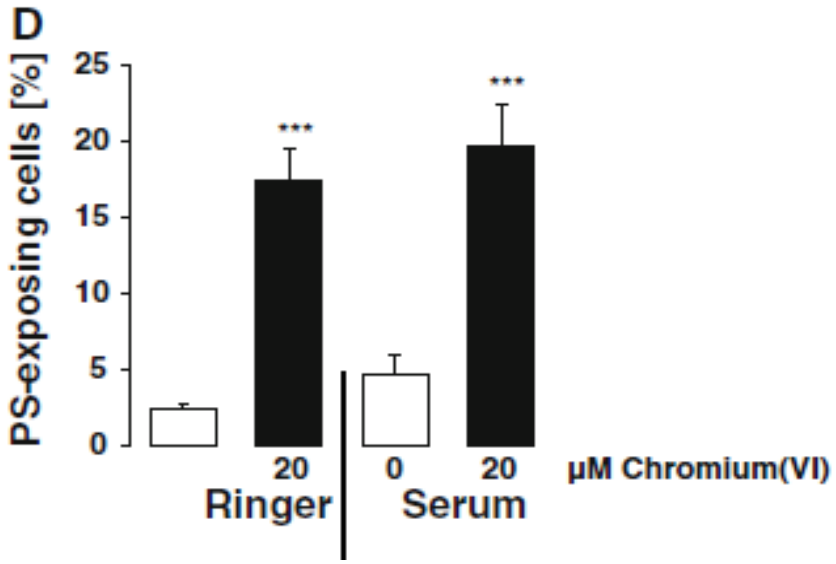

Figure 14d: Effect of chromium (VI) on phosphatidylserine exposure in Ringer or serum.

D) annexin V-binding erythrocytes percentage expressed with arithmetic means ± SEM (n = 4) after a 48 h exposure with Ringer solution (left bars) or serum (right bars) without (white bar) or with (black bars) 20 µM chromium (VI). ***P<0.001 indicates significant difference from the absence of chromium (VI) (ANOVA).

In the Serum, annexin V binding erythrocytes Percentage heads for to be somewhat higher than in the Ringer following exposure to 20-µM chromium (VI), a statistical comparison between them did not show any significant different.

Cellular depletion of ATP is considered eryptosis stimulator [75], In the presence or absence of chromium (VI) the intracellular ATP concentration of erythrocytes was therefore quantified post incubation with chromium (VI). As illustrated in Figure 15, a 48 hours exposure of human erythrocytes to 20µM chromium (VI) resulted in a significant decrease of the cytosolic ATP concentration. Incubation in glucose-free solution served as a positive control (Fig. 15, grey bar).

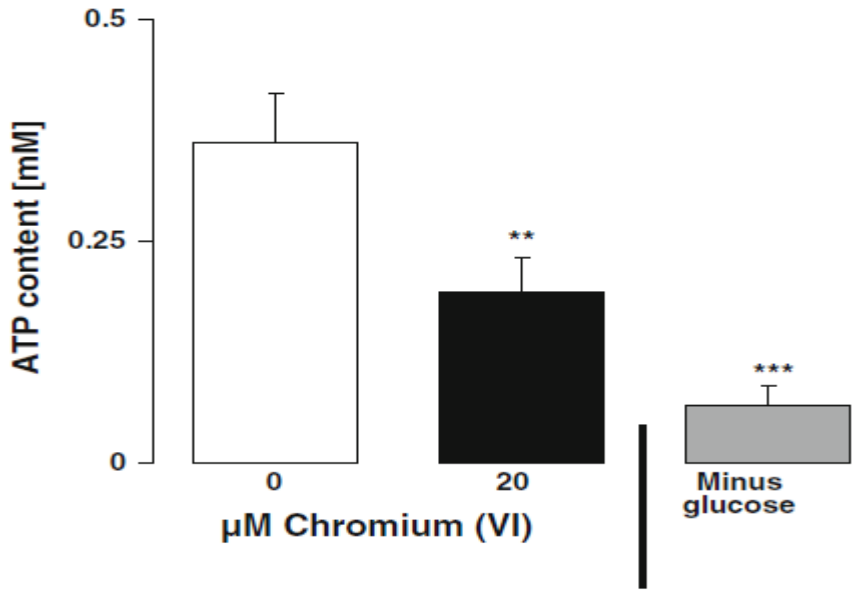

Figure 15: Effect of chromium (VI) on erythrocyte cytosolic ATP content.
Cytosolic ATP concentration as a result of chromium (VI) effect on erythrocyte. ATP concentration were expressed with arithmetic means ± SEM (n = 4) post a 48 h incubation in Ringer solution without (white bar) or with (black bars) 20 µM chromium (VI), or in glucose-depleted Ringer solution (grey bar, minus glucose). **P<0.01, ***P<0.001 indicates significant difference from control (absence of chromium (VI) and presence of glucose) (ANOVA)

Cell membrane scrambling and cell shrinkage could be a subsequent of ceramide enhancing[203], which could thus, in theory, have a significant role in chromium (VI) effect. To determine ceramide formation, FITC-labelled anti-ceramide antibodies was used. A 48 hours exposure of erythrocytes in Ringer solution without (23.5 ± 1.9 a.u., n = 4) and with (21.3 ± 2.4 a.u., n = 4) 20 µM chromium (VI) showed similar readings.

3.2. Involvement of Rotenone in the erythrocyte cell membrane scrambling

To test whether Rotenone increases the cytosolic free Ca^{2+} concentration of erythrocytes, Fluo-3 fluorescence measurements in FACS have been performed in red cells incubated for 48 hours either in the absence or presence of Rotenone (1-10 µM). As illustrated in Fig. 16, the incubation of human erythrocytes in rotenone increased significantly the Fluo-3 fluorescence, pointing to an increase of cytosolic free Ca^{2+} concentration (Fig. 16). At 1 µM rotenone concentration a statistical significance could be detected.

Red cells express Ca^{2+}-sensitive K^{+} channels suggesting that elevated cytosolic Ca^{2+} activity triggers the loss of KCl and osmotically obliged water and thus leads to cell shrinkage, which should be apparent from a decrease of forward scatter in FACS analysis.

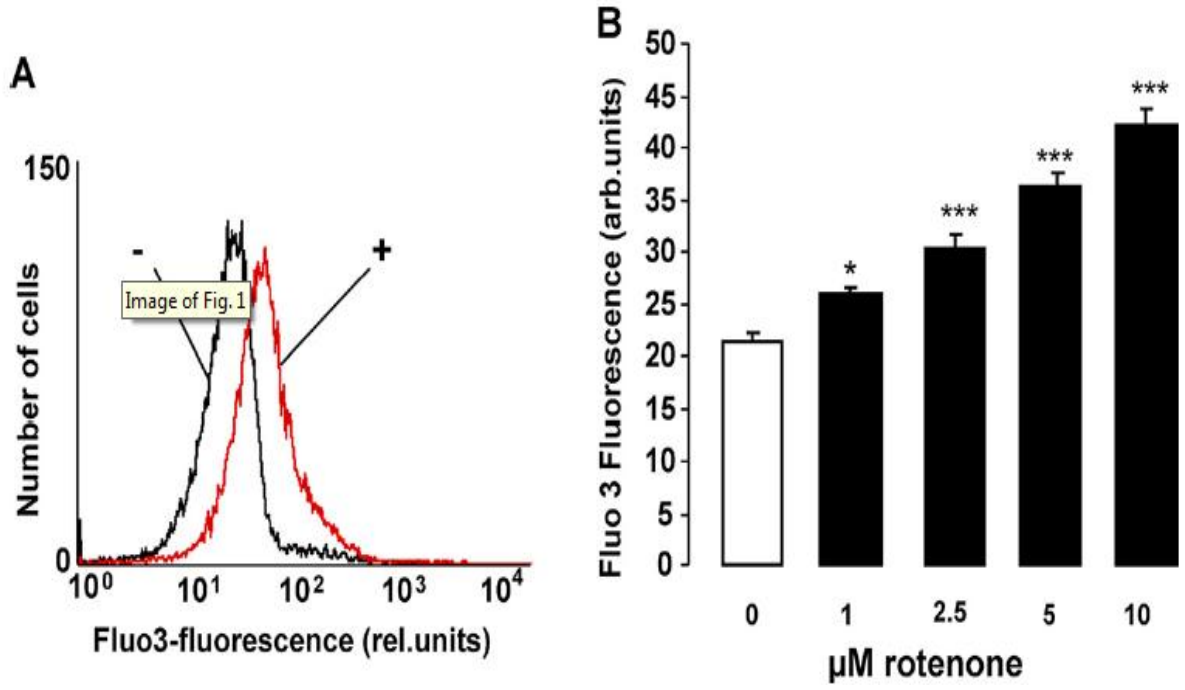

Figure 16: Effect of rotenone on erythrocyte cytosolic Ca^{2+} concentration.

A) Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 10 µM rotenone (indicated as, +)

B) Dose dependence of the effect of rotenone on Fluo3 fluorescence. Arithmetic means ± SEM (n = 10) of the normalized Fluo3 fluorescence of erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to rotenone (black bars) at the indicated concentrations. *p < 0.05, ***p < 0.001 indicate significant difference from values in control Ringer solution (ANOVA).

Fig. 17 indeed demonstrates that exposure to rotenone at concentrations ≥ 2.5 µM led to a decrease of erythrocyte forward scatter in FACS analysis data. At 2.5 µM rotenone concentration a statistically significance could be detected.

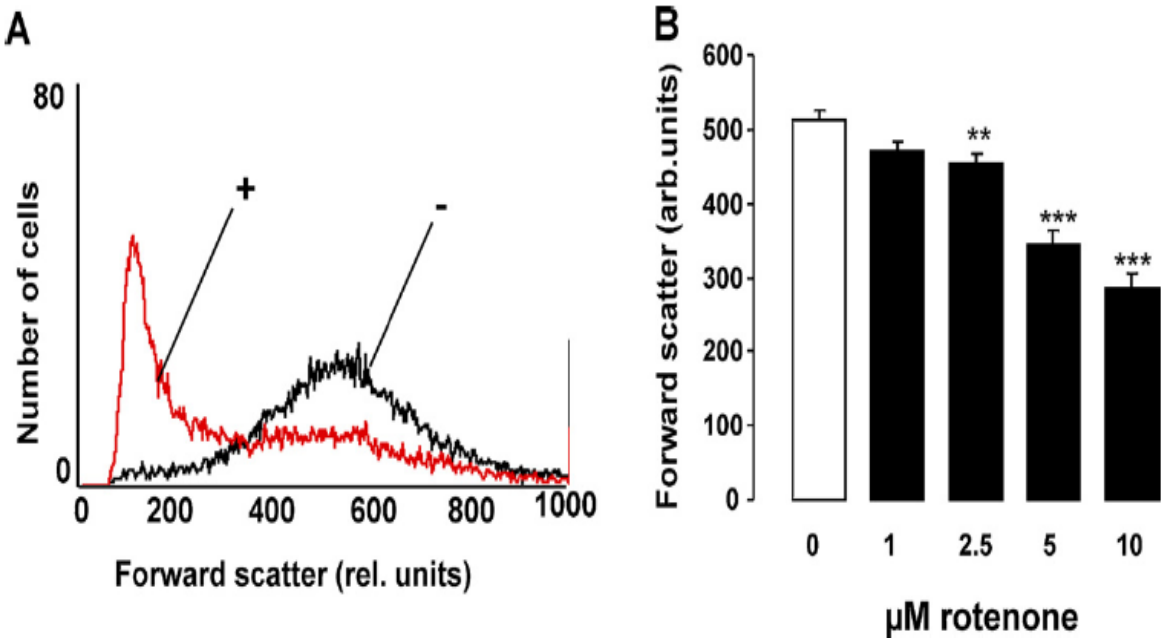

Figure 17: Effect of rotenone on erythrocyte forward scatter.

A) Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 10 µM rotenone (indicated as, +).

B) Effect of rotenone on forward scatter. Arithmetic means ± SEM (n = 10) of the normalized forward scatter of erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to rotenone (black bars). **p < 0.01, ***p < 0.001 indicate significant difference from values in control Ringer solution (ANOVA).

Cell membrane scrambling with subsequent phosphatidylserine exposure at the erythrocytes surface is considered as result of cytosolic Ca^{2+} concentration augmentation, which should further do boosting for erythrocyte annexin V binding. Therefore, a new set of experiments was conducted to investigate whether rotenone has any impact translated into cell membrane scrambling. Fig. 18 shows that a 48 hours exposure to rotenone indeed enhanced the percentage of annexin V binding erythrocytes determined by FACS analysis. The concentration needed for a significant increase of the percentage of annexin V binding erythrocytes was 1 µM.

In order to elucidate, whether rotenone exposure leads to hemolysis, the estimated percentage of hemolysed erythrocytes was quantified from the released haemoglobin in the supernatant. As shown in Fig. 18b, exposure to rotenone for 48 hours increased the hemoglobin concentration in the supernatant, an effect reaching statistical significance at 2.5 µM rotenone concentration.

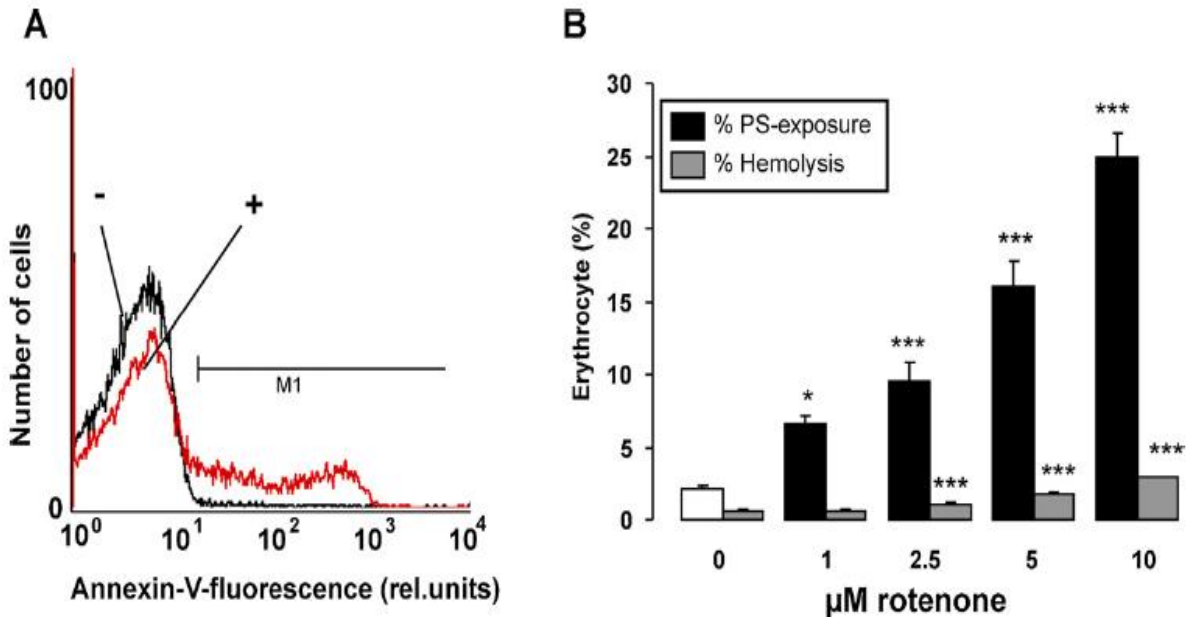

Figure 18: Effect of rotenone on phosphatidylserine exposure and hemolysis.

A) Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 10 µM rotenone (indicated as ,+).

B) Effect of rotenone on phosphatidylserine exposure. Arithmetic means ± SEM (n = 10) of the percentage of annexin V-binding erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to rotenone (black bars). For a purpose of comparison, arithmetic means ± SEM (n = 4) were used to express the percentage of hemolysis presented as grey bars. *p < 0.05, ***p < 0.001 indicates significant difference from values in control Ringer solution (ANOVA).

To explore the significance of rotenone-induced Ca^{2+} entry for PS-exposure and cell shrinkage, the effects of rotenone were tested in the nominal presence and absence of extracellular Ca^{2+} by exposing the erythrocytes to 10 µM rotenone. As shown in Fig. 19, the stimulation of phosphatidylserine exposure by rotenone was significantly blunted in the absence of extracellular Ca^{2+}.

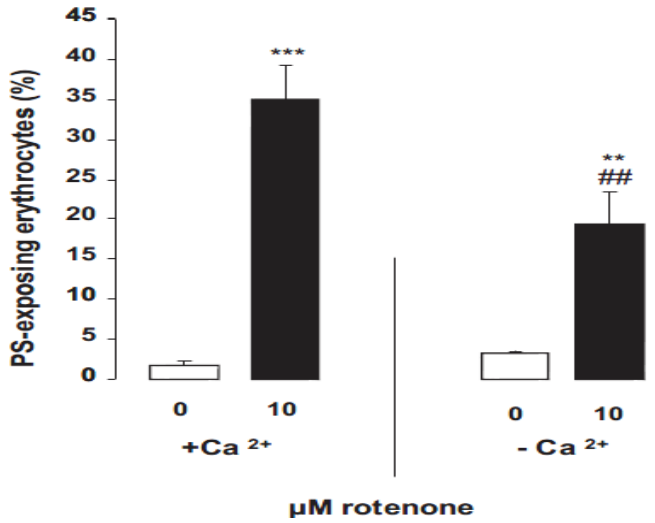

Figure 19: Effect of Ca^{2+} withdrawal on rotenone- induced annexin-V-binding.

Annexin V-binding erythrocytes percentage is expressed with arithmetic means ± SEM (n = 8) post exposeure for 48 hours to plain Ringer solution (white bars) or to 10 µM rotenone (black bars) in the presence (left bars) and absence (right bars) of Ca^{2+} in extracellular fluid. **p < 0.01, ***p < 0.001 indicates significant difference from values in control Ringer solution (ANOVA). ##p < 0.01 indicates significant difference from respective values in the presence of Ca^{2+} (ANOVA).

Since the percentage of phosphatidylserine exposing erythrocytes was remain significantly higher in the presence than in the absence of rotenone, therefore, erythrocyte membrane scrambling triggering in a big part but not absolutely due to Ca^{2+} entry, this end discloses a new involved mechanism.

Removal of extracellular Ca^{2+} did not fully abrogate the breakdown of phosphatidylserine asymmetry of the cell membrane following rotenoe treatment. As sensitivity of cell membrane scrambling to $[Ca^{2+}]I$ is enhanced by ceramide, additional experiments were performed to determine the effect of rotenone on ceramide formation. FITC-labeled anti-ceramide antibodies were employed to identify ceramide abundance at the erythrocyte surface. Figure. 20 illustrated a slightly but significantly increase of ceramide formation post (10µM) rotenone exposing, this end discloses a non-Ca^{2+} mediated mechanism involvement.

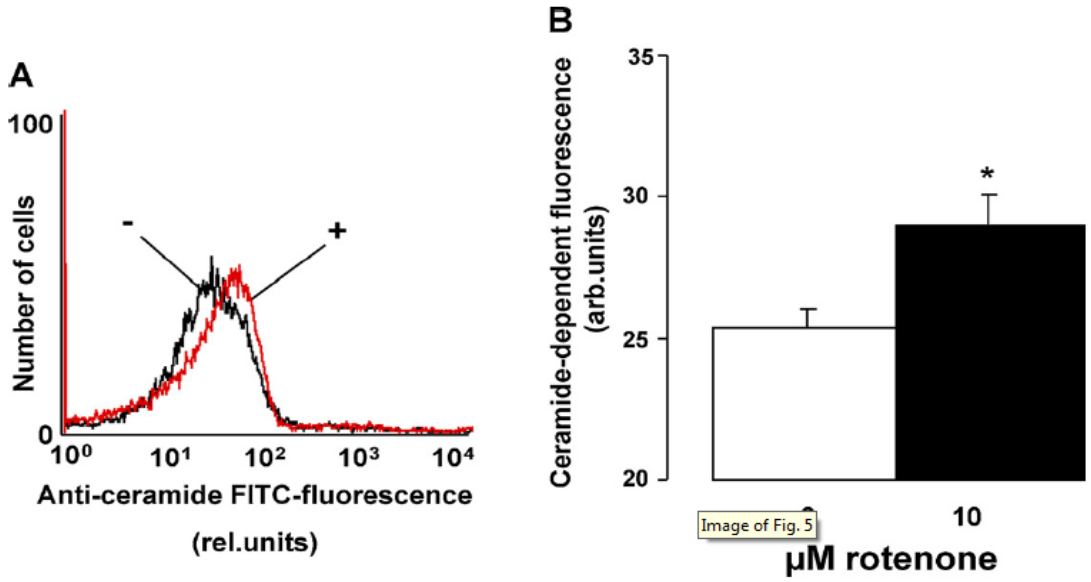

Figure 20: Effect of rotenone on ceramide formation.

A) Histogram of anti-ceramide FITC-fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to Ringer solution without (indicated as, -) or with (indicated as, +) 10 µM rotenone.
B) Arithmetic means ± SEM (n = 4) were used to express ceramide abundance in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) 10 µM rotenone. *p < 0.05 indicates significant difference from values in control Ringer solution (t-test)

3.3. Participation of Apigenin in eryptosis

Both cell shrinkage and cell membrane scrambling could be triggered by increase in the cytosolic Ca^{2+} activity[92]. Accordingly, Fluo3 fluorescence was employed to estimate the cytosolic Ca^{2+} concentration. As illustrated in figure 21 A,B, graded increases of Fluo3 fluorescence was the consequence of human erythrocytes treatment with apigenin, Apigenin Impact reached statistically significant at the employed concentrations (≥1 µM) indicating that apigenin increases cytosolic Ca^{2+} concentration

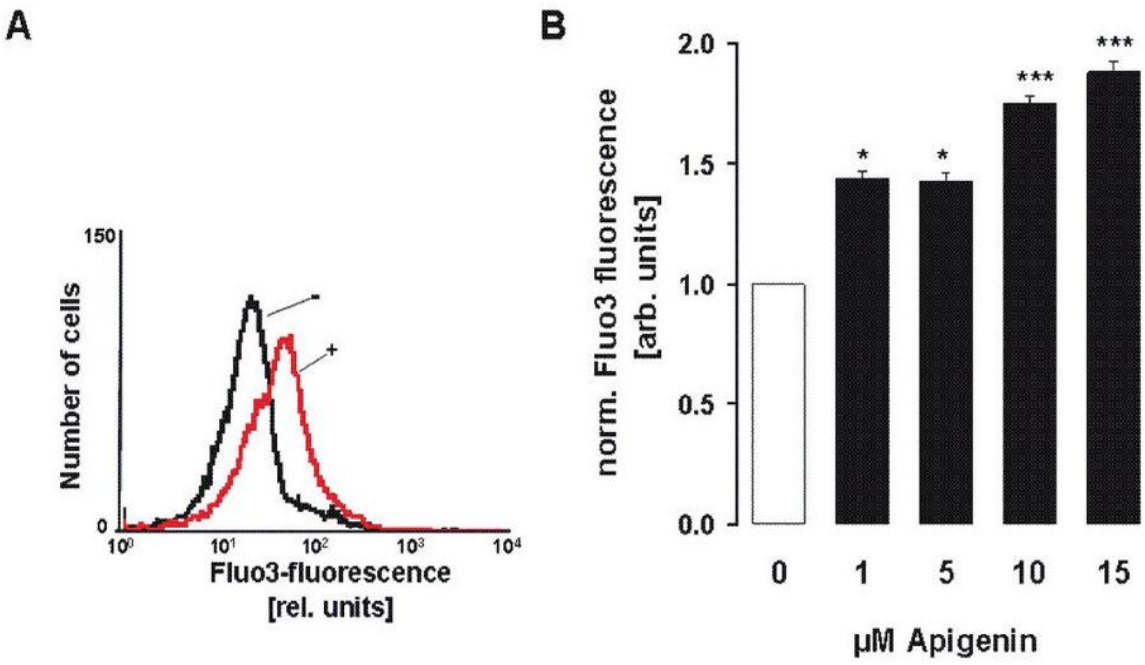

Figure 21: Effect of apigenin on erythrocyte cytosolic Ca^{2+} concentration.

C) Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 15 µM apigenin (indicated as, +)

A) Dose dependence of the effect of apigenin on Fluo3 fluorescence. Arithmetic means ± SEM (n = 12−16) of the normalized Fluo3 fluorescence of erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to apigenin (black bars) at the indicated concentrations. * ($p < 0.05$) and *** ($p < 0.001$) indicate significant difference from values in control Ringer solution (ANOVA

One of the hallmarks of eryptosis is cell shrinkage. To determine, whether apigenine could trigger eryptosis in erythrocytes executing alterations in cell volume which is estimated by forward scatter analysis in FACS. As illustrated in figure 22, graded decrease in forward scatter reflexed respective decreases in cell volumes following apigenin treatment. Apigenin impact reached statistically significant at applied concentrations (≥1 µM).

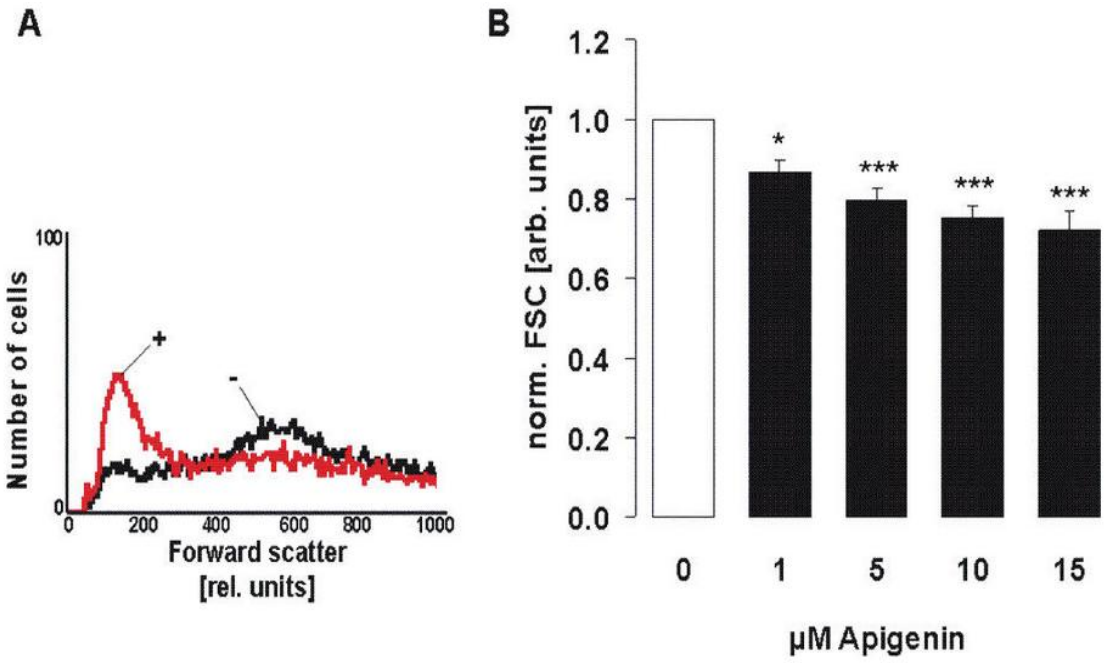

Figure 22: Effect of apigenin on erythrocyte forward scatter.

A) Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 15 µM apigenin (indicated as, +).

B) Effect of apigenin on forward scatter. Arithmetic means ± SEM (12−16) of the normalized forward scatter of erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to apigenin (black bars). * $p < 0.05$ and *** $p < 0.001$ indicate significant difference from values in control Ringer solution (ANOVA).

A further hallmark of eryptosis is cell membrane scrambling, leading to PS exposure at the cell surface. Annexin V-binding was used to quantify PS-exposing erythrocytes by FACS analyis. As shown in figure 23A, graded increases in the percentage of annexin V binding erythrocytes post a 48 h treatment with apigenin. Thus, apigenine impact could reach statistical significance at ≥5 µM.

Apigenin could cause direct lysis of erythrocytes. To test this possibility, the percentage of lysed erythrocytes was determined following a 48h exposure to apigenin. As shown in figure 23B, hemoglobin concentration measurement displayed a slightly increase in the supernatant without reaching statistical significance.

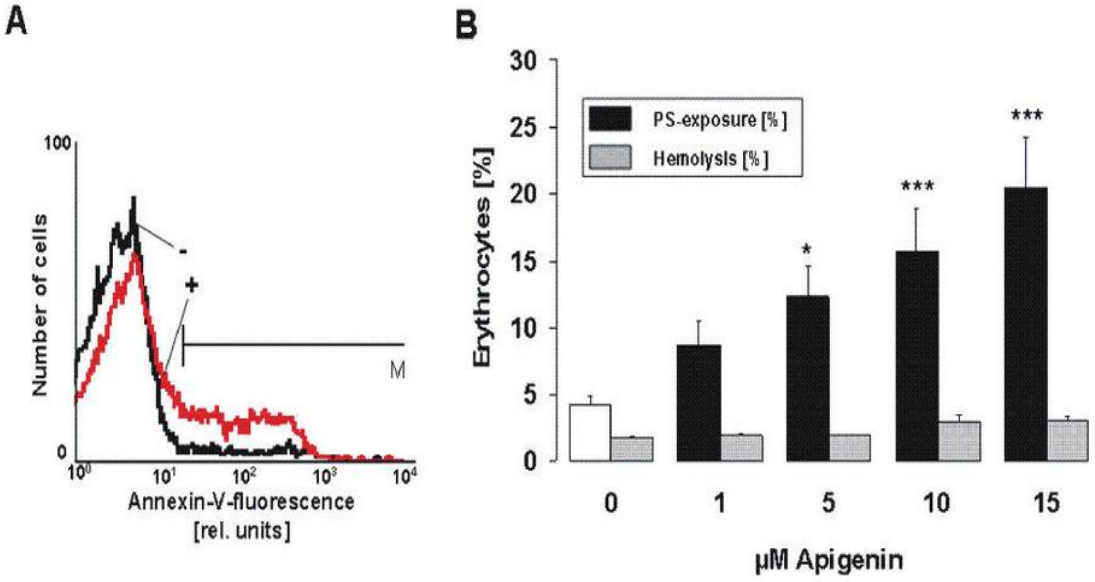

Figure 23: Effect of apigenin on phosphatidylserine exposure.

A) Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 15 µM apigenin (indicated as ,+).

B) Effect of apigenin on phosphatidylserine exposure. Arithmetic means ± SEM (n = 12-16) of the percentage of annexin V-binding erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to apigenin (black bars). For a purpose of comparison, arithmetic means ± SEM (n = 4) were used to express the percentage of hemolysis is shown as

grey bars. * $p < 0.05$ and *** $p < 0.001$ indicates significant difference from values in control Ringer solution (ANOVA).

To test whether apigenin -induced increase in the cytosolic Ca^{2+} concentration is indeed accounts for cell membrane scrambling and phosphatidylserine exposure, Erythrocytes were exposed to 15 µM apigenin in the nominal absence of extracellular Ca^{2+}. As shown in Fig. 24, apigenin -induced phosphatidylserine exposure, where its affect blunted significantly in the nominal absence of extracellular Ca^{2+} confirming the role of Ca^{2+} entry in eryptosis launching.

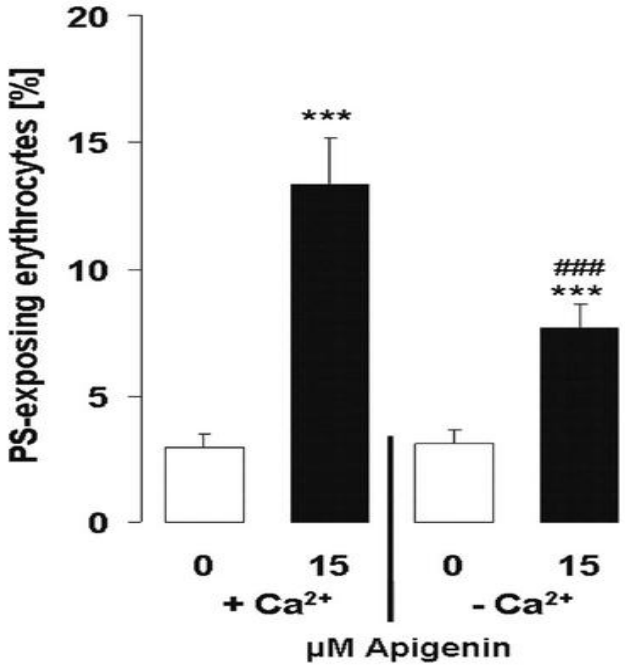

Figure 24: Effect of Ca^{2+} withdrawal on apigenin- induced annexin V binding.
Annexin V-binding erythrocytes percentage was expressed with arithmetic means ± SEM (n = 8) post exposeure for 48 hours to plain Ringer solution (white bars) or to 15 µM apigenin (black bars) in the presence (left bars) and absence (right bars) of Ca^{2+} in extracellular fluid. ***$p < 0.001$ indicates significant difference from values in control Ringer solution (ANOVA). ### $p < 0.001$ indicates significant difference from respective values in the presence of Ca^{2+} (ANOVA).

The percentage of annexin V binding erythrocytes was unable to be completely abrogated in the nominal absence of Ca^{2+} post apigenin exposure, this end discloses indeed additional mechanisms involvement in the stimulation of erythrocyte membrane scrambling by apigenin.

To test whether γ-aminobutyric acid (GABA) able to make any modification on apigenin-induced phosphatidylserine exposure in erythrocytes, extra experiments were applied for this pupose. Thus, incubation of erythrocytes were taken place in the presence and absence of 15 µM apigenin and in the presence and absence of 15 µM GABA as well. The percentage of annexin V binding erythrocytes increased significantly from 2.7 ± 0.5% (n = 6) in the absence of apigenin to 12.3 ± 1.8% (n = 6) in the presence of 15 µM apigenin. No significant modification in annexin V binding percentage of incubated erythrocytes was detectable post addition of 15 µM GABA, in the absence of apigenin (3.5 ± 0.9%, n = 6) nor in the presence of 15 µM apigenin (12.1 ± 2.2%, n = 6).

Further experiments explored whether the PS-exposure triggered by apigenin is reversible or not. Using annexin V binding, PS-exposure was identified after 48 h incubation, Incubated erythrocytes for 48 h with exposure to 15 µM apigenin (19.6 ± 2.3%, n = 4) showed significantly higher PS exposure in comparison to those incubated in the absence of apigenin (3.2 ± 0.5%, n = 4). Tow further aliqutes were taken from the samples, 4 times with Ringer solution was washed the first one and posteriorly incubated for an additional 24 h, while the second aliquot was not washed and only was incubated for an extra 24 h. The PS exposure in the washed aliquot (34.8 ± 2.9%, n = 4) after 72 h did not show any significant differ in comparison to the unwashed one (32.9 ± 4.3%, n = 4) pointing to irreversibile impact of apigenin-induced phosphatidylserine exposure.

Cell membrane scrambling and cell shrinkage could be a subsequent of ceramide enhancing[203], which could thus, in theory, have a significant role in apigenine effect. To determine ceramide formation, FITC-labelled anti-ceramide antibodies was used. As shown in Figures 25A and 25B, a 48 h exposure to Ringer with 15 µM apigenin presented significantly higher ceramide-dependent fluorescence in comparison to those incubated in Ringer solution without apigenin. Thus, apigenin could significantly enhance ceramide formation.

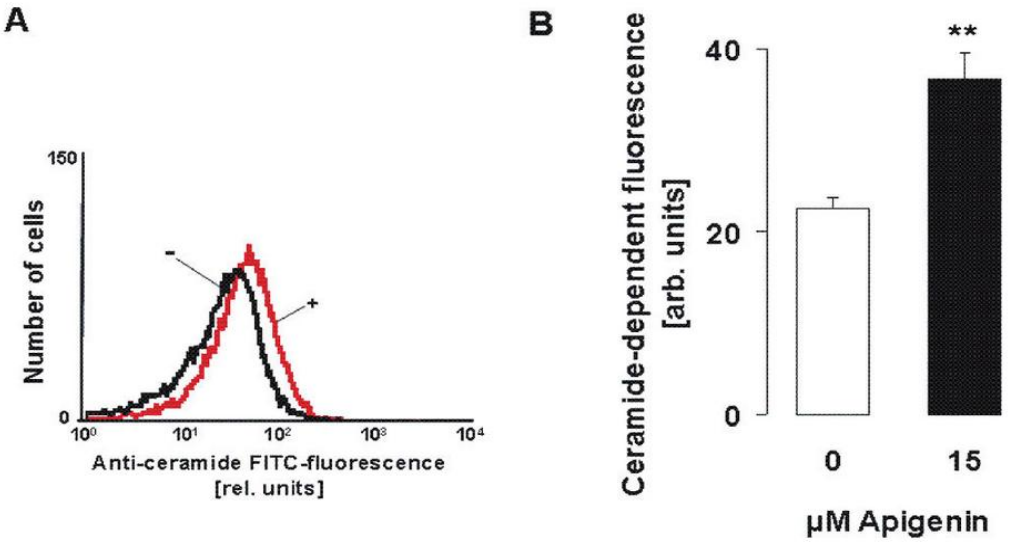

Figure 25. Effect of apigenin on ceramide formation.

A) Histogram of anti-ceramide FITC-fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to Ringer solution without (indicated as, -) or with (indicated as, +) 15 µM apigenin.

B) Arithmetic means ± SEM (n = 4) were used to express ceramide abundance in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) 15 µM apigenin. ** $p < 0.01$ indicates significant difference from values in control Ringer solution (t-test)

Cellular depletion of ATP is considered eryptosis stimulator [75], In the presence or absence of apigenin, the intracellular ATP concentration of erythrocytes was therefore quantified post incubation with apigenin. As illustrated in Figure 26, a 48 hours exposure of human erythrocytes to 15µM apigenin resulted in a significant decrease of the cytosolic ATP concentration. Incubation in glucose-free solution served as a positive control.

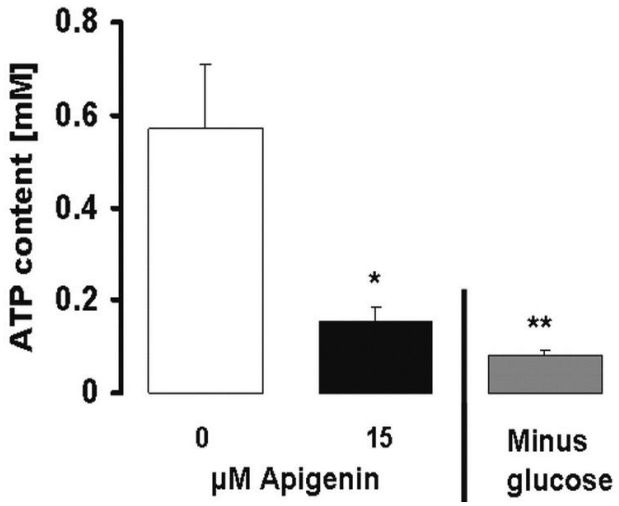

Figure 26: Effect of apigenin on erythrocyte cytosolic ATP content.

Cytosolic ATP concentration as a result of apigenin effect on erythrocyte. ATP concentration were expressed with arithmetic means ± SEM (n = 4) post a 48 h incubation in Ringer solution without (white bar) or with (black bars) 15 µM apigenin, or in glucose-depleted Ringer solution (grey bar, minus glucose). * ($p < 0.05$) and ** ($p < 0.01$) indicates significant difference from control (absence of apigenin and presence of glucose) (ANOVA)

3.4. Honokiol in the triggering of erythrocyte cell membrane scrambling

The present study addressed the effect of Honokiol on eryptosis. Cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$) was determined utilizing Fluo3 fluorescence in an attempt to elucidate the mechanisms underlying the triggering of erythrocyte shrinkage and cell membrane scrambling following Honokiol exposure. To this end, erythrocytes were loaded with Fluo3-AM and Fluo3 fluorescence determined in FACS analysis following incubation in Ringer solution without or with Honokiol (1-15 µM). As illustrated in Figure 27. statistically significant increase of Fluo3 fluorescence as a result of human erythrocytes treatment with Honokiol at only a concentration of 15 µM, while up to 10 µM honokiol remained without significant effect on Fluo3 fluorescence. Observations pointing to an increase of cytosolic Ca^{2+} concentration. Ca^{2+} sensitive K^+ channels is activated as a result of an increase of cytosolic Ca^{2+} concentration which in turn followed by exit of KCl with osmotically obliged water leading to cell shrinkage [203]

In the presence of the calcium ionophore ionomycin as a positive control we measured Fluo3 fluorescence, Erythrocytes were treated with 1 µM ionomycin which was enough to enhance significantly Fluo3 fluorescence from 21 ± 0.9 to 61 ± 2.0 (a.u., n = 4).

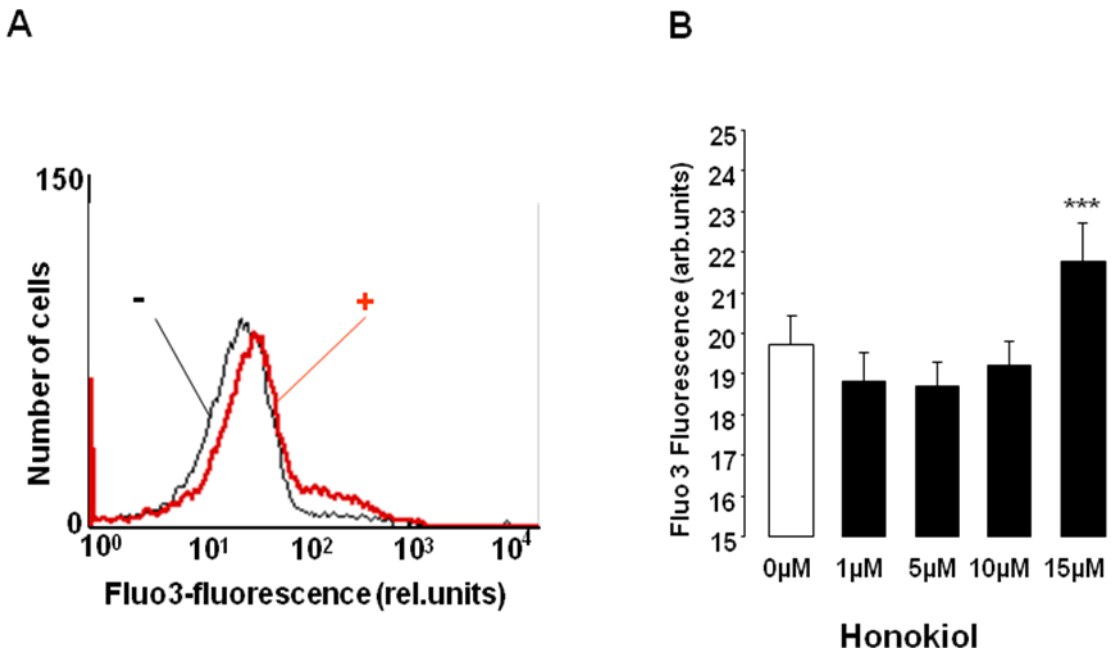

Figure 27: Effect of honokiol on erythrocyte cytosolic Ca^{2+} concentration

a) Histogram of Fluo3 fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 15 µM honokiol (indicated as, +)

b) Dose dependence of the effect of honokiol on Fluo3 fluorescence. Arithmetic means ± SEM (n = 15) of the normalized Fluo3 fluorescence of erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to Honokiol (black bars) at the indicated concentrations. ***p < 0.001 indicate significant difference from values in control Ringer solution (ANOVA)

Cell shrinkage is a hallmark of eryptosis, therefore cell volume was estimated utilizing forward scatter. The forward scatter was determined using flow cytometry. As shown in Figure 28, honokiol treatment was substantially followed by a decrease of forward scatter and reached statistical significance at a concentration ≥5 µM. Susequently, honokiol decreased erythrocyte volume. A further experiment was performed to bolster our results, where the accurate diameter of erythrocyte treated with and without honokiol was determined. As illustrated in the Figure 28C, a significant decrease in the erythrocyte diameter after treatment with 15µM honokiol could recorded.

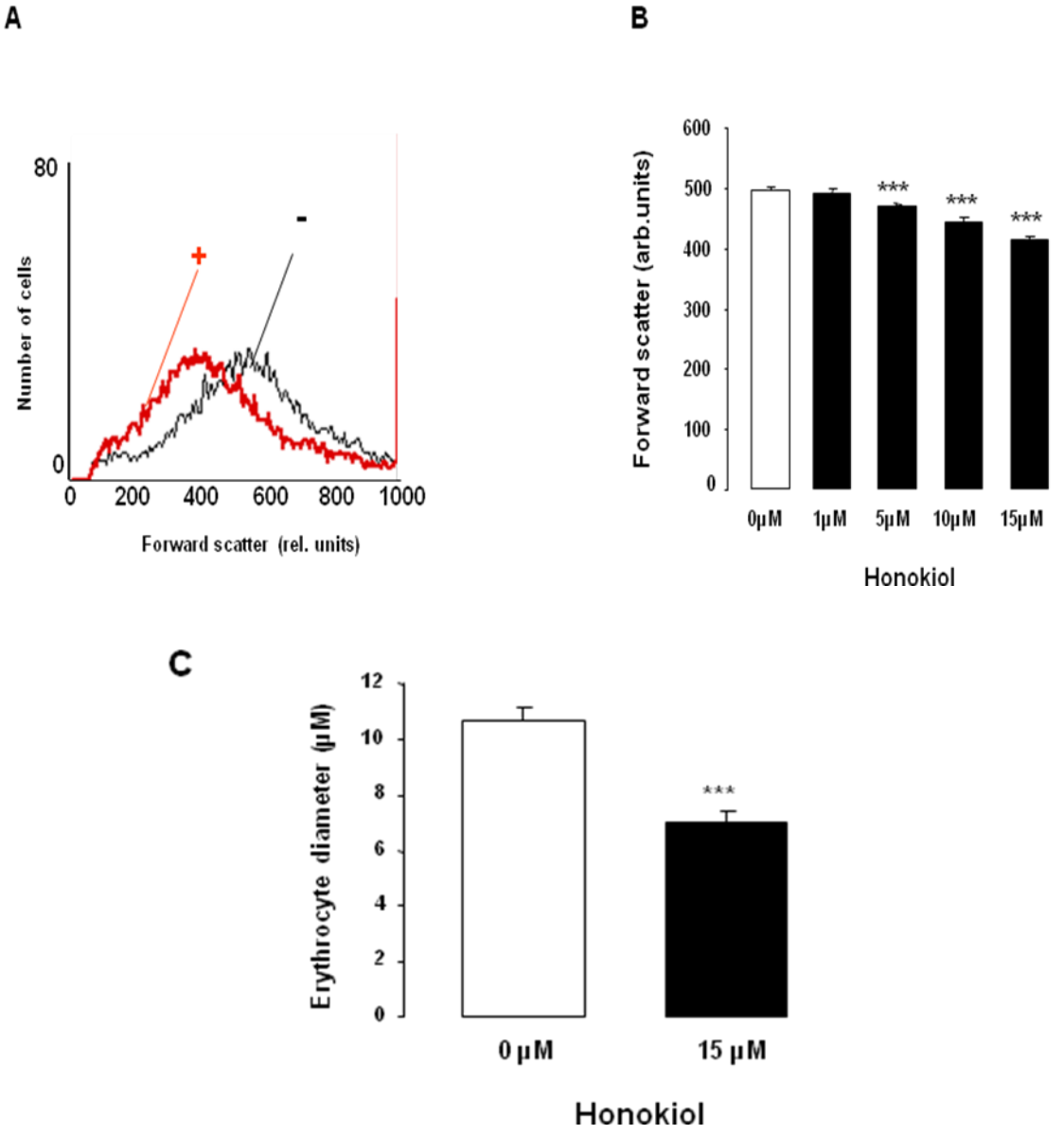

Figure 28: Effect of honokiol on erythrocyte forward scatter and diameter

a) Histogram of forward scatter in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 15 µM honokiol (indicated as, +).

b) Effect of chromium (VI) on forward scatter. Arithmetic means ± SEM (n = 15) of the normalized forward scatter of erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to honokiol (black bars). ***P<0.001 indicate significant difference from values in control Ringer solution (ANOVA).

c) Arithmetic means ± SEM (n = 4) were used to express the normalized erythrocyte diameter post exposure for 48 h to Ringer solution without (white bar) or with (black bars) honokiol (15 µM). *** (p<0.001) indicate significant difference from the absence of honokiol (t test).

Cell membrane scrambling with subsequent phosphatidylserine exposure at the erythrocytes surface is considered as result of cytosolic Ca^{2+}-concentration augmentation, which should further do boosting for erythrocytic annexin V binding. Therefore, a new set of experiments investigated whether honokiol has any impact followed by cell membrane scrambling. Fig. 29 shows that a 48 hours exposure to honokiol indeed enhanced the percentage of annexin V binding erythrocytes determined by FACS analysis. The concentration needed for a significant increase of the percentage of annexin V binding erythrocytes was 5µM. Subsequently, a breakdown of phosphatidylserine asymmetry of the cell membrane was a result of honokiol exposure which is followed by with phosphatidylserine exposure at the cell surface. Honokiol performed a time-dependent increase in phiosphatidylserine exposure (Figure 29C).

in order to elucidate, whether honokiol exposure leads to hemolysis, the estimated percentage of hemolysed erythrocytes was quantified from the released haemoglobin in the supernatant. As shown in Fig. 29b, exposure to honokiol for 48 hours increased the hemoglobin concentration in the supernatant, an effect reaching statistical significance at 10-µM chromium (VI) concentration. Hemolytic erythrocytes percentage is notably still almost one magnitude smaller than the percentage of phosphatidylserine exposing erythrocytes (Fig. 29 b).

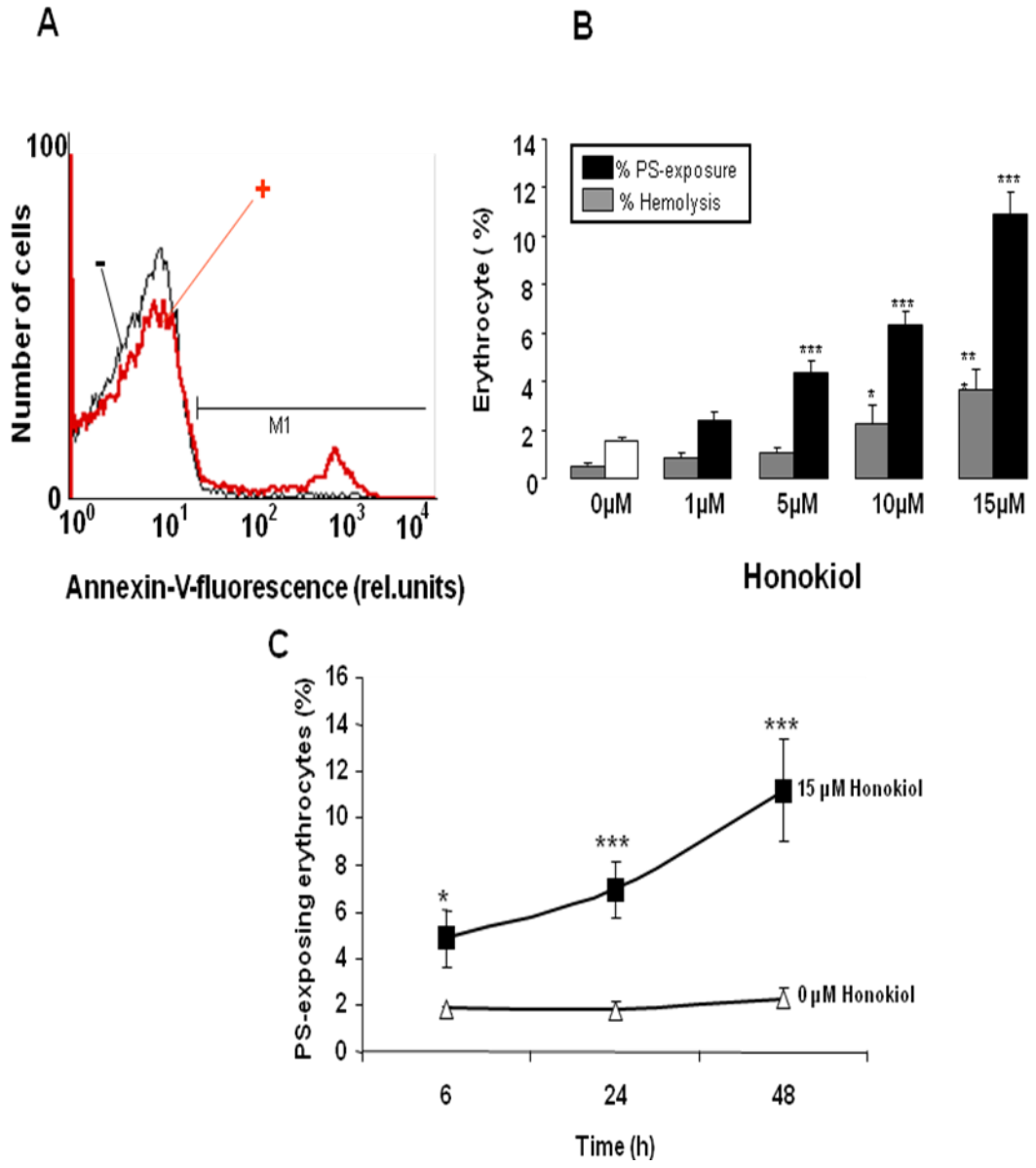

Figure 29: Effect of honokiol on phosphatidylserine exposure and hemolysis

A) Histogram of annexin V-binding in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to plain Ringer solution (indicated as, -) or to 15 µM honokiol (indicated as ,+).

B) Effect of chromium (VI) on phosphatidylserine exposure. Arithmetic means ± SEM (n = 15) of the percentage of annexin V-binding erythrocytes exposed for 48 hours to plain Ringer solution (white bar) or to honokiol (black bars). For a purpose of comparison, arithmetic means ± SEM (n = 4) were used to express the percentage of hemolysis is shown as grey bars. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) indicates significant difference from values in control Ringer solution (ANOVA).

C) Arithmetic means ± SEM (n = 4) were used to express erythrocyte annexin-V-binding post exposure for 6 - 48 h to Ringer solution without (white triangles) or with (black squares) Honokiol (15 µM). * (p

<0.05),*** (p <0.001) indicates significant difference from the absence of Honokiol (t test).

DMSO was the solvent of honokiol, where we studied the effect of DMSO on PS exposure. No significant effect of DMSO could modify PS exposure following erythrocytes treatment with 0.15% DMSO. PS exposure values ranged between 2.6 ± 0.5% in the absence of DMSO and 2.8 ± 0.4% in the presence of DMSO (n=4).

Cell membrane scrambling with subsequent increase of phosphatidylserine abundance at the cell surface is considered the second hallmark of eryptosis. Accordingly, Confocal imaging was performed to visualize eryptosis in the absence and presence of 15 µM honokiol (Fig. 30). The number of annexin V positive erythrocytes in honokiol treated cells was indeed higher than in untreated erythrocytes incubated for 48 hours in Ringer solution.

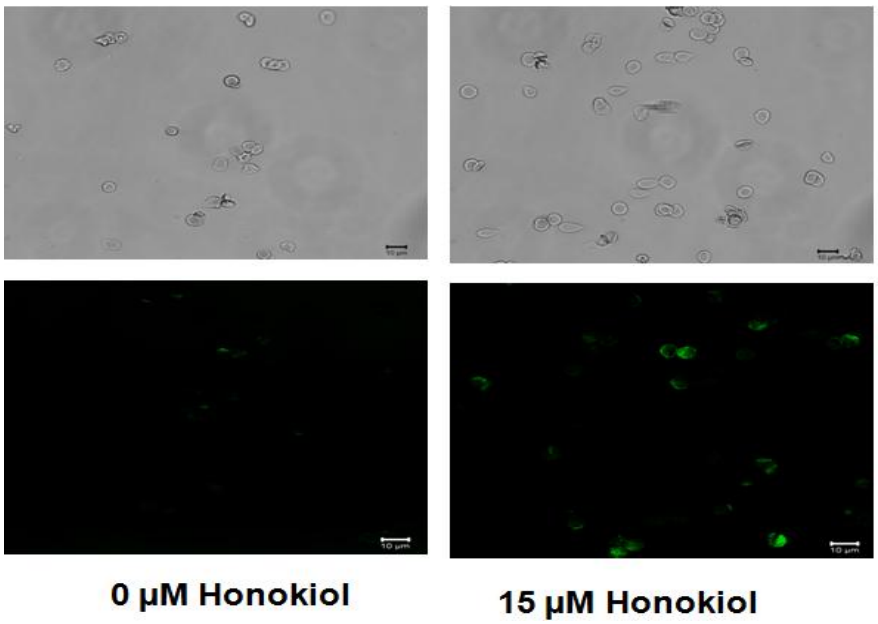

Figure 30: Confocal images of phosphatidylserine exposing erythrocytes with or without honokiol treatment

Confocal microscopy technique was used in tow forms, light microscopy (lower panels) and FITC-dependent fluorescence (upper panels) to visualise stained human erythrocytes with FITC-conjugated annexin-V post 48h incubation in Ringer solution without (left panels) and with (right panels) 15 µM Honokiol.

To test whether honokiol-induced increase in the cytosolic Ca^{2+} concentration is indeed in charge of cell membrane scrambling and phosphatidylserine exposure, Erythrocytes were exposed to 15µM honokiol for a 48h in the nominal absence of extracellular Ca^{2+}. As shown in Fig. 31, honokiol induced phosphatidylserine exposure, where the affect was significantly blunted in the nominal absence of extracellular Ca^{2+}.

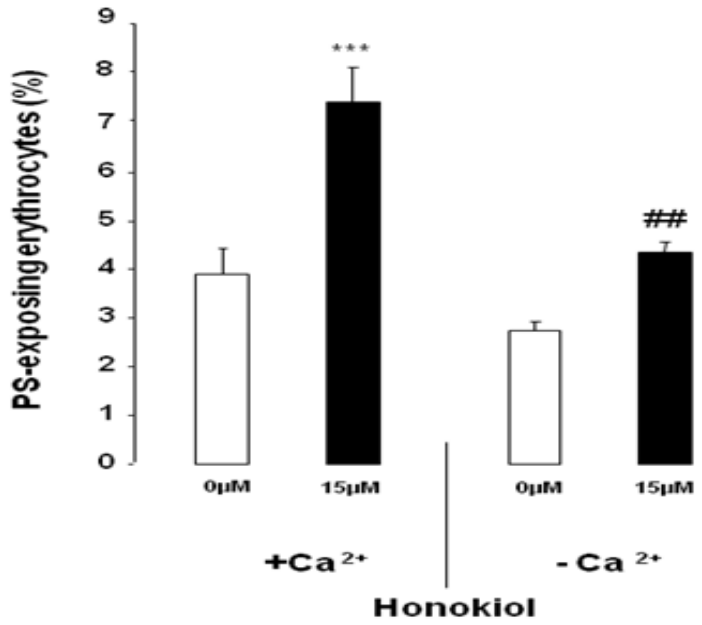

Figure 31: Effect of Ca^{2+} withdrawal on honokiol- induced annexin-V-binding

Annexin V-binding erythrocytes percentage was expressed with arithmetic means ± SEM (n = 8) post exposure for 48 hours to plain

Ringer solution (white bars) or to 15 µM honokiol (black bars) in the presence (left bars) and absence (right bars) of Ca^{2+} in extracellular fluid. ***P<0.001 indicates significant difference from values in control Ringer solution (ANOVA). ## (p<0.01) indicates significant difference from respective values in the presence of Ca^{2+} (ANOVA).

To explore whether calcium-dependent PS exposure induced by honokiol is further enhanced by ionomycin, additional experiment were performed exposing erythrocytes to 15 µM Honokiol in the presence and absence of the calcium ionophore ionomycin. As shown in Figure 32, erythrocytes exposed to both honokiol and 1µM ionomycin have significantly higher intracellular calcium activity than those exposed to honokiol alone.

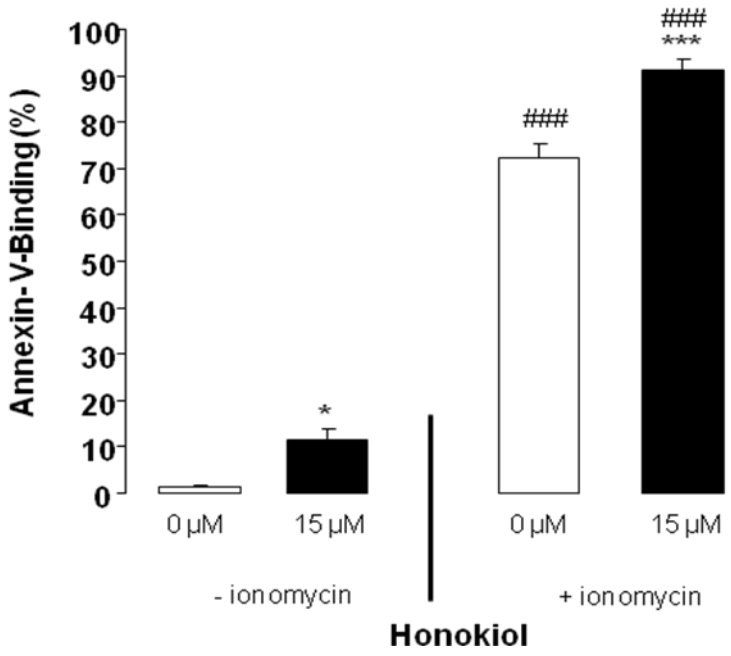

Figure 32: Effect of ionomycin on honokiol- induced annexin-V-binding

Annexin V-binding erythrocytes percentage expressed with arithmetic means ± SEM (n = 4) after a 1 h exposure with Ringer solution without (white bar) or with (black bars) 15 µM honokiol in the absence (left bars)

and presence (right bars) of ionomycin (1 µM) ***P<0.001 indicates significant difference from the absence of honokiol (ANOVA) ### (p<0.001) indicates significant difference from the respective values in the absence of ionomycin.

As a sensitivity of cell membrane scrambling to $[Ca^{2+}]I$ is enhanced by ceramide, additional experiments were performed to determine the effect of honokiol on ceramide formation. FITC-labeled anti-ceramide antibodies was employed to identify ceramide abundance at the erythrocyte surface. Figure. 33 illustrated a slightly but significantly increase of ceramide formation post (15µM) honokiol exposing, this end discloses a non-Ca^{2+} mediated mechanism involvement.

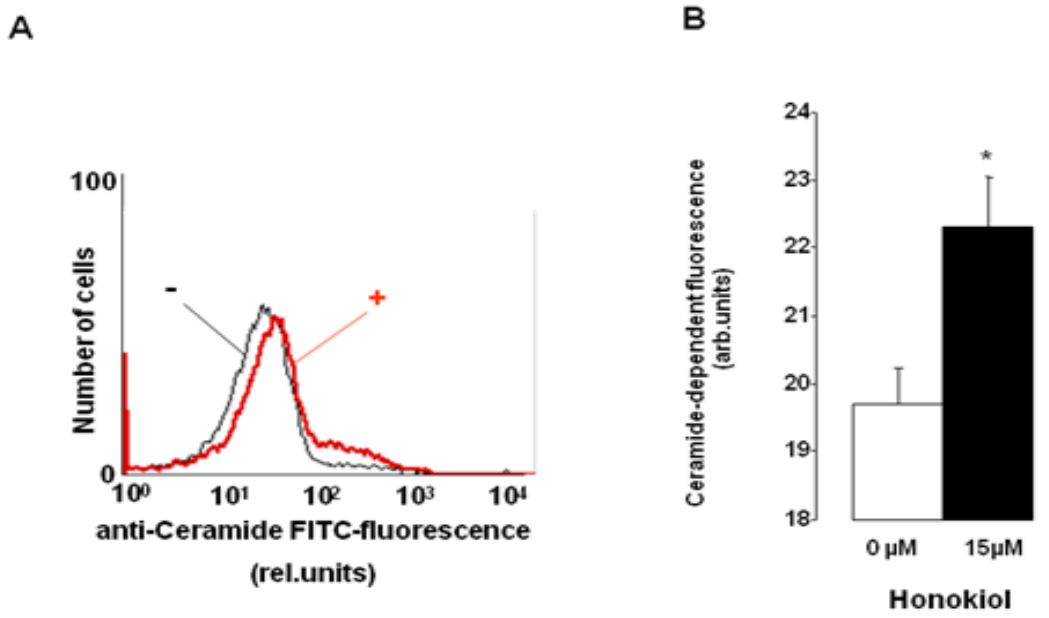

Figure 33: Effect of honokiol on ceramide formation.

A) Histogram of anti-ceramide FITC-fluorescence in a representative experiment of erythrocytes from healthy volunteers exposed for 48 hours to Ringer solution without (indicated as, -) or with (indicated as, +) 15 µM honokiol.

B) Arithmetic means ± SEM (n = 4) were used to express ceramide abundance in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bar) 15 µM honokiol.. *p < 0.05 indicates significant difference from values in control Ringer solution (t-test)

Chapter 4

DISCUSSION

Chapter 4

4. DISCUSSION

4.1. Modulation of erythrocyte survival by Hexavalent Chromium

Unconventional effect of chromium (VI) is disclosed by the present study, where chromium (VI) influences erythrocyte survival by triggering eryptosis, the suicidal death of erythrocytes. Treatment of human erythrocytes with chromium (VI) is followed by decrease of cell volume and cell membrane scrambling with translocation of phosphatidylserine in the cell membrane from inside to the outer surface [11], phosphatidylserine translocation is considered a crucial signal detected by special receptors in macrophages, which in turn bind the eryptotioc erythrocytes, engulf and degrade them [100].

Our observations have shown that Chromium (VI) is able to induce hemolysis, however, its impact pervaded only a restricted percentage of erythrocytes. The affecting concentrations are well in the range of those achieved during chromium intoxication *in vivo*[109]. In severe chromium intoxication some high values have been detected, which approach to 42 µM [204], on the other hand healthy individuals showed chromium serum concentrations less than 0.1 µM [205].

The cell membrane scrambling following chromium (VI) exposure is most likely a result of Ca^{2+} entry with ensuing augment of cytosolic Ca^{2+} activity, where previous observations described the increasing of cytosolic Ca^{2+} activity as a powerful stimulator of cell membrane scrambling [37, 92, 96]. Supposedly, chromium (VI) activates nonselective cation channel, the molecular identity of this channel is still incompletely understood, but somehow involves TRPC6 [77]. This recent Ca^{2+} increase leads to activation Ca^{2+} sensitive K^+ channel, where

opening of those channels fosters exit of K^+ following its chemical gradient, resulting in cell membrane hyperpolarisation, Cl^- exit and thus cellular loss of KCl with osmotically obliged water [206], Accordingly, chromium (VI) provided a decrease of forward scatter, an observation reflecting cell shrinkage.

Chromium (VI) has been proved already its effective cellular impact [109, 118, 207, 208], also the cation channel has been reported to be activated by oxidative stress [209].

The nominal absence of extracellular Ca^{2+} significantly blunted, but did not fully abrogate the scrambling effect of chromium (VI), indicating that Chromium (VI) has to induce cell membrane scrambling not by Ca^{2+} entry but also using some additional further mechanism involved in the apoptosis.

ATP depletion in the erythrocyte is an important factor in eryptosis signaling [75]. According to our observations, chromium (VI) decreases cytosolic ATP concentration, this lack of energy assumably plays an important role by eryptosis stimulation. The energy depletion here is considered a temperate stimulator of eryptosis, thus it has not the fully upper hand by strong stimulation of cell membrane scrambling. Glycolysis could be targeted in erythrocyte by chromium (VI), which is working on ATP generation disruption; its action here could reflect its interference with glycolytic flux and thus on ATP levels.

Ceramide can also stimulate cell membrane scrambling [210], where sensitizes the cell to the proeryptotic effects of increased cytosolic Ca^{2+} activity [98]. Ceramide formation is stimulated by platelet activating factor (PAF), which activates a sphingomyelinase leading to the breakdown of sphingomyelin [211]. Apoptosis may be triggered in same way by ceramide in nucleated cells [212]. However, chromium (VI) could not significantly alter ceramide abundance. Thus, ceramide formation supposedly has no hand by the stimulation of erythrocyte cell membrane scrambling by chromium (VI).

A positive merit of eryptosis, that erythrocytes are removed from circulating blood without release of hemoglobin into plasma. Exposure to

chromium as several other triggers of eryptosis could keep the most treated erythrocytes undergoing eryptosis, instead of hemolysis. Macrophages are usually recognize exposing erythrocytes phosphatidylserine, which clear defective erythrocytes from circulating blood prior to hemolysis.

The rupture of cell membrane is the inevitable result of progressing cell swelling, ultimately followed by release of cellular hemoglobin, which is filtered in renal glomerula with subsequent precipitation in renal tubular fluid and occludes renal tubules [213].

Anemia could be defined, the extent at which enhancing erythrocyte formation cannot compensate the progressing loss of them, accelerated eryptosis may, however, reduce the total circulating erythrocytes, a consequence of accelerated eryptosis is the development of anemia (Lang, Gulbins et al. 2008). Moreover, phosphatidylserine-exposing erythrocytes may adhere to the vascular wall[214, 215] and further stimulate blood clotting[214, 216, 217] . Accordingly, enhanced eryptosis can impair microcirculation and launch involvement in the vascular injury of metabolic syndrome [218] Thus, excessive eryptosis may become pathophysiologically relevant.

Plenty substances can act as eryptosis stimulators [43, 46, 63, 219, 220]. The current findings highligted the effect of chromium (VI) on enucleated cells, therefore, not only trigger apoptosis of nucleated cells but is similarly effective in the absence of nuclei and mitochondria. Finaly, ATP depletion, Ca^{2+} entry are stimulated by chromium (VI) in erythrocytes, which give rise to cell membrane scrambling and cell shrinkage.

4.2. Involvement of Rotenone in the erythrocyte cell membrane scrambling

Triggering of suicidal erythrocyte death or eryptosis is an incoming effect of rotenone, it has been brought to light by the present observations, i.e. cell membrane scrambling, cell shrinkage and phosphatidylserine exposure at the cell surface are a result of human erythrocytes exposure to rotenone. Phosphatidylserine-exposing erythrocytes are engulfed by macrophages equipped with phosphatidylserine receptors[100] and thus eliminated from circulating blood [221]. Earlier observations provided that rotenone plasma concentrations range from 0.4 to 0.9 µM posterior intraperitoneal injections of 2 mg/kg per day[222]. To bring out the effect in our study, it was required a concentration 1 µM, corresponding to approximately 0.4 mg/kg. Applied dosages on animals are ranging between 1 and 10 mg/kg [222] [223] [224, 225] [226]. Rotenone plasma concentrations ranged between 1 and 2 µM have been determined following subcutaneous injection of encapsulating microspheres rotenone[227]. All the above-mentioned observations indicated well relevant confirmation for rotenone treatment in vivo.

Our study revealed that cytosolic Ca^{2+} activity is elevated by rotenone, this increase can be attributable to the involvement of Ca^{2+} permeable cation channels TRPC6 which has proved already in previous other observations its implication in the cation current variations and Ca^{2+} entry into erythrocytes[77]. The cation channels also showed a positive response to oxidative stress[209].

Gardos channels (Ca^{2+}-sensitive K^+ channels) are activated as a result of cytosolic Ca^{2+} augmenting [228] leading to intracellular K^+ loss following its chemical gradient, hyperpolarization and exit of Cl^- ions, these events are usually associated concurrently with a loss of osmotically obliged water ,thus cell shrinkage[37]. As a matter of fact, rotenone decreases the forward scatter, as findings reflect cell shrinkage, which was apparent in our observations.

The effect of rotenone on cell membrane scrambling results at least in part from an increase of cytosolic Ca^{2+} concentration, which is well known to trigger erythrocyte membrane scrambling resulting in phosphatidylserine exposure at the erythrocyte surface [37, 92, 96]. Rotenone- induced cell membrane scrambling is significantly blunted by removal of extracellular Ca^{2+}, indicating that rotenone is at least partially effective by stimulating Ca^{2+} -entry from extracellular space. Removal of extracellular Ca^{2+} does, however, not fully abrogate the rotenone induced cell membrane scrambling, indicating that one or more additional mechanisms may be involved in the stimulation of cell membrane scrambling by rotenone.

Besides stimulating Ca^{2+} entry, rotenone triggers the formation of ceramide, which is known to enhance the sensitivity of the scrambling machinery to $[Ca^{2+}]i$ [229] . Ceramide is produced by a sphingomyelinase, which is activated by platelet activating factor[229]. Ceramide similarly triggers apoptosis of nucleated cells[212]. Ceramide thus contributes to the pathophysiology of several clinical disorders, such as, Wilson's disease [230], multiple sclerosis [231], major depression [212], Parkinson's disease [232], Alzheimer's disease [212, 233], cardiovascular disease [234], cystic fibrosis [235], lung inflammation, fibrosis and infection [236].

Excessive eryptosis may, however, be pathophysiologically relevant [237]. Phosphatidylserine exposing erythrocytes further adhere to endothelial CXCL16/SR-PSO [238]. The adherance of phosphatidylserine exposing erythrocytes to the vascular wall is expected to compromise microcirculation[214, 215, 238, 239, 240] and foster blood clotting [214, 216, 217]. Thus, rotenone-induced exaggerated eryptosis increases the risk of thrombosis. Phosphatidylserine exposing erythrocytes are further engulfed and thus cleared from circulating blood [203, 218]. Accordingly, excessive eryptosis may lead to anemia [203, 218].

In conclusion, Ca^{2+} entry and ceramide formation are the result of exposing erythrocytes to rotenone with inevitable consequences represented in triggering of cell membrane scrambling and cell shrinkage. Thus, the suicidal

death of erythrocytes is stimulated by retonene regardless the presence of mitochondria.

4.3. Participation of Apigenin in eryptosis

The current study discloses an innovative function of apigenin, i.e. the stimulation of eryptosis. Specifically, apigenin activates cation channels leading to influx of Ca^{2+}, cell shrinkage, and phosphatidylserine exposure at the erythrocyte surface. The range of concentrations observed in vivo included our concentration needed to trigger cell membrane scrambling and PS exposure.

Stimulating Ca^{2+} entry with further increase of cytosolic Ca^{2+} activity is the first pathway employed by apigenin to demonstrate its effective impact on erythrocytes. Cation nonselective channels are supposedly atctivated by apigenin, which have formerly been shown to involve TRPC6 [77]. Oxidative stress is a considered a main trigger of cation channel activity [209]. Phosphatidylserine exposure at the erythrocyte surface as shown previously [37, 92, 96] is a result of cytosolic Ca^{2+} activity increasing which in turn stimulates cell membrane scrambling, The scrambling effect of apigenin is significantly blunted in the nominal absence of extracellular Ca^{2+}, indicating that apigenin triggered cell membrane scrambling at least partialy by augmenting Ca^{2+} entry. However, even in the nominal absence of Ca^{2+}, apigenin could significantly enhance phosphatidylserine exposure. Thus, apigenin must have been effective by additional mechanisms.

Apigenin treatment was indeed followed by an increase of ceramide formation, an effect never shown before. Ceramide stimulates cell membrane scrambling in erythrocytes and apoptosis of nucleated cells [98, 210], which is similarly known to trigger apoptosis of nucleated cells[212].

Excessive ceramide formation participates in the pathophysiology of diverse diseases, such as lung inflammation, fibrosis and infection[236], cystic

fibrosis [235], cardiovascular disease [234, 241], Wilson's disease[230]. Thus, ceramide formation supposedly involved in the stimulation of cell membrane scrambling.

Further mechanisms known to trigger eryptosis include energy depletion [75]. As a matter of fact, apigenin indeed decreased cytosolic ATP concentration, an effect again boost the stimulation of eryptosis. Erythrocyte ATP generation is somehow dependent on glycolysis, where the effect of apigenin on cytosolic ATP levels may reflect an interference with glycolytic flux either by utilization or through impaired formation of ATP. In theory, energy consuming associated with Ca^{2+} export during cation nonselective channels activation could considered as direct cause of ATP depletion. However, removal of extracellular Ca^{2+} could not blunt the decreasing of cytosolic Ca^{2+} concentration post apigenin treatment.

Apigenin increased cytosolic Ca^{2+} activity which is expected to result in cell shrinkage by activation of Ca^{2+}-sensitive K^+ channels [20, 228]. The opening of those channels leads exit of K+ following its chemical gradient, cell membrane hyperpolarization, Cl^- exit due to increased electrical driving force and thus cellular loss of KCl with osmotically obliged water [242].

Apigenin as wide range of xenobiotics proved its proeryptotic activity by triggering eryptosis [219, 220]. Morover, eryptosis is enhanced in diverse clinical disorders [203], including diabetes [243, 244], renal insufficiency [67], hemolytic uremic syndrome[245], sepsis[246], sickle cell disease[247].malaria,[248, 249], Wilson's disease[230], iron deficiency[221], phosphate depletion[250] and presumably metabolic syndrome[251].

In general, erythrocytes which expose phosphatidylserine must be quickly cleared from circulating blood, Anemia could result from excessive eryptosis [218] .Moreover, microcirculation could be impaired by the adherence phosphatidylserine-exposing erythrocytes to the vascular wall[214, 240] and by stimulating blood clotting[214, 217].

Ca^{2+} entry, ceramide formation and ATP depletion all are triggered by apigenin, which is ultimately launch eryptosis, characterized by cell shrinkage and cell membrane scrambling of erythrocytes.

4.4. Honokiol in the triggering of erythrocyte cell membrane scrambling

The present study explored, whether honokiol triggers eryptosis, the suicidal death of erythrocytes. The results indicate that using honokiol to treat erythrocytes drawn from healthy volunteers is followed by breakdown of phosphatidylserine asymmetry of the cell membrane and erythrocyte shrinkage expressing hallmarks of eryptosis. The concentrations required to trigger eryptosis (5 µM) are well in the range of those concentrations (10 µM) applied on tumor cells to trigger apoptosis [189] . Other obsevations showed that lower concentrations of honokiol (0.125-1 µM) have in contrast the ability to counteract apoptosis [194]

plasma honokiol concentration augmented up to 1 g/l (≈ 4 mM) following in vivo administration of 250 mg/kg[252]. Moreover, the therapeutic plasma concentration was closing to 10 mg/l (≈ 40 µM) [252, 253]. therefore, the apoptotic applied concentrations are indeed well pertinent to in vivo one.

The erythrocyte shrinkage following honokiol treatment is most likely the result of increased cytosolic Ca^{2+} activity, which activates Ca^{2+} sensitive K^+ channels [20, 228] leading to cell membrane hyperpolarization. The increased electrical driving force drives Cl^- exit and thus leads to cellular loss of KCl with osmotically obliged water [206] .

The absence of extracellular Ca^{2+} resulted in a significant blunting of phosphatidylserine asymmetry breakingdown of the erythrocyte cell membrane, this blunting at least in part due to increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]i$). Cell membrane scrambling with phosphatidylserine translocation from the inner leaflet of the cell membrane to the outer leaflet of the cell membrane is well

admitted of being stimulated by an increase of $[Ca^{2+}]i$ [203]. Fundamental mechanisms of Ca^{2+} entry comprise Ca^{2+} permeable non selective cation channels comprising the transient receptor potential channel TRPC6 [77] . Oxidative stress can activate the Ca^{2+} permeable erythrocyte cation channels as well [209] .

All the same, high concentrations (15 µM) of honkiol were in demand to increase $[Ca^{2+}]i$ appreciably. However, in the current study to some extent astonishingly at lower honokiol concentrations, the presence of Ca^{2+} plays crucial role in the honokiol impact on phosphatidylserine translocation without necessary requirement for $[Ca^{2+}]i$ increase.

Honokiol-induced phosphatidylserine translocation is blunted but not completely abolished in the nominal absence of extracellular Ca^{2+}, indicating to involvement of additional mechanisms. Those mechanisms include formation of ceramide, Ceramide is well known having the ability to enhance Ca^{2+} sensitivity of phosphatidylserne translocation [210]. In reality, honokiol treatment could stimulate an increase of ceramide formation, where ceramide is known to trigger breakdown of cell membrane phosphatidylserine asymmetry in erythrocytes [210].

Consequences of enhanced eryptosis include anemia. In vivo, eryptotic erythrocytes are followed by clearance of eryptotic erythrocytes from circulating blood where trapped in the spleen and thus rapidly removed [218]. Anemia development aggravates gradually unless the loss of erythrocytes by triggering of eryptosis is not well compensated with a similar enhancement of erythropoiesis[218].

Excessive enhanced eryptosis can further causes adhesion of phosphatidylserine exposing erythrocytes to endothelial CXCL16/SR PSO [238] . At least in theory, accordingly, microcirculation and interfere with blood flow could be compromized by the adhesion of erythrocytes to the vascular wall [214, 215, 238, 239, 254, 255]. The effect may be compounded by the stimulating effect of phosphatidylserine exposure on blood clotting, which may bring up the

development of thrombosis[214, 216, 217] . Based on the above, honokiol treatement may be accompanied with an augmented risk of thromboembolic events

.

5. SUMMARY

Eryptosis, the suicidal death of erythrocytes, is characterized by erythrocyte shrinkage, membrane blebbing, cell membrane phospholipid scrambling with eventually phosphatidylserine exposure at the red cell surface. Increased cytosolic Ca^{2+} activity triggers eryptosis, where this extra activity may explained by Ca^{2+} entry through Ca^{2+} -permeable cation channels. Phosphatidylserine-exposing erythrocytes are rapidly cleared from circulating blood, where respective receptors of macrophages can recognize the exposing death signal of eryptotic cells, which give further the order for engulfing and degrading.

As a result, excessive eryptosis can play a crucial physiological role of determining erythrocyte fate, therefore, accelerated cases of eryptosis may participate in anemia unless the considerd loss of erythrocytes is compensated by enough enhanced generation of new erythrocytes.

In an industrialized society, residential, commercial and industrial sources discharge daily various forms of contaminants to the environment. However, accumulated significant discharge of a contaminant/hazardous substance has dual potential effect on human health and the surrounding environment.

The first study explored, whether chromium (VI) as a environmental contaminants is capable to trigger eryptosis, a possible reason for anemia, and if so, whether the chromium (VI) induced eryptosis using a hall mark alterations of cytosolic Ca^{2+} activity, ceramide formation and/or cytosolic ATP levels. According to annexin-V-binding in FACS analysis, chromium (VI) (10 µM) stimulated phosphatidylserine exposure, this effect significantly blunted in the nominal absence of Ca^{2+}. According to Fluo3 fluorescence, chromium (VI) increased cytosolic Ca^{2+} activity and decreases cytosolic ATP concentration, both are considered to be presumably major contributers to the stimulation of eryptosis, Where ceramide abundance was not significantly modified. In conclusion, the present observations may shed additional light on the effects of

chromium (VI) on enucleated cells, chromium (VI) stimulates Ca^{2+} entry and ATP depletion in erythrocytes, which in turn lead to cell membrane scrambling and cell shrinkage, similarly in nucleated cells.

The second study explored, whether the pesticide intoxication rotenone, a cause of Parkinson's disease stimulates apoptosis and elucidate underlying mechanisms. Following exposure of human erythrocytes to rotenone, it was indeed able to scramble the cell membrane in addition to cell shrinkage at required concentration for this effect 1 µM. Rotenone increases cytosolic Ca^{2+} activity due to activation of Ca^{2+} permeable cation channels, which further leads to Ca^{2+} sensitive K^+ channels activation followed by many cellular events such KCl loss, exit of osmotically obliged water and thus cell shrinkage. The effect of rotenone is, however, not fully abrogated by removal of extracellular Ca^{2+}, pointing to the participation of an additional mechanism related to stimulation of ceramide formation. Ceramide sensitizes erythrocytes to the scrambling effect of Ca^{2+} and thus stimulates cell membrane scrambling in erythrocytes.

The third and fourth studies explored the involvement of two natural products in the regulation of eryptosis. They are chemical substance produced by a living organism that have distinctive pharmacological effects. By applying Apigenin and Honokiol, Fluo3 fluorescence was employed to estimate cytosolic Ca^{2+} concentration, forward scatter to analyse cell volume and annexin V-binding to disclose phosphatidylserine exposure by Facs analysis, as a result Apigenin and Honokiol share thier proeryptotic activity with a wide variety of xenobiotics similarly triggering eryptosis. Apigenin and Honokiol are partially effective by stimulating Ca^{2+} entry with subsequent activating nonselective cation channels, Cell membrane scrambling is stimulated by ceramide, which is similarly known to trigger apoptosis of nucleated cells. The effect of apigenin on decreasing cytosolic ATP concentration could result from enhanced ATP utilization or impaired formation of ATP by interference with glycolytic flux.

Taken together, the study displays the functional significance of some xenobiotics which directly or indirectly regulate the suicidal erythrocyte death and survival.

6. REFERENCES

1. Bessis M, Delpech G. Discovery of the red blood cell with notes on priorities and credits of discoveries, past, present and future. *Blood Cells* 1981, **7**(3)**:** 447-480.

2. Tavassoli M. Red cell delivery and the function of the marrow-blood barrier: a review. *Exp Hematol* 1978, **6**(3)**:** 257-269.

3. Lew VL, Bookchin RM. Ion transport pathology in the mechanism of sickle cell dehydration. *Physiol Rev* 2005, **85**(1)**:** 179-200.

4. Steck TL. The organization of proteins in the human red blood cell membrane. A review. *J Cell Biol* 1974, **62**(1)**:** 1-19.

5. Zwaal RFS, A. J. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* 1997, **89**(4)**:** 1121-1132.

6. Bennett V. Proteins involved in membrane--cytoskeleton association in human erythrocytes: spectrin, ankyrin, and band 3. *Methods Enzymol* 1983, **96:** 313-324.

7. Nicolas V. Rh-RhAG/ankyrin-R, a new interaction site between the membrane bilayer and the red cell skeleton, is impaired by Rh(null)-associated mutation. *Journal of Biological Chemistry* 2003.

8. Reid ME, Takakuwa Y, Conboy J, Tchernia G, Mohandas N. Glycophorin C content of human erythrocyte membrane is regulated by protein 4.1. *Blood* 1990, **75**(11)**:** 2229-2234.

9. Maher AD, Kuchel PW. The Gardos channel: a review of the Ca2+-activated K+ channel in human erythrocytes. *Int J Biochem Cell Biol* 2003, **35**(8)**:** 1182-1197.

10. Garrahan PJ, Rega AF. Comparison between plasma membrane Ca2+ and Na,K-ATPases: short review. *Braz J Med Biol Res* 1988, **21**(6)**:** 1261-1267.

11. Lang KS, Lang PA, Bauer C, Duranton C, Wieder T, Huber SM*, et al.* Mechanisms of suicidal erythrocyte death. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2005, **15**(5)**:** 195-202.

12. Bonilla S, Goecke IA, Bozzo S, Alvo M, Michea L, Marusic ET. Effect of chronic renal failure on Na,K-ATPase alpha 1 and alpha 2 mRNA transcription in rat skeletal muscle. *J Clin Invest* 1991, **88**(6)**:** 2137-2141.

13. Skou JC. Enzymatic Basis for Active Transport of Na+ and K+ across Cell Membrane. *Physiol Rev* 1965, **45:** 596-617.

14. Lew VL, Muallem S, Seymour CA. Properties of the Ca2+-activated K+ channel in one-step inside-out vesicles from human red cell membranes. *Nature* 1982, **296**(5859)**:** 742-744.

15. Alvarez J, Garcia-Sancho J. An estimate of the number of Ca2+-dependent K+ channels in the human red cell. *Biochim Biophys Acta* 1987, **903**(3)**:** 543-546.

16. Brugnara C, De Franceschi L, Alper SL. Ca(2+)-activated K+ transport in erythrocytes. Comparison of binding and transport inhibition by scorpion toxins. *The Journal of biological chemistry* 1993, **268**(12)**:** 8760-8768.

17. Gardos G. The function of calcium in the potassium permeability of human erythrocytes. *Biochim Biophys Acta* 1958, **30**(3)**:** 653-654.

18. Simons TJ. Calcium-dependent potassium exchange in human red cell ghosts. *J Physiol* 1976, **256**(1)**:** 227-244.

19. Lew VL, Ferreira HG. Calcium Transport and the Properties of a Calcium-Activated Potassium Channel in Red Cell Membranes. In: Felix B, Arnost K (eds). *Current Topics in Membranes and Transport*, vol. Volume 10. Academic Press, 1978, pp 217-277.

20. Brugnara C, de Franceschi L, Alper SL. Inhibition of Ca(2+)-dependent K+ transport and cell dehydration in sickle erythrocytes by clotrimazole and other imidazole derivatives. *JClinInvest* 1993, **92**(1)**:** 520-526.

21. Lauf PK, Bauer J, Adragna NC, Fujise H, Zade-Oppen AM, Ryu KH*, et al.* Erythrocyte K-Cl cotransport: properties and regulation. *Am J Physiol* 1992, **263**(5 Pt 1)**:** C917-932.

22. Gillen CM, Brill S, Payne JA, Forbush B, 3rd. Molecular cloning and functional expression of the K-Cl cotransporter from rabbit, rat, and human. A new member of the cation-chloride cotransporter family. *The Journal of biological chemistry* 1996, **271**(27)**:** 16237-16244.

23. Jennings ML, Adame MF. Direct estimate of 1:1 stoichiometry of K(+)-Cl(-) cotransport in rabbit erythrocytes. *American journal of physiology Cell physiology* 2001, **281**(3)**:** C825-832.

24. Reipert S, Reipert BM, Hickman JA, Allen TD. Nuclear pore clustering is a consistent feature of apoptosis in vitro. *Cell death and differentiation* 1996, **3**(1)**:** 131-139.

25. Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *J Intern Med* 2005, **258**(6)**:** 479-517.

26. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 2007, **35**(4)**:** 495-516.

27. Lowe SW, Bodis S, Bardeesy N, McClatchey A, Remington L, Ruley HE*, et al.* Apoptosis and the prognostic significance of p53 mutation. *Cold Spring Harb Symp Quant Biol* 1994, **59:** 419-426.

28. Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death. *Nat Rev Mol Cell Biol* 2007, **8**(5)**:** 405-413.

29. Zimmermann KC, Bonzon C, Green DR. The machinery of programmed cell death. *Pharmacol Ther* 2001, **92**(1)**:** 57-70.

30. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 1998, **94**(4)**:** 481-490.

31. Li H, Zhu H, Xu CJ, Yuan J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 1998, **94**(4)**:** 491-501.

32. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ*, et al.* Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 1998, **17**(6)**:** 1675-1687.

33. LeBlanc HN, Ashkenazi A. Apo2L/TRAIL and its death and decoy receptors. *Cell death and differentiation* 2003, **10**(1)**:** 66-75.

34. Bracci R, Perrone S, Buonocore G. Oxidant injury in neonatal erythrocytes during the perinatal period. *Acta Paediatr Suppl* 2002, **91**(438)**:** 130-134.

35. Lang KS, Roll B, Myssina S, Schittenhelm M, Scheel-Walter HG, Kanz L*, et al.* Enhanced erythrocyte apoptosis in sickle cell anemia, thalassemia and glucose-6-phosphate dehydrogenase deficiency. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2002, **12**(5-6)**:** 365-372.

36. Barvitenko NN, Adragna NC, Weber RE. Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2005, **15**(1-4)**:** 1-18.

37. Lang KS, Duranton C, Poehlmann H, Myssina S, Bauer C, Lang F*, et al.* Cation channels trigger apoptotic death of erythrocytes. *Cell death and differentiation* 2003, **10**(2)**:** 249-256.

38. Mandal D, Mazumder A, Das P, Kundu M, Basu J. Fas-, caspase 8-, and caspase 3-dependent signaling regulates the activity of the aminophospholipid translocase and phosphatidylserine externalization in human erythrocytes. *The Journal of biological chemistry* 2005, **280**(47)**:** 39460-39467.

39. Niemoeller OM, Foller M, Lang C, Huber SM, Lang F. Retinoic acid induced suicidal erythrocyte death. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2008, **21**(1-3)**:** 193-202.

40. Sopjani M, Foller M, Dreischer P, Lang F. Stimulation of eryptosis by cadmium ions. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2008, **22**(1-4)**:** 245-252.

41. Kempe DS, Lang PA, Eisele K, Klarl BA, Wieder T, Huber SM*, et al.* Stimulation of erythrocyte phosphatidylserine exposure by lead ions. *American journal of physiology Cell physiology* 2005, **288**(2)**:** C396-402.

42. Nicolay JP, Gatz S, Lang F, Lang UE. Lithium-induced suicidal erythrocyte death. *J Psychopharmacol* 2010, **24**(10)**:** 1533-1539.

43. Mahmud H, Foller M, Lang F. Arsenic-induced suicidal erythrocyte death. *Arch Toxicol* 2009, **83**(2)**:** 107-113.

44. Sopjani M, Foller M, Gulbins E, Lang F. Suicidal death of erythrocytes due to selenium-compounds. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2008, **22**(5-6)**:** 387-394.

45. Sopjani M, Foller M, Haendeler J, Gotz F, Lang F. Silver ion-induced suicidal erythrocyte death. *J Appl Toxicol* 2009, **29**(6)**:** 531-536.

46. Braun M, Foller M, Gulbins E, Lang F. Eryptosis triggered by bismuth. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2009, **22**(3)**:** 453-460.

47. Kiedaisch V, Akel A, Niemoeller OM, Wieder T, Lang F. Zinc-induced suicidal erythrocyte death. *Am J Clin Nutr* 2008, **87**(5)**:** 1530-1534.

48. Foller M, Geiger C, Mahmud H, Nicolay J, Lang F. Stimulation of suicidal erythrocyte death by amantadine. *Eur J Pharmacol* 2008, **581**(1-2)**:** 13-18.

49. Foller M, Sopjani M, Mahmud H, Lang F. Vanadate-induced suicidal erythrocyte death. *Kidney Blood Press Res* 2008, **31**(2)**:** 87-93.

50. Nicolay JP, Bentzen PJ, Ghashghaeinia M, Wieder T, Lang F. Stimulation of erythrocyte cell membrane scrambling by amiodarone. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2007, **20**(6)**:** 1043-1050.

51. Mahmud H, Mauro D, Qadri SM, Foller M, Lang F. Triggering of suicidal erythrocyte death by amphotericin B. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2009, **24**(3-4)**:** 263-270.

52. Akel A, Hermle T, Niemoeller OM, Kempe DS, Lang PA, Attanasio P*, et al.* Stimulation of erythrocyte phosphatidylserine exposure by chlorpromazine. *Eur J Pharmacol* 2006, **532**(1-2)**:** 11-17.

53. Niemoeller OM, Mahmud H, Foller M, Wieder T, Lang F. Ciglitazone and 15d-PGJ2 induced suicidal erythrocyte death. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2008, **22**(1-4)**:** 237-244.

54. Bentzen PJ, Lang E, Lang F. Curcumin induced suicidal erythrocyte death. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2007, **19**(1-4)**:** 153-164.

55. Bunchman TE, Brookshire CA. Cyclosporine-induced synthesis of endothelin by cultured human endothelial cells. *J Clin Invest* 1991, **88**(1)**:** 310-314.

56. Foller M, Sopjani M, Schlemmer HP, Claussen CD, Lang F. Triggering of suicidal erythrocyte death by radiocontrast agents. *Eur J Clin Invest* 2009, **39**(7)**:** 576-583.

57. Gatidis S, Foller M, Lang F. Hemin-induced suicidal erythrocyte death. *Ann Hematol* 2009, **88**(8)**:** 721-726.

58. Lang PA, Kaiser S, Myssina S, Birka C, Weinstock C, Northoff H*, et al.* Effect of Vibrio parahaemolyticus haemolysin on human erythrocytes. *Cell Microbiol* 2004, **6**(4)**:** 391-400.

59. Foller M, Shumilina E, Lam R, Mohamed W, Kasinathan R, Huber S*, et al.* Induction of suicidal erythrocyte death by listeriolysin from Listeria monocytogenes. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2007, **20**(6)**:** 1051-1060.

60. Mahmud H, Foller M, Lang F. Stimulation of erythrocyte cell membrane scrambling by methyldopa. *Kidney Blood Press Res* 2008, **31**(5)**:** 299-306.

61. Schneider J, Nicolay JP, Foller M, Wieder T, Lang F. Suicidal erythrocyte death following cellular K+ loss. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2007, **20**(1-4)**:** 35-44.

62. Qadri SM, Eberhard M, Mahmud H, Foller M, Lang F. Stimulation of ceramide formation and suicidal erythrocyte death by vitamin K(3) (menadione). *Eur J Pharmacol* 2009, **623**(1-3)**:** 10-13.

63. Eberhard M, Foller M, Lang F. Effect of phytic acid on suicidal erythrocyte death. *Journal of agricultural and food chemistry* 2010, **58**(3)**:** 2028-2033.

64. Qadri SM, Mahmud H, Foller M, Lang F. Thymoquinone-induced suicidal erythrocyte death. *Food Chem Toxicol* 2009, **47**(7)**:** 1545-1549.

65. Nicolay JP, Liebig G, Niemoeller OM, Koka S, Ghashghaeinia M, Wieder T*, et al.* Inhibition of suicidal erythrocyte death by nitric oxide. *Pflugers Arch* 2008, **456**(2)**:** 293-305.

66. Lang PA, Kempe DS, Myssina S, Tanneur V, Birka C, Laufer S*, et al.* PGE(2) in the regulation of programmed erythrocyte death. *Cell death and differentiation* 2005, **12**(5)**:** 415-428.

67. Myssina S, Huber SM, Birka C, Lang PA, Lang KS, Friedrich B*, et al.* Inhibition of erythrocyte cation channels by erythropoietin. *J Am Soc Nephrol* 2003, **14**(11)**:** 2750-2757.

68. Qadri SM, Foller M, Lang F. Inhibition of suicidal erythrocyte death by resveratrol. *Life Sci* 2009, **85**(1-2)**:** 33-38.

69. Kasinathan RS, Foller M, Koka S, Huber SM, Lang F. Inhibition of eryptosis and intraerythrocytic growth of Plasmodium falciparum by flufenamic acid. *Naunyn Schmiedebergs Arch Pharmacol* 2007, **374**(4)**:** 255-264.

70. Floride E, Foller M, Ritter M, Lang F. Caffeine inhibits suicidal erythrocyte death. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2008, **22**(1-4)**:** 253-260.

71. Kucherenko Y, Geiger C, Shumilina E, Foller M, Lang F. Inhibition of cation channels and suicidal death of human erythrocytes by zidovudine. *Toxicology* 2008, **253**(1-3)**:** 62-69.

72. Mahmud H, Qadri SM, Foller M, Lang F. Inhibition of suicidal erythrocyte death by vitamin C. *Nutrition* 2010, **26**(6)**:** 671-676.

73. Mahmud H, Mauro D, Foller M, Lang F. Inhibitory effect of thymol on suicidal erythrocyte death. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2009, **24**(5-6)**:** 407-414.

74. Niemoeller OM, Bentzen PJ, Lang E, Lang F. Adenosine protects against suicidal erythrocyte death. *Pflugers Arch* 2007, **454**(3)**:** 427-439.

75. Klarl BA, Lang PA, Kempe DS, Niemoeller OM, Akel A, Sobiesiak M*, et al.* Protein kinase C mediates erythrocyte "programmed cell death" following glucose depletion. *American journal of physiology Cell physiology* 2006, **290**(1)**:** C244-253.

76. Lang KS, Myssina S, Lang PA, Tanneur V, Kempe DS, Mack AF*, et al.* Inhibition of erythrocyte phosphatidylserine exposure by urea and Cl. *Am J Physiol Renal Physiol* 2004, **286**(6)**:** F1046-1053.

77. Foller M, Kasinathan RS, Koka S, Lang C, Shumilina E, Birnbaumer L*, et al.* TRPC6 contributes to the Ca(2+) leak of human erythrocytes. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2008, **21**(1-3)**:** 183-192.

78. Kaestner L, Bernhardt I. Ion channels in the human red blood cell membrane: their further investigation and physiological relevance. *Bioelectrochemistry* 2002, **55**(1-2)**:** 71-74.

79. Lang F, Huber SM, Szabo I, Gulbins E. Plasma membrane ion channels in suicidal cell death. *Arch Biochem Biophys* 2007, **462**(2)**:** 189-194.

80. Lang F, Lang KS, Lang PA, Huber SM, Wieder T. Mechanisms and significance of eryptosis. *Antioxid Redox Signal* 2006, **8**(7-8)**:** 1183-1192.

81. Gottlieb RA, Gruol DL, Zhu JY, Engler RL. Preconditioning rabbit cardiomyocytes: role of pH, vacuolar proton ATPase, and apoptosis. *J Clin Invest* 1996, **97**(10)**:** 2391-2398.

82. Thangaraju M, Sharma K, Leber B, Andrews DW, Shen SH, Srikant CB. Regulation of acidification and apoptosis by SHP-1 and Bcl-2. *The Journal of biological chemistry* 1999, **274**(41)**:** 29549-29557.

83. Lang F, Madlung J, Bock J, Lukewille U, Kaltenbach S, Lang KS*, et al.* Inhibition of Jurkat-T-lymphocyte Na+/H+-exchanger by CD95(Fas/Apo-1)-receptor stimulation. *Pflugers Arch* 2000, **440**(6)**:** 902-907.

84. Waibel M, Kramer S, Lauber K, Lupescu A, Manns J, Schulze-Osthoff K*, et al.* Mitochondria are not required for death receptor-mediated cytosolic acidification during apoptosis. *Apoptosis* 2007, **12**(3)**:** 623-630.

85. Bilmen S, Aksu TA, Gumuslu S, Korgun DK, Canatan D. Antioxidant capacity of G-6-PD-deficient erythrocytes. *Clin Chim Acta* 2001, **303**(1-2)**:** 83-86.

86. Mavelli I, Ciriolo MR, Rossi L, Meloni T, Forteleoni G, De Flora A*, et al.* Favism: a hemolytic disease associated with increased superoxide dismutase and decreased glutathione peroxidase activities in red blood cells. *Eur J Biochem* 1984, **139**(1)**:** 13-18.

87. Duranton C, Huber SM, Lang F. Oxidation induces a Cl(-)-dependent cation conductance in human red blood cells. *J Physiol* 2002, **539**(Pt 3)**:** 847-855.

88. Huber SM, Uhlemann AC, Gamper NL, Duranton C, Kremsner PG, Lang F. Plasmodium falciparum activates endogenous Cl(-) channels of human erythrocytes by membrane oxidation. *EMBO J* 2002, **21**(1-2)**:** 22-30.

89. Tanneur V, Duranton C, Brand VB, Sandu CD, Akkaya C, Kasinathan RS*, et al.* Purinoceptors are involved in the induction of an osmolyte permeability in

malaria-infected and oxidized human erythrocytes. *FASEB J* 2006, **20**(1)**:** 133-135.

90. Myssina S, Lang PA, Kempe DS, Kaiser S, Huber SM, Wieder T*, et al.* Cl-channel blockers NPPB and niflumic acid blunt Ca(2+)-induced erythrocyte 'apoptosis'. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2004, **14**(4-6)**:** 241-248.

91. Matarrese P, Straface E, Pietraforte D, Gambardella L, Vona R, Maccaglia A*, et al.* Peroxynitrite induces senescence and apoptosis of red blood cells through the activation of aspartyl and cysteinyl proteases. *FASEB J* 2005, **19**(3)**:** 416-418.

92. Bratosin D, Estaquier J, Petit F, Arnoult D, Quatannens B, Tissier JP*, et al.* Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. *Cell death and differentiation* 2001, **8**(12)**:** 1143-1156.

93. Mandal D, Moitra PK, Saha S, Basu J. Caspase 3 regulates phosphatidylserine externalization and phagocytosis of oxidatively stressed erythrocytes. *FEBS Lett* 2002, **513**(2-3)**:** 184-188.

94. Mandal D, Baudin-Creuza V, Bhattacharyya A, Pathak S, Delaunay J, Kundu M*, et al.* Caspase 3-mediated proteolysis of the N-terminal cytoplasmic domain of the human erythroid anion exchanger 1 (band 3). *The Journal of biological chemistry* 2003, **278**(52)**:** 52551-52558.

95. Weil M, Jacobson MD, Raff MC. Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes. *Journal of cell science* 1998, **111 (Pt 18):** 2707-2715.

96. Berg CP, Engels IH, Rothbart A, Lauber K, Renz A, Schlosser SF*, et al.* Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. *Cell death and differentiation* 2001, **8**(12)**:** 1197-1206.

97. Kaestner L, Tabellion W, Lipp P, Bernhardt I. Prostaglandin E2 activates channel-mediated calcium entry in human erythrocytes: an indication for a blood clot formation supporting process. *Thromb Haemost* 2004, **92**(6)**:** 1269-1272.

98. Lang KS, Myssina S, Brand V, Sandu C, Lang PA, Berchtold S*, et al.* Involvement of ceramide in hyperosmotic shock-induced death of erythrocytes. *Cell death and differentiation* 2004, **11**(2)**:** 231-243.

99. Garay R, Braquet P. Involvement of K+ movements in the membrane signal induced by PAF-acether. *Biochemical pharmacology* 1986, **35**(16)**:** 2811-2815.

100. Boas FE, Forman L, Beutler E. Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. *Proc Natl Acad Sci U S A* 1998, **95**(6)**:** 3077-3081.

101. Kiefer CR, Snyder LM. Oxidation and erythrocyte senescence. *Curr Opin Hematol* 2000, **7**(2)**:** 113-116.

102. Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E*, et al.* Functional significance of cell volume regulatory mechanisms. *Physiol Rev* 1998, **78**(1)**:** 247-306.

103. Sillix DH, McDonald FD. Acute renal failure. *Crit Care Clin* 1987, **3**(4)**:** 909-925.

104. Foller M, Huber SM, Lang F. Erythrocyte programmed cell death. *IUBMB Life* 2008, **60**(10)**:** 661-668.

105. Kirk K. Membrane transport in the malaria-infected erythrocyte. *Physiol Rev* 2001, **81**(2)**:** 495-537.

106. Prager JC. Environmental Contaminant Reference Databook. 1998, **1-3:** 3810.

107. Velma V, Vutukuru SS, Tchounwou PB. Ecotoxicology of hexavalent chromium in freshwater fish: a critical review. *Rev Environ Health* 2009, **24**(2)**:** 129-145.

108. Ramirez-Diaz MI, Diaz-Perez C, Vargas E, Riveros-Rosas H, Campos-Garcia J, Cervantes C. Mechanisms of bacterial resistance to chromium compounds. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2008, **21**(3)**:** 321-332.

109. Thompson CM, Haws LC, Harris MA, Gatto NM, Proctor DM. Application of the U.S. EPA mode of action Framework for purposes of guiding future research: a case study involving the oral carcinogenicity of hexavalent chromium. *Toxicol Sci* 2011, **119**(1)**:** 20-40.

110. Oze C, Bird DK, Fendorf S. Genesis of hexavalent chromium from natural sources in soil and groundwater. *Proc Natl Acad Sci U S A* 2007, **104**(16)**:** 6544-6549.

111. Fernandez-Minano E, Ortiz C, Vicente A, Calvo JL, Ortiz AJ. Metallic ion content and damage to the DNA in oral mucosa cells of children with fixed orthodontic appliances. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2011, **24**(5)**:** 935-941.

112. Hedberg Y, Hedberg J, Liu Y, Wallinder IO. Complexation- and ligand-induced metal release from 316L particles: importance of particle size and crystallographic structure. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2011, **24**(6)**:** 1099-1114.

113. Meyskens FL, Yang S. Thinking about the role (largely ignored) of heavy metals in cancer prevention: hexavalent chromium and melanoma as a case in point. *Recent Results Cancer Res* 2011, **188:** 65-74.

114. Pereira Mdo C, Pereira Mde L, Sousa JP. Individual study of chromium in the stainless steel implants degradation: an experimental study in mice. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 1999, **12**(3)**:** 275-280.

115. Das AP, Singh S. Occupational health assessment of chromite toxicity among Indian miners. *Indian J Occup Environ Med* 2011, **15**(1)**:** 6-13.

116. Chiu A, Shi XL, Lee WK, Hill R, Wakeman TP, Katz A*, et al.* Review of chromium (VI) apoptosis, cell-cycle-arrest, and carcinogenesis. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 2010, **28**(3)**:** 188-230.

117. Holmes AL, Wise SS, Wise JP, Sr. Carcinogenicity of hexavalent chromium. *Indian J Med Res* 2008, **128**(4)**:** 353-372.

118. McCarroll N, Keshava N, Chen J, Akerman G, Kligerman A, Rinde E. An evaluation of the mode of action framework for mutagenic carcinogens case study II: chromium (VI). *Environ Mol Mutagen* 2010, **51**(2)**:** 89-111.

119. Nickens KP, Patierno SR, Ceryak S. Chromium genotoxicity: A double-edged sword. *Chem Biol Interact* 2010, **188**(2)**:** 276-288.

120. Stout MD, Herbert RA, Kissling GE, Collins BJ, Travlos GS, Witt KL*, et al.* Hexavalent chromium is carcinogenic to F344/N rats and B6C3F1 mice after chronic oral exposure. *Environ Health Perspect* 2009, **117**(5)**:** 716-722.

121. Asatiani N, Abuladze M, Kartvelishvili T, Kulikova N, Asanishvili L, Holman HY*, et al.* Response of antioxidant defense system to chromium (VI)-induced cytotoxicity in human diploid cells. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2010, **23**(1)**:** 161-172.

122. Hartwig A. Current aspects in metal genotoxicity. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 1995, **8**(1)**:** 3-11.

123. WH G. Tissue distribution and elimination of rotenone in rainbow trout. *Aquat Toxicol* 1986, **8:** 27-40.

124. Isman MB. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. *Annu Rev Entomol* 2006, **51:** 45-66.

125. Ali SF, Binienda ZK, Imam SZ. Molecular aspects of dopaminergic neurodegeneration: gene-environment interaction in parkin dysfunction. *Int J Environ Res Public Health* 2011, **8**(12)**:** 4702-4713.

126. Gosalvez M. Carcinogenesis with the insecticide rotenone. *Life Sci* 1983, **32**(8)**:** 809-816.

127. Nistico R, Mehdawy B, Piccirilli S, Mercuri N. Paraquat- and rotenone-induced models of Parkinson's disease. *Int J Immunopathol Pharmacol* 2011, **24**(2)**:** 313-322.

128. Giasson BI, Lee VM. A new link between pesticides and Parkinson's disease. *Nat Neurosci* 2000, **3**(12)**:** 1227-1228.

129. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* 2000, **3**(12)**:** 1301-1306.

130. Hirata Y, Suzuno H, Tsuruta T, Oh-hashi K, Kiuchi K. The role of dopamine transporter in selective toxicity of manganese and rotenone. *Toxicology* 2008, **244**(2-3)**:** 249-256.

131. Hirata Y, Nagatsu T. Rotenone and CCCP inhibit tyrosine hydroxylation in rat striatal tissue slices. *Toxicology* 2005, **216**(1)**:** 9-14.

132. Kim HJ, Park HJ, Park HK, Chung JH. Tranexamic acid protects against rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells. *Toxicology* 2009, **262**(2)**:** 171-174.

133. Swarnkar S, Singh S, Mathur R, Patro IK, Nath C. A study to correlate rotenone induced biochemical changes and cerebral damage in brain areas with neuromuscular coordination in rats. *Toxicology* 2010, **272**(1-3)**:** 17-22.

134. Duty S, Jenner P. Animal models of Parkinson's disease: a source of novel treatments and clues to the cause of the disease. *Br J Pharmacol* 2011, **164**(4)**:** 1357-1391.

135. Greenamyre JT, Cannon JR, Drolet R, Mastroberardino PG. Lessons from the rotenone model of Parkinson's disease. *Trends Pharmacol Sci* 2010, **31**(4)**:** 141-142; author reply 142-143.

136. Reiter RJ, Manchester LC, Tan DX. Neurotoxins: free radical mechanisms and melatonin protection. *Curr Neuropharmacol* 2010, **8**(3)**:** 194-210.

137. Molnar J, Engi H, Hohmann J, Molnar P, Deli J, Wesolowska O*, et al.* Reversal of multidrug resitance by natural substances from plants. *Curr Top Med Chem* 2010, **10**(17)**:** 1757-1768.

138. Fiskum G, Starkov A, Polster BM, Chinopoulos C. Mitochondrial mechanisms of neural cell death and neuroprotective interventions in Parkinson's disease. *Ann N Y Acad Sci* 2003, **991:** 111-119.

139. Koopman WJ, Nijtmans LG, Dieteren CE, Roestenberg P, Valsecchi F, Smeitink JA*, et al.* Mammalian mitochondrial complex I: biogenesis, regulation, and

reactive oxygen species generation. *Antioxid Redox Signal* 2010, **12**(12)**:** 1431-1470.

140. Nakagawa Y, Nakajima K, Suzuki T. Chlorpropham induces mitochondrial dysfunction in rat hepatocytes. *Toxicology* 2004, **200**(2-3)**:** 123-133.

141. Naoi M, Maruyama W, Shamoto-Nagai M, Yi H, Akao Y, Tanaka M. Oxidative stress in mitochondria: decision to survival and death of neurons in neurodegenerative disorders. *Mol Neurobiol* 2005, **31**(1-3)**:** 81-93.

142. Scatena R, Martorana GE, Bottoni P, Giardina B. Mitochondrial dysfunction by synthetic ligands of peroxisome proliferator activated receptors (PPARs). *IUBMB Life* 2004, **56**(8)**:** 477-482.

143. Segura Aguilar J, Kostrzewa RM. Neurotoxins and neurotoxic species implicated in neurodegeneration. *Neurotox Res* 2004, **6**(7-8)**:** 615-630.

144. Ren W, Qiao Z, Wang H, Zhu L, Zhang L. Flavonoids: promising anticancer agents. *Med Res Rev* 2003, **23**(4)**:** 519-534.

145. Beara IN, Lesjak MM, Jovin ED, Balog KJ, Anackov GT, Orcic DZ*, et al.* Plantain (Plantago L.) species as novel sources of flavonoid antioxidants. *Journal of agricultural and food chemistry* 2009, **57**(19)**:** 9268-9273.

146. Yu YS, Hsu CL, Yen GC. Anti-inflammatory effects of the roots of Alpinia pricei Hayata and its phenolic compounds. *Journal of agricultural and food chemistry* 2009, **57**(17)**:** 7673-7680.

147. Soares R, Azevedo I. Apigenin: is it a pro- or anti-inflammatory agent? *Am J Pathol* 2006, **168**(5)**:** 1762; author reply 1762-1763.

148. Fuchs J, Milbradt R. Skin anti-inflammatory activity of apigenin-7-glucoside in rats. *Arzneimittel-Forschung* 1993, **43**(3)**:** 370-372.

149. Singh JP, Selvendiran K, Banu SM, Padmavathi R, Sakthisekaran D. Protective role of Apigenin on the status of lipid peroxidation and antioxidant defense against hepatocarcinogenesis in Wistar albino rats. *Phytomedicine* 2004, **11**(4)**:** 309-314.

150. Bektic J, Guggenberger R, Spengler B, Christoffel V, Pelzer A, Berger AP*, et al.* The flavonoid apigenin inhibits the proliferation of prostatic stromal cells via the MAPK-pathway and cell-cycle arrest in G1/S. *Maturitas* 2006, **55, Supplement 1**(0)**:** S37-S46.

151. Lindenmeyer F, Li H, Menashi S, Soria C, Lu H. Apigenin acts on the tumor cell invasion process and regulates protease production. *Nutr Cancer* 2001, **39**(1)**:** 139-147.

152. Choi EJ, Kim GH. Apigenin Induces Apoptosis through a Mitochondria/Caspase-Pathway in Human Breast Cancer MDA-MB-453 Cells. *J Clin Biochem Nutr* 2009, **44**(3)**:** 260-265.

153. Ruela-de-Sousa RR, Fuhler GM, Blom N, Ferreira CV, Aoyama H, Peppelenbosch MP. Cytotoxicity of apigenin on leukemia cell lines: implications for prevention and therapy. *Cell Death Dis* 2010, **1:** e19.

154. Czeczot H, Tudek B, Kusztelak J, Szymczyk T, Dobrowolska B, Glinkowska G*, et al.* Isolation and studies of the mutagenic activity in the Ames test of flavonoids naturally occurring in medical herbs. *Mutation research* 1990, **240**(3)**:** 209-216.

155. Shukla S, Gupta S. Apigenin: a promising molecule for cancer prevention. *Pharm Res* 2010, **27**(6)**:** 962-978.

156. Bruno A, Siena L, Gerbino S, Ferraro M, Chanez P, Giammanco M*, et al.* Apigenin affects leptin/leptin receptor pathway and induces cell apoptosis in lung adenocarcinoma cell line. *Eur J Cancer* 2011, **47**(13)**:** 2042-2051.

157. Choi AY, Choi JH, Lee JY, Yoon KS, Choe W, Ha J*, et al.* Apigenin protects HT22 murine hippocampal neuronal cells against endoplasmic reticulum stress-induced apoptosis. *Neurochem Int* 2010, **57**(2)**:** 143-152.

158. Lu HF, Chie YJ, Yang MS, Lu KW, Fu JJ, Yang JS*, et al.* Apigenin induces apoptosis in human lung cancer H460 cells through caspase- and mitochondria-dependent pathways. *Hum Exp Toxicol* 2011, **30**(8)**:** 1053-1061.

159. Liu LZ, Fang J, Zhou Q, Hu X, Shi X, Jiang BH. Apigenin inhibits expression of vascular endothelial growth factor and angiogenesis in human lung cancer cells: implication of chemoprevention of lung cancer. *Mol Pharmacol* 2005, **68**(3)**:** 635-643.

160. Xu HL, Tang W, Du GH, Kokudo N. Targeting apoptosis pathways in cancer with magnolol and honokiol, bioactive constituents of the bark of Magnolia officinalis. *Drug discoveries & therapeutics* 2011, **5**(5)**:** 202-210.

161. Bai X, Cerimele F, Ushio-Fukai M, Waqas M, Campbell PM, Govindarajan B*, et al.* Honokiol, a small molecular weight natural product, inhibits angiogenesis in vitro and tumor growth in vivo. *The Journal of biological chemistry* 2003, **278**(37)**:** 35501-35507.

162. Watanabe K, Watanabe HY, Goto Y, Yamamoto N, Yoshizaki M. Studies on the active principles of magnolia bark. Centrally acting muscle relaxant activity of magnolol and honokiol. *Jpn J Pharmacol* 1975, **25**(5)**:** 605-607.

163. Konoshima T, Kozuka M, Tokuda H, Nishino H, Iwashima A, Haruna M*, et al.* Studies on inhibitors of skin tumor promotion, IX. Neolignans from Magnolia officinalis. *J Nat Prod* 1991, **54**(3)**:** 816-822.

164. Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. *Free Radic Biol Med* 2007, **43**(1)**:** 4-15.

165. Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radic Biol Med* 1997, **22**(5)**:** 749-760.

166. Son HJ, Lee HJ, Yun-Choi HS, Ryu JH. Inhibitors of nitric oxide synthesis and TNF-alpha expression from Magnolia obovata in activated macrophages. *Planta Med* 2000, **66**(5)**:** 469-471.

167. Lee J, Jung E, Park J, Jung K, Lee S, Hong S*, et al.* Anti-inflammatory effects of magnolol and honokiol are mediated through inhibition of the downstream pathway of MEKK-1 in NF-kappaB activation signaling. *Planta Med* 2005, **71**(4)**:** 338-343.

168. Nagase H, Ikeda K, Sakai Y. Inhibitory effect of magnolol and honokiol from Magnolia obovata on human fibrosarcoma HT-1080. Invasiveness in vitro. *Planta Med* 2001, **67**(8)**:** 705-708.

169. Yang SE, Hsieh MT, Tsai TH, Hsu SL. Down-modulation of Bcl-XL, release of cytochrome c and sequential activation of caspases during honokiol-induced apoptosis in human squamous lung cancer CH27 cells. *Biochemical pharmacology* 2002, **63**(9)**:** 1641-1651.

170. Chiu JH, Ho CT, Wei YH, Lui WY, Hong CY. In vitro and in vivo protective effect of honokiol on rat liver from peroxidative injury. *Life Sci* 1997, **61**(19)**:** 1961-1971.

171. Ou HC, Chou FP, Lin TM, Yang CH, Sheu WH. Protective effects of honokiol against oxidized LDL-induced cytotoxicity and adhesion molecule expression in endothelial cells. *Chem Biol Interact* 2006, **161**(1)**:** 1-13.

172. Arora S, Singh S, Piazza GA, Contreras CM, Panyam J, Singh AP. Honokiol: a novel natural agent for cancer prevention and therapy. *Current molecular medicine* 2012.

173. Cheng N, Xia T, Han Y, He QJ, Zhao R, Ma JR. Synergistic antitumor effects of liposomal honokiol combined with cisplatin in colon cancer models. *Oncology letters* 2011, **2**(5)**:** 957-962.

174. Lee JD, Lee JY, Baek BJ, Lee BD, Koh YW, Lee WS*, et al.* The inhibitory effect of honokiol, a natural plant product, on vestibular schwannoma cells. *The Laryngoscope* 2012, **122**(1)**:** 162-166.

175. Lee YJ, Lee YM, Lee CK, Jung JK, Han SB, Hong JT. Therapeutic applications of compounds in the Magnolia family. *Pharmacology & therapeutics* 2011, **130**(2)**:** 157-176.

176. Li Y, Huang W, Huang S, Du J, Huang C. Screening of anti-cancer agent using zebrafish: comparison with the MTT assay. *Biochemical and biophysical research communications* 2012, **422**(1)**:** 85-90.

177. Liu X, Han Y, Peng K, Liu Y, Li J, Liu H. Effect of traditional Chinese medicinal herbs on Candida spp. from patients with HIV/AIDS. *Advances in dental research* 2011, **23**(1)**:** 56-60.

178. Mannal PW, Schneider J, Tangada A, McDonald D, McFadden DW. Honokiol produces anti-neoplastic effects on melanoma cells in vitro. *Journal of surgical oncology* 2011, **104**(3)**:** 260-264.

179. Ponnurangam S, Mammen JM, Ramalingam S, He Z, Zhang Y, Umar S*, et al.* Honokiol in combination with radiation targets notch signaling to inhibit colon cancer stem cells. *Molecular cancer therapeutics* 2012, **11**(4)**:** 963-972.

180. Shen JL, Man KM, Huang PH, Chen WC, Chen DC, Cheng YW*, et al.* Honokiol and magnolol as multifunctional antioxidative molecules for dermatologic disorders. *Molecules* 2010, **15**(9)**:** 6452-6465.

181. Steinmann P, Walters DK, Arlt MJ, Banke IJ, Ziegler U, Langsam B*, et al.* Antimetastatic activity of honokiol in osteosarcoma. *Cancer* 2012, **118**(8)**:** 2117-2127.

182. Wang X, Duan X, Yang G, Zhang X, Deng L, Zheng H*, et al.* Honokiol crosses BBB and BCSFB, and inhibits brain tumor growth in rat 9L intracerebral gliosarcoma model and human U251 xenograft glioma model. *PloS one* 2011, **6**(4)**:** e18490.

183. Weng TI, Wu HY, Chen BL, Liu SH. Honokiol attenuates the severity of acute pancreatitis and associated lung injury via acceleration of acinar cell apoptosis. *Shock* 2012, **37**(5)**:** 478-484.

184. Chen XR, Lu R, Dan HX, Liao G, Zhou M, Li XY*, et al.* Honokiol: a promising small molecular weight natural agent for the growth inhibition of oral squamous cell carcinoma cells. *International journal of oral science* 2011, **3**(1)**:** 34-42.

185. Arora S, Bhardwaj A, Srivastava SK, Singh S, McClellan S, Wang B*, et al.* Honokiol arrests cell cycle, induces apoptosis, and potentiates the cytotoxic effect of gemcitabine in human pancreatic cancer cells. *PloS one* 2011, **6**(6)**:** e21573.

186. Chilampalli C, Guillermo R, Kaushik RS, Young A, Chandrasekher G, Fahmy H*, et al.* Honokiol, a chemopreventive agent against skin cancer, induces cell cycle arrest and apoptosis in human epidermoid A431 cells. *Exp Biol Med (Maywood)* 2011, **236**(11)**:** 1351-1359.

187. He Z, Subramaniam D, Ramalingam S, Dhar A, Postier RG, Umar S*, et al.* Honokiol radiosensitizes colorectal cancer cells: enhanced activity in cells with

mismatch repair defects. *American journal of physiology Gastrointestinal and liver physiology* 2011, **301**(5)**:** G929-937.

188. Ishikawa C, Arbiser JL, Mori N. Honokiol induces cell cycle arrest and apoptosis via inhibition of survival signals in adult T-cell leukemia. *Biochimica et biophysica acta* 2012, **1820**(7)**:** 879-887.

189. Jeong JJ, Lee JH, Chang KC, Kim HJ. Honokiol exerts an anticancer effect in T98G human glioblastoma cells through the induction of apoptosis and the regulation of adhesion molecules. *International journal of oncology* 2012.

190. Lin JW, Chen JT, Hong CY, Lin YL, Wang KT, Yao CJ*, et al.* Honokiol traverses the blood-brain barrier and induces apoptosis of neuroblastoma cells via an intrinsic bax-mitochondrion-cytochrome c-caspase protease pathway. *Neuro-oncology* 2012, **14**(3)**:** 302-314.

191. Marin GH, Mansilla E. Apoptosis induced by Magnolia Grandi fl ora extract in chlorambucil-resistant B-chronic lymphocytic leukemia cells. *Journal of cancer research and therapeutics* 2010, **6**(4)**:** 463-465.

192. Wang Y, Yang Z, Zhao X. Honokiol induces paraptosis and apoptosis and exhibits schedule-dependent synergy in combination with imatinib in human leukemia cells. *Toxicology mechanisms and methods* 2010, **20**(5)**:** 234-241.

193. Hoi CP, Ho YP, Baum L, Chow AH. Neuroprotective effect of honokiol and magnolol, compounds from Magnolia officinalis, on beta-amyloid-induced toxicity in PC12 cells. *Phytotherapy research : PTR* 2010, **24**(10)**:** 1538-1542.

194. Sheu ML, Chiang CK, Tsai KS, Ho FM, Weng TI, Wu HY*, et al.* Inhibition of NADPH oxidase-related oxidative stress-triggered signaling by honokiol suppresses high glucose-induced human endothelial cell apoptosis. *Free radical biology & medicine* 2008, **44**(12)**:** 2043-2050.

195. Tang X, Yao K, Zhang L, Yang Y, Yao H. Honokiol inhibits H(2)O(2)-induced apoptosis in human lens epithelial cells via inhibition of the mitogen-activated protein kinase and Akt pathways. *European journal of pharmacology* 2011, **650**(1)**:** 72-78.

196. Fried LE, Arbiser JL. Honokiol, a multifunctional antiangiogenic and antitumor agent. *Antioxidants & redox signaling* 2009, **11**(5)**:** 1139-1148.

197. Rajendran P, Li F, Shanmugam MK, Vali S, Abbasi T, Kapoor S*, et al.* Honokiol inhibits signal transducer and activator of transcription-3 signaling, proliferation, and survival of hepatocellular carcinoma cells via the protein tyrosine phosphatase SHP-1. *J Cell Physiol* 2012, **227**(5)**:** 2184-2195.

198. Yu C, Zhang Q, Zhang HY, Zhang X, Huo X, Cheng E*, et al.* Targeting the intrinsic inflammatory pathway: honokiol exerts proapoptotic effects through STAT3 inhibition in transformed Barrett's cells. *American journal of physiology Gastrointestinal and liver physiology* 2012, **303**(5)**:** G561-569.

199. Leeman-Neill RJ, Cai Q, Joyce SC, Thomas SM, Bhola NE, Neill DB, *et al.* Honokiol inhibits epidermal growth factor receptor signaling and enhances the antitumor effects of epidermal growth factor receptor inhibitors. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2010, **16**(9)**:** 2571-2579.

200. Liu SH, Wang KB, Lan KH, Lee WJ, Pan HC, Wu SM, *et al.* Calpain/SHP-1 interaction by honokiol dampening peritoneal dissemination of gastric cancer in nu/nu mice. *PloS one* 2012, **7**(8)**:** e43711.

201. Liu SH, Shen CC, Yi YC, Tsai JJ, Wang CC, Chueh JT, *et al.* Honokiol inhibits gastric tumourigenesis by activation of 15-lipoxygenase-1 and consequent inhibition of peroxisome proliferator-activated receptor-gamma and COX-2-dependent signals. *British journal of pharmacology* 2010, **160**(8)**:** 1963-1972.

202. Andree HA, Reutelingsperger CP, Hauptmann R, Hemker HC, Hermens WT, Willems GM. Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. *The Journal of biological chemistry* 1990, **265**(9)**:** 4923-4928.

203. Lang F, Gulbins E, Lerche H, Huber SM, Kempe DS, Foller M. Eryptosis, a window to systemic disease. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2008, **22**(5-6)**:** 373-380.

204. Schiffl H, Weidmann P, Weiss M, Massry SG. Dialysis treatment of acute chromium intoxication and comparative efficacy of peritoneal versus hemodialysis in chromium removal. *Miner Electrolyte Metab* 1982, **7**(1)**:** 28-35.

205. Chernecky CC BB. Laboratory tests and diagnostic procedures. 1997.

206. Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM. Role of Ca2+-activated K+ channels in human erythrocyte apoptosis. *Am J Physiol Cell Physiol* 2003, **285**(6)**:** C1553-C1560.

207. Wise SS, Holmes AL, Wise JP, Sr. Hexavalent chromium-induced DNA damage and repair mechanisms. *Rev Environ Health* 2008, **23**(1)**:** 39-57.

208. Yao H, Guo L, Jiang BH, Luo J, Shi X. Oxidative stress and chromium(VI) carcinogenesis. *J Environ Pathol Toxicol Oncol* 2008, **27**(2)**:** 77-88.

209. Brand VB, Sandu CD, Duranton C, Tanneur V, Lang KS, Huber SM, *et al.* Dependence of Plasmodium falciparum in vitro growth on the cation permeability of the human host erythrocyte. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2003, **13**(6)**:** 347-356.

210. Lang F, Gulbins E, Lang PA, Zappulla D, Foller M. Ceramide in suicidal death of erythrocytes. *Cell Physiol Biochem* 2010, **26**(1)**:** 21-28.

211. Lang PA, Kempe DS, Tanneur V, Eisele K, Klarl BA, Myssina S*, et al.* Stimulation of erythrocyte ceramide formation by platelet-activating factor. *Journal of cell science* 2005, **118**(Pt 6)**:** 1233-1243.

212. Kornhuber J, Tripal P, Reichel M, Muhle C, Rhein C, Muehlbacher M*, et al.* Functional Inhibitors of Acid Sphingomyelinase (FIASMAs): a novel pharmacological group of drugs with broad clinical applications. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2010, **26**(1)**:** 9-20.

213. Burdmann EA, Woronik V, Prado EB, Abdulkader RC, Saldanha LB, Barreto OC*, et al.* Snakebite-induced acute renal failure: an experimental model. *The American journal of tropical medicine and hygiene* 1993, **48**(1)**:** 82-88.

214. Andrews DA, Low PS. Role of red blood cells in thrombosis. *CurrOpinHematol* 1999, **6**(2)**:** 76-82.

215. Closse C, Dachary-Prigent J, Boisseau MR. Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. *BrJ Haematol* 1999, **107**(2)**:** 300-302.

216. Chung SM, Bae ON, Lim KM, Noh JY, Lee MY, Jung YS*, et al.* Lysophosphatidic acid induces thrombogenic activity through phosphatidylserine exposure and procoagulant microvesicle generation in human erythrocytes. *ArterioscIerThrombVascBiol* 2007, **27**(2)**:** 414-421.

217. Zwaal RF, Comfurius P, Bevers EM. Surface exposure of phosphatidylserine in pathological cells. *Cell Mol Life Sci* 2005, **62**(9)**:** 971-988.

218. Lang F, Gulbins E, Lerche H, Huber SM, Kempe DS, F"ller M. Eryptosis, a window to systemic disease. *Cell Physiol Biochem* 2008, **22** (5-6)**:** 373-380.

219. Bhavsar SK, Bobbala D, Xuan NT, Foller M, Lang F. Stimulation of suicidal erythrocyte death by alpha-lipoic acid. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2010, **26**(6)**:** 859-868.

220. Ghashghaeinia M, Toulany M, Saki M, Bobbala D, Fehrenbacher B, Rupec R*, et al.* The NFkB pathway inhibitors Bay 11-7082 and parthenolide induce programmed cell death in anucleated Erythrocytes. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2011, **27**(1)**:** 45-54.

221. Kempe DS, Lang PA, Duranton C, Akel A, Lang KS, Huber SM*, et al.* Enhanced programmed cell death of iron-deficient erythrocytes. *FASEB J* 2006, **20**(2)**:** 368-370.

222. He Y, Imam SZ, Dong Z, Jankovic J, Ali SF, Appel SH*, et al.* Role of nitric oxide in rotenone-induced nigro-striatal injury. *J Neurochem* 2003, **86**(6)**:** 1338-1345.

223. Lima MM, Andersen ML, Reksidler AB, Ferraz AC, Vital MA, Tufik S. Paradoxical sleep deprivation modulates tyrosine hydroxylase expression in the nigrostriatal pathway and attenuates motor deficits induced by dopaminergic depletion. *CNS Neurol Disord Drug Targets* 2012, **11**(4)**:** 359-368.

224. Shinomol GK, Mythri RB, Srinivas Bharath MM, Muralidhara. Bacopa monnieri extract offsets rotenone-induced cytotoxicity in dopaminergic cells and oxidative impairments in mice brain. *Cell Mol Neurobiol* 2012, **32**(3)**:** 455-465.

225. Wu YN, Johnson SW. Dopamine oxidation facilitates rotenone-dependent potentiation of N-methyl-D-aspartate currents in rat substantia nigra dopamine neurons. *Neuroscience* 2011, **195:** 138-144.

226. Zaitone SA, Abo-Elmatty DM, Shaalan AA. Acetyl-L-carnitine and alpha-lipoic acid affect rotenone-induced damage in nigral dopaminergic neurons of rat brain, implication for Parkinson's disease therapy. *Pharmacol Biochem Behav* 2012, **100**(3)**:** 347-360.

227. Marella M, Seo BB, Nakamaru-Ogiso E, Greenamyre JT, Matsuno-Yagi A, Yagi T. Protection by the NDI1 gene against neurodegeneration in a rotenone rat model of Parkinson's disease. *PLoS One* 2008, **3**(1)**:** e1433.

228. Bookchin RM, Ortiz OE, Lew VL. Activation of calcium-dependent potassium channels in deoxygenated sickled red cells. *ProgClinBiolRes* 1987, **240:** 193-200.

229. Lang F, Gulbins E, Lang PA, Zappulla D, Foller M. Ceramide in suicidal death of erythrocytes. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2010, **26**(1)**:** 21-28.

230. Lang PA, Schenck M, Nicolay JP, Becker JU, Kempe DS, Lupescu A*, et al.* Liver cell death and anemia in Wilson disease involve acid sphingomyelinase and ceramide. *Nature medicine* 2007, **13**(2)**:** 164-170.

231. Walter S, Fassbender K. Spingolipids in Multiple Sclerosis. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2010, **26**(1)**:** 49-56.

232. Arboleda G, Huang TJ, Waters C, Verkhratsky A, Fernyhough P, Gibson RM. Insulin-like growth factor-1-dependent maintenance of neuronal metabolism through the phosphatidylinositol 3-kinase-Akt pathway is inhibited by C2-ceramide in CAD cells. *Eur J Neurosci* 2007, **25**(10)**:** 3030-3038.

233. de la Monte SM. Insulin resistance and Alzheimer's disease. *BMB Rep* 2009, **42**(8)**:** 475-481.

234. Kuebler WM, Yang Y, Samapati R, Uhlig S. Vascular barrier regulation by PAF, ceramide, caveolae, and NO - an intricate signaling network with discrepant effects in the pulmonary and systemic vasculature. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2010, **26**(1)**:** 29-40.

235. Becker KA, Grassme H, Zhang Y, Gulbins E. Ceramide in Pseudomonas aeruginosa infections and cystic fibrosis. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2010, **26**(1)**:** 57-66.

236. Dhami R, He X, Schuchman EH. Acid sphingomyelinase deficiency attenuates bleomycin-induced lung inflammation and fibrosis in mice. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2010, **26**(4-5)**:** 749-760.

237. Lang E, Qadri SM, Lang F. Killing me softly - suicidal erythrocyte death. *Int J Biochem Cell Biol* 2012, **44**(8)**:** 1236-1243.

238. Borst O, Abed M, Alesutan I, Towhid ST, Qadri SM, Foller M*, et al.* Dynamic adhesion of eryptotic erythrocytes to endothelial cells via CXCL16/SR-PSOX. *Am J Physiol Cell Physiol* 2012, **302**(4)**:** C644-C651.

239. Gallagher PG, Chang SH, Rettig MP, Neely JE, Hillery CA, Smith BD*, et al.* Altered erythrocyte endothelial adherence and membrane phospholipid asymmetry in hereditary hydrocytosis. *Blood* 2003, **101**(11)**:** 4625-4627.

240. Wood BL, Gibson DF, Tait JF. Increased erythrocyte phosphatidylserine exposure in sickle cell disease: flow-cytometric measurement and clinical associations. *Blood* 1996, **88**(5)**:** 1873-1880.

241. Li X, Becker KA, Zhang Y. Ceramide in redox signaling and cardiovascular diseases. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2010, **26**(1)**:** 41-48.

242. Lang PA, Kaiser S, Myssina S, Wieder T, Lang F, Huber SM. Role of Ca2+-activated K+ channels in human erythrocyte apoptosis. *American journal of physiology Cell physiology* 2003, **285**(6)**:** C1553-1560.

243. Calderon-Salinas JV, Munoz-Reyes EG, Guerrero-Romero JF, Rodriguez-Moran M, Bracho-Riquelme RL, Carrera-Gracia MA*, et al.* Eryptosis and oxidative damage in type 2 diabetic mellitus patients with chronic kidney disease. *Mol Cell Biochem* 2011, **357**(1-2)**:** 171-179.

244. Nicolay JP, Schneider J, Niemoeller OM, Artunc F, Portero-Otin M, Haik G, Jr.*, et al.* Stimulation of suicidal erythrocyte death by methylglyoxal. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2006, **18**(4-5)**:** 223-232.

245. Lang PA, Beringer O, Nicolay JP, Amon O, Kempe DS, Hermle T*, et al.* Suicidal death of erythrocytes in recurrent hemolytic uremic syndrome. *J Mol Med (Berl)* 2006, **84**(5)**:** 378-388.

246. Kempe DS, Akel A, Lang PA, Hermle T, Biswas R, Muresanu J*, et al.* Suicidal erythrocyte death in sepsis. *J Mol Med (Berl)* 2007, **85**(3)**:** 273-281.

247. Lang PA, Kasinathan RS, Brand VB, Duranton C, Lang C, Koka S*, et al.* Accelerated clearance of Plasmodium-infected erythrocytes in sickle cell trait and annexin-A7 deficiency. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2009, **24**(5-6)**:** 415-428.

248. Siraskar B, Ballal A, Bobbala D, Foller M, Lang F. Effect of amphotericin B on parasitemia and survival of plasmodium berghei-infected mice. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2010, **26**(3)**:** 347-354.

249. Bobbala D, Alesutan I, Foller M, Huber SM, Lang F. Effect of anandamide in Plasmodium Berghei-infected mice. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology* 2010, **26**(3)**:** 355-362.

250. Birka C, Lang PA, Kempe DS, Hoefling L, Tanneur V, Duranton C*, et al.* Enhanced susceptibility to erythrocyte "apoptosis" following phosphate depletion. *Pflugers Arch* 2004, **448**(5)**:** 471-477.

251. Zappulla D. Environmental stress, erythrocyte dysfunctions, inflammation, and the metabolic syndrome: adaptations to CO2 increases? *J Cardiometab Syndr* 2008, **3**(1)**:** 30-34.

252. Chen F, Wang T, Wu YF, Gu Y, Xu XL, Zheng S*, et al.* Honokiol: a potent chemotherapy candidate for human colorectal carcinoma. *World journal of gastroenterology : WJG* 2004, **10**(23)**:** 3459-3463.

253. Jiang QQ, Fan LY, Yang GL, Guo WH, Hou WL, Chen LJ*, et al.* Improved therapeutic effectiveness by combining liposomal honokiol with cisplatin in lung cancer model. *BMC cancer* 2008, **8:** 242.

254. Pandolfi A, Di Pietro N, Sirolli V, Giardinelli A, Di Silvestre S, Amoroso L*, et al.* Mechanisms of uremic erythrocyte-induced adhesion of human monocytes to cultured endothelial cells. *J Cell Physiol* 2007, **213**(3)**:** 699-709.

255. Wood BL, Gibson DF, Tait JF. Increased erythrocyte phosphatidylserine exposure in sickle cell disease: flow-cytometric measurement and clinical associations. *Blood* 1996, **88**(5)**:** 1873-1880.

256. Lupescu A, Jilani K, Zelenak C, Zbidah M, Qadri SM, Lang F. Hexavalent chromium-induced erythrocyte membrane phospholipid asymmetry.

Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine 2012, **25**(2)**:** 309-318.

257. Lupescu A, Jilani K, Zbidah M, Lang F. Induction of apoptotic erythrocyte death by rotenone. *Toxicology* 2012, **300**(3)**:** 132-137.

258. Zbidah M, Lupescu A, Jilani K, Fajol A, Michael D, Qadri SM*, et al.* Apigenin-nduced suicidal erythrocyte death. *Journal of agricultural and food chemistry* 2012, **60**(1)**:** 533-538.

259. Zbidah M, Lupescu A, Herrmann T, Yang W, Foller M, Jilani K*, et al.* Effect of honokiol on erythrocytes. *Toxicology in vitro : an international journal published in association with BIBRA* 2013, **27**(6)**:** 1737-1745.

7. Zusammenfassung

Eryptose, der suizidale Zelltod von Erythrozyten, ist durch Zellschrumpfung, Ausknospung der Zellmembran („Blebbing“) sowie durch die Exposition von Phosphatidylserin an der Zelloberfläche, als Folge der Umlagerung der Phospholipide der Zellmembran („Scrambling“), gekennzeichnet.

Eine erhöhte intrazelluläre Ca^{2+}- Aktivität löst Eryptose aus, wobei dieser zusätzliche Ca^{2+}-Einstrom durch den erythrozytären Ca^{2+} durchlässigen Kationenkanal erklärt wird. Phosphatidylserin exponierende Erythrozyten werden schnell aus dem zirkulierenden Blut eliminiert, da Makrophagen das exponierte Phosphatidylserin als Todessignal der Erythrozyten erkennen und deren Abbau durch Phagozytose einleiten.

Eine übermäßige Eryptose spielt eine entscheidende physiologische Rolle bezüglich des Schicksals von Erythrozyten. Deshalb trägt eine erhöhte Eryptose möglicherweise zu Anämie bei, wenn der Verlust von Erythrozyen nicht durch die Bildung neuer Erythrozyten ausgeglichen wird.

In der industrialisierten Gesellschaft führen wohn-, gewerbliche und industrielle Quellen täglich zu einem Eintrag verschiedener Formen von Schadstoffen in die Umwelt. Jedoch hat eine Akkumulation von gefährlichen Substanzen eine potentiell schlimmere Wirkung auf die menschliche Gesundheit und deren Umgebung.

In der ersten Studie wurde untersucht, ob Chrom (VI) als Umweltschadstoff Eryptose auslöst, welche ein möglicher Grund für Anämie ist, und wenn ja, ob Chrom (VI) Eryptose mit den für diesen Vorgang charakteristischen Veränderungen der cytosolischen Ca^{2+}-Aktivität, der Ceramidbildung und/ oder des zytosolischen ATPs induziert.

Der Annexin-V-Bindung im FACS zufolge, stimulierte Chrom (VI) (10 uM) die Phosphatidylserin-Exposition, wobei dieser Effekt bei Abwesenheit von Ca^{2+}

signifikant abgeschwächt war. Bei Betrachten der Fluo3 Fluoreszenz, erhöhte Chrom (VI) die intrazelluläre Ca^{2+}-Aktivität und verminderte die cytosolische ATP Konzentration, wobei beide wichtige Faktoren für die Stimulation der Eryptose sind. Die Ceramidbildung wurde jedoch durch das Vorhandensein von Chrom nicht beeinflusst.

Schlussfolgernd lässt sich sagen, dass diese Beobachtungen zur Aufklärung der Auswirkung von Chrom (VI) auf Zellen ohne Zellkern beitragen, wobei Chrom (VI) den Ca^{2+}-Einstrom und den ATP-Abbau in Erythrozyten stimuliert, was wiederum zur Umlagerung der Phospholipide der Zellmembran (”Scrambling”) und zum Zellschrumpfen, ähnlich wie bei kernhaltigen Zellen, führt.

In der zweiten Studie wurde untersucht, ob das Pestizid Rotenon , ein Verursacher der Parkinson-Krankheit, Apoptose stimuliert, wobei die zugrunde liegenden Mechanismen aufgeklärt werden sollten. Nach Exposition menschlicher Erythrozyten mit 1 uM Rotenon, führte dies zur Umlagerung der Membran und zum Zellschrumpfen. Rotenon erhöhte ebenfalls die intrazelluläre Ca^{2+} - Konzentration durch Aktivierung Ca^{2+} durchlässiger Kationenkanäle, was wiederum zur Aktivierung Ca^{2+} sensitiver K^{+} Kanäle und zum Austritt von KCl und Wasser und somit zum Zellschrumpfen beiträgt.

Die Wirkung von Rotenon wird jedoch nicht vollständig durch das Entfernen von extrazellulärem Ca^{2+} aufgehoben, was auf die Beteiligung eines weiteren Mechanismus im Zusammenhang mit der Stimulation der Ceramid-Bildung deutet. Ceramid sensibilisiert Erythrozyten in Bezug auf den Umlagerungseffekt von Ca^{2+} und stimuliert daher die Umlagerung der Membran in Erythrozyten.

Die dritte und vierte Studie untersuchten die Beteiligung von zwei natürlichen Substanzen in der Regulation der Eryptose. Beide sind chemische Substanzen, die von einem lebenden Organismus erzeugt werden und eine gezielte pharmakologische Wirkung haben. Um die Wirkung von Apigenin und Honokiol zu untersuchen, wurde Fluo3 für die Bestimmung der intrazellulären

Ca^{2+} Konzentration eingesetzt, das Vorwärtsstreulicht für das Zellvolumen und die Annexin V-Bindung zum Nachweis von Phosphatidylserin.

Apigenin und Honokiol sind Teil einer Vielzahl von Xenobiotika, die Eryptose auslösen. Apigenin und Honokiol sind teilweise wirksam durch die Stimulation des Ca^{2+} Einstroms mit anschließender Aktivierung nicht-selektiver Kationen-Kanäle. Weiterhin führen sie zur Umlagerung der Zellmembran durch Ceramidbildung, wobei bekannt ist, dass Ceramid zur Apoptose kernhaltiger Zellen beiträgt. Die Wirkung von Apigenin auf die Verminderung der zytosolischen ATP-Konzentration könnte durch eine verbesserte ATP Nutzung oder durch eine eingeschränkte Bildung von ATP durch Interferenz mit dem glykolytischen Fluss erklärt werden. Insgesamt zeigt die Studie die Bedeutung einiger Xenobiotika, die direkt oder indirekt den suizidalen Erythrozytentod oder deren Überleben regulieren.

8. Author's declaration

I hereby declare that this thesis is my own work and effort, and is a record of work performed by me under the supervision of Professor Florian Lang, except where I will state as following:

- Confocal images of phosphatidylserine-exposing erythrocytes with or without chromium (VI) treatment was performed By Dr. Christine Zelenak and cells were prepared by me (Figure 13).
- Confocal images of phosphatidylserine-exposing erythrocytes with or without Honokiol were performed By Dr.Wenting Yang. and cells were prepared by me (Figure 30).
- The percentage of annexin V-binding erythrocytes after a 48 h treatment with chromium (VI), Rotenone, Apigenin and honokiol in Ringer solution in the presence and absence of calcium was measured by Kashif Jilani and cells were prepared by me (Figures 14C,19, 24,31).
- Cytosolic ATP concentration measurement as a result of apigenin effect on erythrocyte was performed by Abul Fajol and cells were prepared by me (Figure 26).

I want to commend here on teamwork, which reflects the spirit of constructive work and helps in the blending of diverse expertise and concerted efforts for the success of any outstanding achievement.

Mohanad Zbidah

(2013)

9. ACKNOWLEDGEMENTS

I wish to express my most sincere gratitude and appreciation to my Supervisor ***Professor Florian Lang,*** the one who has guided me into the fascinating world of research. I wish to express my thanks for his guidance, patience and encouragement throughout the development of the project. I gratefully acknowledge the enthusiastic supervision of ***Dr. Syed Qadri*** for his positive and constructive advice and support throughout and for always being helpful.

The work would not have been possible without the support and the encouragement of my colleagues, ***Dr. Adrian Lupescu,*** and very special thanks to ***M.Phil. Kashif Jilani*** for his generosity and kindness by helping, also to ***Dr. Christine Zelenak, Dr. Nazneen shaik, Ms. Abul Fajol.***

Most importantly, thanks and love to my wonderful parents ***Bakri*** and ***khadija***. watched me from a distance while I worked towards my degree. They have wished so long for this thesis, waited and supported me throughout these years; to them I dedicate this work.

Deeply from my heart with love and faith, I would like to thank my beloved brothers **Husam** and **Yaman** for their encouragement and supporting throughout my life.

To my wife, If at all I am successful in life, it is not sheer luck or my brilliance, it is all my loving wife Rama's support and endless love. She takes care of me, starting from what I eat what I wear and so on and so forth. Whether I was working on the Thesis or not, she always took care of the kids, her extracurricular activities amidst of her busy work schedule. Thanks ***Rama***, I am very fortunate to have you in my life. To my kids ***Maya and Yazan*** – they are the source of my energy and happiness.

Finally I would like to thank my friends : ***Dr. Buni, Dr. alkhaled, Dr. Shehata, Dr. Hindawi, Dr. Ebied, Dr. Abdelraouf*** for making my stay in Tübingen a pleasant and memorable one .

Mohanad Zbidah

(2014)