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The inhibitory effect of sodium nitroprusside and N-acetyl-L-cysteine on desipramine-induced eryptosis

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ACER2	Alkaline Ceramidase 2
Ach	Acetylcholine
AchE	Acetylcholinesterase
AchR	Acetylcholine receptor
Ax-WB	Annexin wash buffer
ASMase	Acid sphingomyelinase
Bcl-2	B cell CLL/lymphoma-2
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
$[Ca^{2+}]_i$	Intracellular free Ca^{2+} concentration
CADs	Cationic amphiphilic drugs
Cat	Catalase
CER	Ceramide
Cl^-	Chloride
CNS	Central nervous system
CO_2	Carbon dioxide
CYP2C19	Cytochrome 450 2C 19
CYP2D6	Cytochrome P450 2D6
Des	Desipramine hydrochloride
DMSO	Dimethyl sulfoxide
EC	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
eNOS	Endothelial nitric oxide synthase
F6P	Fructose-6-phosphate
FACS	Fluorescence-activated cell sorter
G3P	Glyceraldehyde-3-phosphate
G6P	Glucose-6-phosphate

G6PD	Glucose-6-phosphate dehydrogenase
G6PDH	6-phosphate dehydrogenase
GBD	Global burden of disease
GPx	Glutathione peroxidase
GR	Glutathione reductase
Grx	GSH-glutaredoxin
GS	Glutathione synthase
GSH	Glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
Hct	Hematocrit
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRBC	Human erythrocyte
IC_{50}	Half maximal inhibitory concentration
K^+	Potassium
$[K^+]_i$	Intracellular potassium concentration
KCl	Potassium chloride
L-Cys	L-cysteine
Mg^{2+}	Magnesium
$MgSO_4$	Magnesium sulfate
Na^+	Sodium
NAC	N-acetyl-L-cysteine
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NaOH	Sodium hydroxide
NE	Norepinephrine
NEM	N-Ethylmaleimide
NET	Norepinephrine transporter
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase

O_2	Oxygen
$O_2^{\bullet-}$	Superoxide anion
PGE_2	Prostaglandin E_2
PPP	Pentose phosphate pathway
PS	Phosphatidylserine
ROS	Reactive oxygen species
Ru5P	Ribulose-5-phosphate
RVD	Regulatory volume decrease
RVI	Regulatory volume increase
S-1-P	Sphingosine -1-P
SER	Serotonin
SERT	Serotonin transporter
SM	Sphingomyelin
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SPH	Sphingosine
TCA	Tricyclic antidepressant
Trx	Thioredoxin
WBC	White blood cell
XO	Xanthine oxidase
γ-GCS	γ-glutamylcysteine synthetase
6PGDH	6-phosphogluconate dehydrogenase
6PGL	Phosphogluconolactonase

1 Introduction

1.1 Depression

Depression, the most common mental illness, is a kind of mood disorder, which leads to a constant feeling of fatigue, sadness, sleep disturbance, and loss of interest (Uher et al., 2014, Rakel, 1999). The incidence of depression is very high and tends to increase yearly. According to the Global Burden of Disease (GBD) 1990 epidemiological study, the incidence of depression is 29 per 100,000 women and 16 per 100,000 men per year (Murray and Lopez, 1996), while GBD 2000 epidemiological data shows 49 per 100,000 women and 31 per 100,000 men per year (Ustun et al., 2004).

The burden of disease associated with depression is enormous, like other diseases that commonly cause disability and death, depression is no less dangerous than heart disease and stroke, and it imposes a huge economic burden and suffering on families and society every year, and arguably, the damage caused by depression may even be greater. It imposes a non-fatal burden that accounts for nearly 12 % of all years lived with disability worldwide (Ustun et al., 2004). In the latest statistical survey data from the World Health Organization, by 2017, approximately 322 million people worldwide were suffering from depression, representing 4.4% of the global population. And globally, depression is ranked as the largest contributor to non-fatal diseases (Organization, 2017). However, due to shame, inadequate mental health resources, and lack of effective treatments, depression is undiagnosed and untreated (Smith and De Torres, 2014), and the prevalence of depression may be worse than the statistics.

For the treatment of depression, there were no effective antidepressants at the beginning. It was not until the 1950s that two major antidepressants, Iproniazid (monoamine oxidase inhibitor) and Imipramine (tricyclic

antidepressants), were discovered for the first time in human history (Hindmarch, 2002). Since then, the "monoamine theory of depression" concept was gradually developed and dominated the neurochemical system hypothesis of antidepressants (Van Praag, 2001).

1.2 Monoamine theory of depression

Of all the studies regarding the classic "monoamine theory of depression", two hypotheses are the most representative and controversial. One was “the catecholamine hypothesis of affective disorders: a review of supporting evidence” published in the American Journal of Psychiatry by American psychiatrist Joseph Schildkraut, while the other was "The biochemistry of affective disorders" published in the British Journal of Psychiatry by British psychiatrist Alec Coppen (Mulinari, 2012). The former proposed that the alterations of catecholamine levels in the brain, particularly norepinephrine (NE) depletion, may be responsible for the alteration of emotional states (Schildkraut, 1965). However, the latter attributed depression to serotonin (SER) deficiency rather than catecholamine deficiency (Coppen, 1967). Although these two types of hypotheses express their respective views, these were collectively referred to as the monoamine theory of depression since both catecholamines and serotonin belong to the monoamines (Mulinari, 2012). Since then, most therapeutic drug development and application for depression were based on the monoamine theory.

1.3 Desipramine

Desipramine ([10,11-Dihydro-5-[3-(methylamino)propyl]-5H-dibenz[b,f] azepine monohydrochloride]; Des), an amine tricyclic antidepressant (TCA) used primarily for the treatment of depression (Guemei et al., 2008) has also been used for the treatment of attention deficit hyperactivity disorder, and neuropathic pain (Hearn et al., 2014).

According to the monoamine theory of depression, depression may be caused by dysfunction that leads to decreased monoamine availability in the central nervous system (CNS). Imipramine is a TCA derived from benzodiazepines and, like all TCAs, is the prototype of a non-selective monoamine uptake inhibitor (Ban, 2001). Imipramine is metabolized primarily by mono-N-demethylation and di-N-demethylation, which are catalyzed by hepatic cytochrome 450 2C 19 (CYP2C19), to form the active metabolite Des (Nguyen et al., 2016) (**Figure 1**).

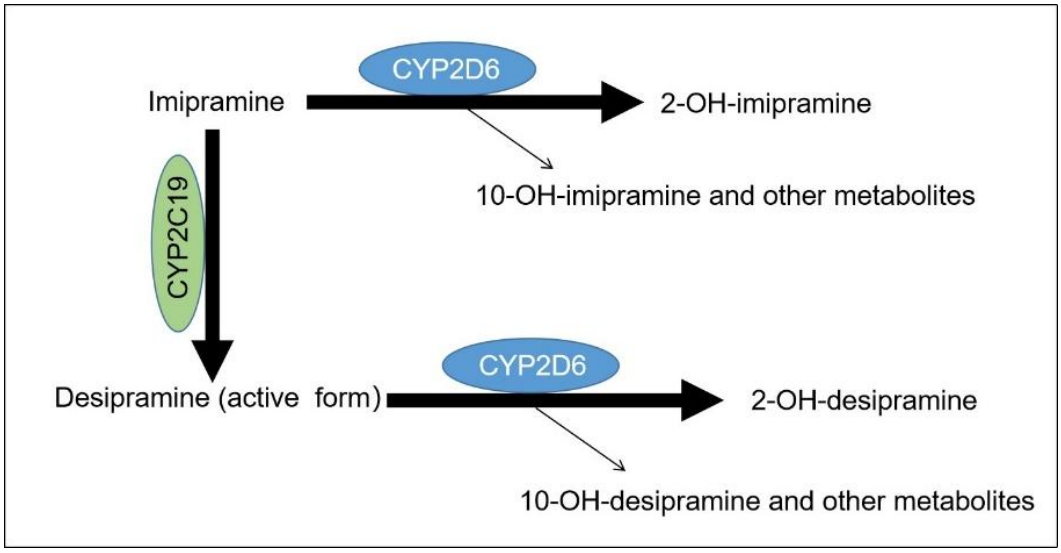


Figure 1: Metabolism of imipramine and desipramine in humans. Both imipramine and its derivative desipramine are metabolized by CYP2D6, mostly to 2-OH-imipramine/-desipramine, respectively and to a small extent to 10-OH-imipramine/-desipramine and further metabolites. CYP2D6: Cytochrome P450 2D6, CYP2C19: Cytochrome P450 2C19.

As the main metabolite of imipramine, Des also has the function of inhibiting the uptake of NE and SER (Maan et al., 2021). After oral administration, Des is barely absorbed in the stomach, and approximately 95% of Des is absorbed as it passes through the alkaline environment of the small intestine (Gram and Christiansen, 1975). After absorption by the body, Des can regulate appetite, emotion, mood, and sleep in humans by directly inhibiting the sodium- and chloride ion-dependent neurotransmitter transporters of SER (SERT) and NE (NET) in the presynaptic membrane (Zhou et al., 2007), thereby inhibiting the reuptake of NE and SER into the presynaptic neuron (Torres et al., 2003,

Iversen, 2006), which terminate neuronal transmission in the CNS through reuptake mechanisms (Schloss and Williams, 1998) (**Figure 2**). Eventually, Des is metabolized through cytochrome P450 2D6 (CYP2D6) by two metabolic pathways, the main metabolic pathway and the alternative metabolic pathway. The former metabolizes Des to the inactive metabolite 2-OH-Des while the latter is metabolized Des to 10-OH-Des and demethylation to didesipramine further (Dahl et al., 1993, Brøsen et al., 1993).

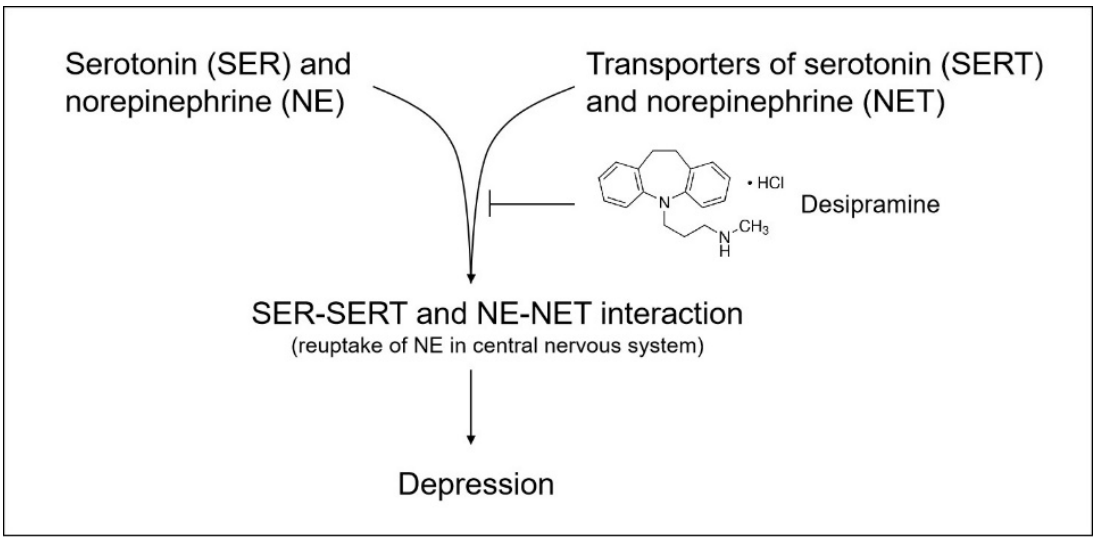


Figure 2: Serotonin and norepinephrine reuptake in the brain and the antidepressant mechanism of desipramine. TCAs among them also desipramine have at least two primary targets, a) serotonin and norepinephrine transporters (SERT, NET) (Zhou et al., 2007). and b) acidic sphingomyelinase (ASMase) (Hurwitz et al., 1994, Carpinteiro et al., 2020). Desipramine interaction with NET preferentially increase norepinephrine (NE) transmission by inhibiting NE reuptake, thus relieving depressive symptoms (Hyman and Nestler, 1996).

1.4 High volume distribution of desipramine

The basic-lipophilic Des shows a high volume of distribution (Minder et al., 1994), meaning most of the drug is outside the plasma compartment. Des shows an extremely high binding affinity to human muscle tissue (Fichtl and Kurz, 1978), lungs, liver, kidneys (Clausen and Bickel, 1993, Morin et al., 1984), brain (Fišar et al., 1996) and also has a high erythrocyte/plasma ratio (Linnoila et al., 1978). Comparison between erythrocytes and ghosts indicated a high enrichment of imipramine and Des in the erythrocyte plasma membrane. Of note, enrichment of imipramine and Des in the brain

membrane is much higher than in the erythrocytes membrane (Fišar et al., 1996). This suggests that tissue distribution, brain membrane/Des and erythrocyte/Des (antidepressant) ratio more likely correlate with the outcome of treatment than the total drug concentration in plasma.

1.5 Cell membrane and intracellular distribution of desipramine

Des localization/distribution is not only restricted to the cell membrane. Moreover, Des influences the functions of subcellular compartments, e.g. lysosomes (Kölzer et al., 2004, Kuzu et al., 2017, Allemailem et al., 2021), mitochondria (Luo et al., 2014, Corda et al., 2001, Levitsky et al., 2020, Abdel-Razaq et al., 2011, Chan et al., 2020, Hroudova and Fisar, 2010, Hroudová and Fišar, 2011), endoplasmic reticulum (Ma et al., 2011, Mao et al., 2019) and nucleus (Pallis et al., 2001), for more details see **Figure 3**.

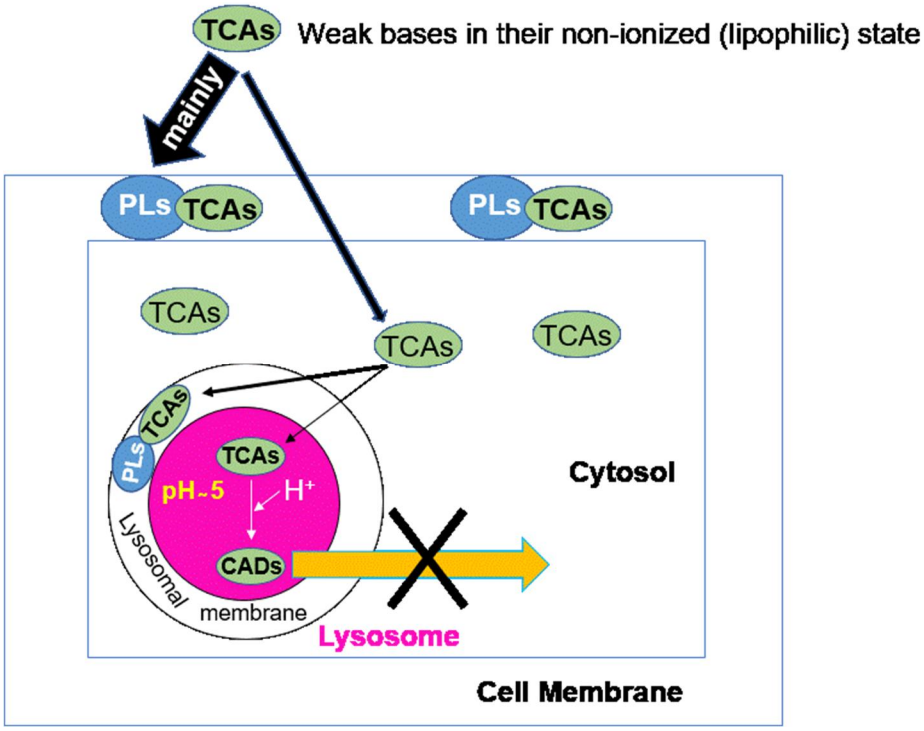


Figure 3: Desipramine localization/distribution is not only restricted to the cell membrane. Both non-specific binding to membrane phospholipids (PLs) as well as ion-trapping inside cytoplasm and acidic cellular compartments (TCAs + H^+ → CAD) contribute to desipramine uptake (Daniel et al., 1995, Kuzu et al., 2017).

1.6 Pharmacokinetics of desipramine

The half-life of Des is 14 to 62 h, with peak blood concentration occurring 4-6 h after administration. The binding rate of Des to plasma proteins is ranging from 73% to 91%, and the Des in plasma is ranging from 9% to 27% (Foye, 2008, Sallee and Pollock, 1990). Autopsies of individuals who died from Des overdose have detected plasma Des concentrations as low as 500 ng/ml (Robinson et al., 1974). The clinically recommended dose of Des is 50-300 mg/d (adults) with plasma concentrations of 125-300 ng/ml (Amitai and Frischer, 2004). Caution and close supervision are needed for the administration of Des to toddlers. Studies have shown that exposure to Des at less than 5 mg/kg is probably safe, however, it is lethal when the dose exceeds 15 mg/kg (Rosenbaum and Kou, 2005). Des affects a variety of ion channels, e.g. Sodium (Na^+) channels with half maximal inhibitory concentration (IC_{50}) of 1.52 µM and L-type Ca^{2+} channels with IC_{50} of 1.71 µM (Mirams et al., 2011).

1.7 Some common targets of desipramine in nucleated cells and human erythrocytes

Caspase-3 has a pro-apoptotic function in hRBCs (Mandal et al., 2002, Maellaro et al., 2013). An overwhelming number of publications show a direct correlation between agonists (e.g. etoposide or cathepsin-D)-mediated acid sphingomyelinase (ASMase) activation, the resulting ceramide generation, activation of caspase-3 and -9 and ultimate induction of apoptosis in nucleated cells (Sawada et al., 2000, Heinrich et al., 2004). Des by itself activates the pro-apoptotic caspase-3 and diminishes the expression of the anti-apoptotic molecule B cell CLL/lymphoma-2 (Bcl-2), thus promoting the apoptotic machinery (Kinjo et al., 2010). Several studies report ASMase-mediated apoptosis occurring via caspase-3 activation and Bcl-2

downregulation (Ion et al., 2006, Ma et al., 2011). Therefore, it is not surprising that Des, as a highly specific ASMase inhibitor (Hurwitz et al., 1994), exhibits anti-apoptotic properties in combination with ASMase activators. Caspase-3 is not the only common target of Des in nucleated cells and hRBCs. Sodium-hydrogen exchanger-1 (NHE-1), nitric oxide synthase (NOS) and acetylcholine receptor (AchR) are also among them. Other common targets of Des are not excluded. NHE-1 is a major regulator of intracellular pH ($[pH]_i$) in both hRBCs (Ceolotto et al., 1997) and nucleated cells and is considered as a driving force for cellular transformation, invasion and metastasis (Beaty et al., 2014, Gatenby et al., 2007, Cong et al., 2015, Brisson et al., 2013). Des directly inhibits the Na^+/H^+ antiport activity (Choi et al., 2006) and should therefore be considered not only as an anti-depressant but also as an anti-cancer drug.

Of note, hRBCs possess functional endothelial nitric oxide synthase (eNOS) (Deliconstantinos et al., 1995, Chen and Mehta, 1998, Kleinbongard et al., 2006, Kuck et al., 2022, Kuck et al., 2020) using L-arginine to produce nitric oxide (NO) and L-citrulline. The binding of neurotransmitters such as acetylcholine on receptors located on endothelial cells (EC) leads to eNOS-dependent NO production. Subsequently, NO diffuses to the adjacent muscle layer, acts on vascular smooth muscle cells and induces relaxation (Furchgott, 1999, Wilson et al., 2016). Both Imipramine and Des have inhibitory effects on nicotinic AchR (Rana et al., 1993, Shytle et al., 2002). Of note, hRBCs possess functional AchR (Bennekou, 1993, Huestis and McConnell, 1974). In addition to this, hRBCs bear the disulfide-linked dimer and amphipathic acetylcholinesterase (AchE) on the exterior site of their membrane (Anstee, 1990, Rosenberry and Scoggin, 1984, Heller and Hanahan, 1972). One of the main functions of AchE is to hydrolyze acetylcholine, thus terminating cholinergic neurotransmission (AchE + acetylcholine → choline + acetate) (Soreq and Seidman, 2001). From the combination of all these remarkable

publications, we were then convinced that Des might affect the NO synthesis machinery of hRBCs.

1.8 Overdose of desipramine

While the serious situation of the impact of depression on the global human population has been mentioned above, we also need to notice that depression, is also the leading cause of death by suicide, with the number of suicides approaching 800,000 per year, accounting for nearly 1.5% of all global deaths (Organization, 2017). Especially for patients with major depression, approximately 25% of patients are suicidal, and roughly 15% of patients eventually die by suicide (Dumais et al., 2005).

Since many depressed patients have a high tendency to commit suicide, antidepressant treatment (e.g. TCA, selective serotonin reuptake inhibitor (SSRI), selective noradrenaline reuptake inhibitors (SNRI) and so on) for depressed patients should be administered with extra caution. Des, as a lipophilic TCA, after being taken orally, is rapidly absorbed by the intestine. Moreover, Des has anticholinergic- and antihistaminic-mediated effects, which can lead to a series of symptoms with overdose, the former leading to seizures, and the latter leading to tachycardia, intestinal obstruction, dilated pupils, urinary retention, confusion, and delirium, while alpha-adrenergic and muscarinic blockade can lead to tachycardia and hypotension, and eventually, patients will experience CNS depression and coma (Yates et al., 2014, Maan et al., 2021).

The most serious complication of toxic doses of Des is fatal non-perfusing arrhythmias. Resuscitation of cardiac arrest due to this condition is extremely difficult, and it is the main cause of death from TCA overdose (O'Sullivan et al., 2014). Compared to other TCAs, Des is more toxic and the risk of death is greater for depressed patients who take toxic doses of Des to commit suicide (Amitai and Frischer, 2006). Currently, no specific antidote is available for TCA

poisoning; therefore, further studies on Des may provide new therapeutic possibilities for clinical Des overdose.

1.9 Apoptosis

Apoptosis, a highly regulated process of programmed death, is performed to maintain the homeostasis of cell numbers in multicellular organisms, a dynamic balance between the rate of new cells that were generated through mitosis and the rate of damaged or unrequired cells (D'Arcy, 2019). Morphologically, apoptosis exhibits cell shrinkage, pyknosis, dense cytoplasm, membrane blebbing, tight-packed organelles, distended endoplasmic reticulum, and formation of cytoplasmic vacuoles and apoptotic bodies (Barman et al., 2018). There are two main apoptosis pathways: intrinsic and extrinsic pathways. The former is characterized by non-receptor-mediated initiation and mitochondrial regulation, thus it is also known as the mitochondrial pathway, and the latter is characterized by receptor-mediated initiation (Xu et al., 2019, Daniel et al., 2001). Apoptosis plays a critical role in coordinating programmed cell death mechanisms and their impact on homeostasis.

1.10 Human erythrocytes

Erythrocytes are the most abundant cells in the human body. An adult male has about 30×10^{12} cells, of which about 24.5×10^{12} are erythrocytes(Sender et al., 2016). The main function of erythrocytes is to act as a carrier to transport oxygen from the lungs to peripheral tissues, and from peripheral tissues to transport carbon dioxide to the lungs for excretion (Helms et al., 2018). The average lifespan of erythrocytes is about 120 days, and senescent erythrocytes are phagocytosed and eliminated by macrophages (Seki et al., 2020).

1.11 Role of ion-transport ATPases in human erythrocytes

Membrane potential of human erythrocytes (hRBCs) is low (-7 mV). Chloride transport is maintained by (perhaps) an unverified anion exchanger, whereas the low intracellular sodium $[Na^+]_I$ and high intracellular potassium concentration $[K^+]_I$ is provided by membrane-bound enzyme Na^+/K^+-ATPase. Furthermore, calcium (Ca^{2+})- stimulated and Magnesium (Mg^{2+})-dependent ATPase (Ca^{2+}-ATPase) ensures the extremely low intracellular calcium concentration $[Ca^{2+}]_I$ in intact hRBCs (**Figure 4**). These ATPases activities are important basis prerequisites for maintaining the vitality of hRBCs.

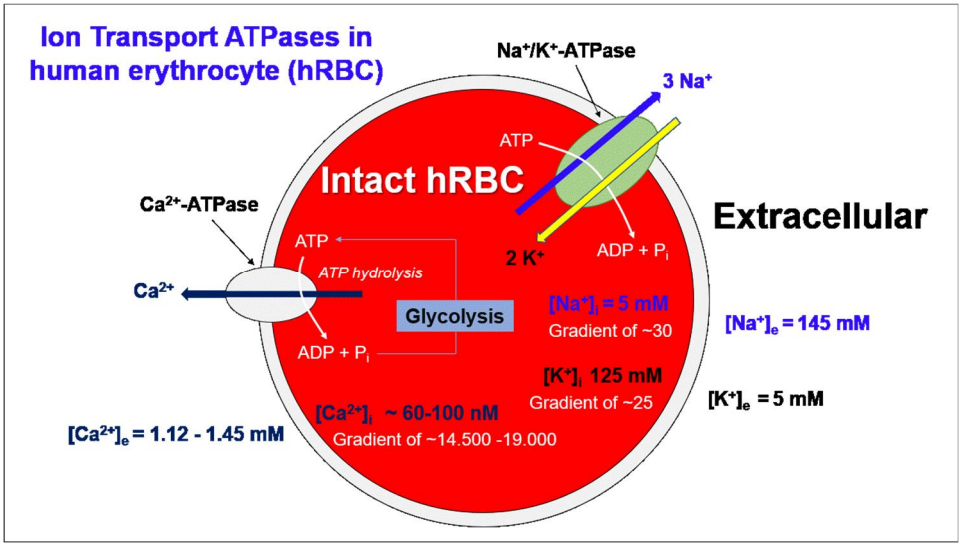


Figure 4: Ion transport ATPases in human erythrocytes (hRBCs). Shortened version of hRBCs ion transport pathways involved in transmembrane distribution of sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}). By means of ATP hydrolysis, the Na^+/K^+ and Ca^{2+} ATPases, respectively, maintain the intracellular concentrations of Na^+, K^+, and Ca^{2+} in the physiological range. This is one of the most basic elementary process to maintain the function of the hRBCs. The required energy (ATP) is generated from the glycolysis pathway.

1.12 Eryptosis

Different terms such as eryptosis, apoptosis, senescence, hemolysis, and necrosis describe the demise of a cell. Physiological range of intracellular calcium concentration $[Ca^{2+}]_i$ allows the ATP-dependent enzyme translocase to transport the phosphatidylserine (PS) molecules uni-directionally into the inner surface of the cell membrane. However, sustained increase in $[Ca^{2+}]_i$ is

associated with the activation of ATP-independent enzyme scramblase and thus unspecific PS translocation to the outer and inner layers of the erythrocyte membrane. And this is the prelude of PS-dependent eryptosis machinery and phagocytosis of erythrocytes.

For erythrocytes, due to the lack of organelles such as nuclei and mitochondria, erythrocytes cannot enter apoptosis. But similar to apoptosis in nucleated cells, erythrocytes can undergo eryptosis (Lang et al., 2005c). Similar to the typical morphologic features of apoptotic nucleated cells, eryptotic cells exhibit cell shrinkage, membrane blebbing, and PS exposure (Berg et al., 2001, Bratosin et al., 2001). In the early stages of eryptosis, the concentration of cytosolic Ca^{2+} increases, and increased cytosolic Ca^{2+} concentration eventually leads to cell membrane PS externalization, cell membrane blebbing, and cell shrinkage (Foller et al., 2009).

The mechanism of the increased cytosolic Ca^{2+} concentration is not clear but context-dependent is associated with the over-activation of non-selective Ca^{2+}-permeable cation channels (Lang et al., 2003a, Lang et al., 2006a) (**Figure 5**). Activation of the channels is caused by oxidative stress, ceramide, energy depletion and other triggers (Lang et al., 2005a). Furthermore, the increased cytosolic Ca^{2+} concentration activates Ca^{2+}-sensitive potassium (K^+) channels first described by Gardos (Gardos, 1958, Brugnara et al., 1993). Membrane potential of hRBCs is low (- 7mV). Activation of Gardos potassium channels results in strong hyperpolarization of the membrane potential towards the electrochemical equilibrium potential of potassium (E_K). This drives the efflux of the counterion chloride (Cl^-) through a background conductance (anion exchanger?) The loss of Cl^- together with K^+ through Gardos channels and the parallel loss of isosmotically obliged H_2O through aquaporins shrinks the erythrocytes resulting in echinocyte formation (Foller et al., 2009, Lang et al., 2003b) and **Figure 5**.

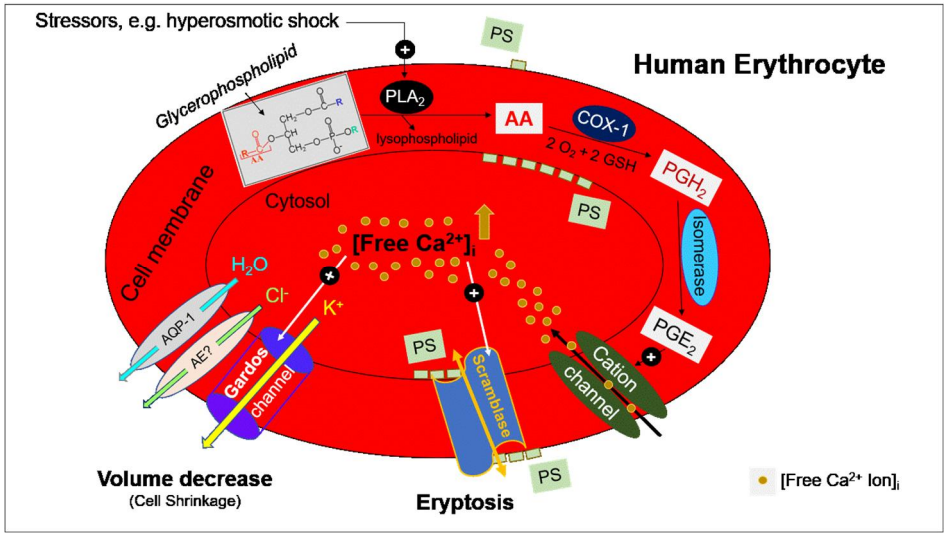


Figure 5: Impact of hyperosmotic shock (Hs)-induced and prostaglandin E_2 (PGE_2-) mediated activation of non-selective (Ca^{2+}-permeable) cation channels (NSC) in eryptosis and cell shrinkage of human erythrocyte. In the first step, Hs-induced phospholipase A_2 (PLA_2) activation leads to the release of arachidonic acid (AA) from membrane phospholipid pools (i.e. glycerophospholipid). In the second step the enzyme cyclooxygenase-1 (COX-1) catalyzes the oxygenation of AA to produce the cyclic endoperoxide prostaglandin H_2 (PGH_2). Finally, the isomerase takes over the enzymatic rearrangement of PGH_2 structure to gain PGE_2. The latter represents one of the many bioactive products of the AA metabolism. PGE_2 mediated activation of NSC evokes Ca^{2+} influx leading to an increase of cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_i\uparrow$. Subsequently, Ca^{2+} activates the enzyme scramblase which initiates phosphatidylserine (PS) exposure to the outer and inner leaflet of the plasma membrane. This is the prelude for the in vivo engulfment of hRBCs by the macrophages. In parallel, Ca^{2+}-dependent K^+ channels (Gardos channels) are activated. The efflux of K^+ ions lead to the hyperpolarization of the cell membrane. This drives the efflux of the counterion Cl^- through a background conductance (anion exchanger?). Finally, according to the osmosis principle water molecules exit erythrocytes through the water channel aquaporin-1 (AQP-1) (Preston et al., 1992, Walz et al., 1997, Sidel and Solomon, 1957) resulting in cell volume decrease and shrinkage. Interestingly, AQP-1 is also actively involved in CO_2 transport across the hRBCs membrane (Endeward et al., 2006).

1.13 The triggers of eryptosis and related clinical disorders

Eryptosis can be induced by a multitude of endogenous and exogenous small molecules or substances. Prominent examples are platelet-activating factor (Lang et al., 2005c), prostaglandin E_2 (PGE_2) (Lang et al., 2005b), carbon monoxide (Lang et al., 2012), chlorpromazine (Akel et al., 2006), celecoxib (Lupescu et al., 2013), aristolochic acid (Malik et al., 2014), amiodarone

(Nicolay et al., 2007), paclitaxel (Lang et al., 2006b), ribavirin (Oswald et al., 2014) and radiocontrast agents (Föller et al., 2009).

A wide range of clinical disorders can also stimulate eryptosis, more common diseases such as iron deficiency (Kempe et al., 2006), dehydration (Abed et al., 2013), hypoxia (Weiss et al., 2012), diabetes mellitus (Fırat et al., 2012), metabolic syndrome (Zappulla, 2008), heart failure (Mahmud et al., 2013), renal insufficiency (Polak-Jonkisz and Purzyc, 2012), hemolytic anemia (Banerjee et al., 2008), sepsis (Kempe et al., 2007). Some genetic diseases such as sickle cell disease (Lang et al., 2002), thalassemia (Ayi et al., 2004), and glucose-6-phosphate dehydrogenase (G6PD) deficiency (Cappadoro et al., 1998) can also stimulate eryptosis.

1.14 Desipramine-induced apoptosis

TCAs have been proposed to act through apoptosis against different types of cancers (Levkovitz et al., 2005, Arimochi and Morita, 2006, Xia et al., 1999). The potency of TCAs acting as cationic amphiphilic drugs (CADs) (TCAs + H^+ → CADs), their subsequent interaction with polar lipids and the resulting impairment or collapse of lysosomal lipid metabolism, might expand the spectrum of TCAs and CADs clinical applicability, especially for cancer (Ellegaard et al., 2016, Petersen et al., 2013, Arimochi and Morita, 2008) and anti-viral (Pakkanen et al., 2009, Salata et al., 2017) therapies. However, some retrospective studies using large population-representative databases raise doubts about the anti-cancer effect of TCAs (Boursi et al., 2015, Chen et al., 2020, Abdel Karim et al., 2019). There are two main pathways for apoptosis induced by various conditions, the endogenous pathway induced by caspases through the activation of mitochondria, and the exogenous pathway induced by caspases through the activation of extracellular signals (Wu and Bratton, 2013, Ashkenazi, 2008, Daniel et al., 2001). Caspases are the main effectors of apoptosis. When upstream caspases are activated, they will

activate downstream caspase-3 and -7, which will cleave many proteins involved in cellular functions and participate in the coordinated destruction of important organelles and cellular structures, thus producing apoptosis (Taylor et al., 2008, Salvesen and Riedl, 2008). Des, as one of the classical TCAs, has also been shown to have cytotoxic effects in many malignant cells. It induces apoptosis in rat gliomas through the endoplasmic reticulum stress (Ma et al., 2011) and in human HT29 colon carcinoma cells through non-mitochondrial and mitochondrial pathways (Arimochi and Morita, 2008), and also shows anti-tumor proliferative effects in human PC3 prostate cancer cells and human hepatoma Hep3B cells (Chang et al., 2008, Yang and Kim, 2017).

1.15 Desipramine-induced eryptosis?

To date, there is no publication on Des-induced eryptosis. However, Des may trigger hemolysis in rats (Sheppard et al., 1969). TCAs, e.g. Des exert concentration-dependent different effects on the stability of the erythrocyte membrane, stabilizing at low and destabilizing at high concentrations (Yasuhara et al., 1985). Des has a high erythrocyte/plasma ratio and the plasma concentration correlates with the concentration of Des in the brain (Bogema Jr, 1983, Linnoila et al., 1978). In addition, it has been shown that Des can induce apoptosis in certain tumor cells (e.g. C6 glioma cells and PC3 prostate cancer cells) by activating caspase-3 (Qi et al., 2002, Chang et al., 2008). As mentioned in section 1.7, hRBCs possess caspase-3 and other known targets which also occur in nucleated cells (Galtieri et al., 2010, Mandal et al., 2002), thus we anticipated that Des could induce eryptosis.

1.16 Nitric oxide (NO) and NO donor sodium nitroprusside (SNP)

1.16.1 NO

NO, a soluble gas, is synthesized in the vascular endothelium from amino acid L-arginine, under the catalysis of Ca^{2+}/calmodulin-dependent enzyme

NOS (Garthwaite et al., 1988). In addition, erythrocytes, platelets, neutrophils, and macrophages have also been shown to be capable of synthesizing and releasing NO (Eligini et al., 2013). NO has a wide range of biological properties in maintaining vascular homeostasis, it has the function of inducing vasodilation and therefore plays an important role in the regulation of vascular tone (Baskurt et al., 2011), and it also exerts biological effects in regulating inflammation and oxidative stress by generating reactive oxygen species (ROS) (Tousoulis et al., 2012). In addition, NO is a potent inhibitor of platelet function, which inhibits platelet aggregation and can have a protective effect on blood vessels (Mellion et al., 1981). The half-life of NO is very short, only 3 to 6 seconds (Dhir and Kulkarni, 2011). NO elimination is easily accomplished by its oxidation ($NO \rightarrow NO_3^-$) or reduction ($NO \rightarrow N_2O$) (BORUTAITÉ and Brown, 1996). As a consequence, NO concentration decreases, whereby oxidation is the main mechanism of NO depletion in the vasculature (Thomas, 2015).

1.16.2 The relationship between NO and apoptosis and eryptosis

NO is a double-edged sword for apoptosis, and its effective concentration and regulation of apoptosis vary greatly in different cells. Most of the time, prolonged exposure of cells to high concentrations of NO can induce apoptosis, and the specific mechanism is not clear yet. For example, it may be associated with the activation of certain apoptotic factors or pathways by oxidative stress (Kim et al., 2001, Li and Wogan, 2005, Shen et al., 1998). However, low physiological concentrations of NO have an inhibitory effect on apoptosis, and various mechanisms have been proposed for this inhibition effect, which may be mediated by cGMP-dependent pathways or cGMP-independent pathways (Taylor et al., 2003, Sata et al., 2000, Wang et al., 2002).

Similar to nucleated cells, the stimulation or inhibition of eryptosis by NO is concentration-dependent (Lang and Lang, 2015), and at low doses NO is a very effective inhibitor of eryptosis (Nicolay et al., 2008), as has been also

demonstrated in numerous articles (Ghashghaeinia et al., 2017, Nader et al., 2020). The doses of NO that exert the inhibitory function in erythrocytes are even lower than those that inhibit apoptosis in nucleated cells (William et al., 2005, Malan et al., 2003). The discrimination between stimulation or inhibition of eryptosis by NO is not well understood yet. However, it is assumed that the mechanisms are similar to apoptosis of nucleated cells. One mechanism may be associated with the induction of S-nitrosylation of thioredoxin, an anti-apoptotic enzyme that is activated in reaction to antioxidant stress (Haendeler et al., 2002). However, high doses of NO also stimulate eryptosis which is supposed to be associated with oxidative stress (Pacher et al., 2007, Lang and Lang, 2015).

1.16.3 Sodium nitroprusside

Sodium nitroprusside ($Na_2[Fe(CN)_5NO]\cdot 2H_2O$; SNP), a water-soluble sodium salt discovered by Playfair in 1849, is a vasodilator that is still widely used in clinical practice because of its effective vasodilatory effects on both arteries and veins (Playfair, 1849, Hottinger et al., 2014). In terms of the chemical structure, SNP is composed of a Fe^{2+} in the center and surrounded by nitrosonium ion (NO^+) and 5 cyanide anions (Zoupa and Pitsikas, 2021). After being ingested by the human body, SNP can react with sulfhydryl groups on hRBCs to generate NO (Ivankovich et al., 1978). Since SNP is more stable than NO, it is widely used as a NO donor in molecular biology and physiology research.

1.17 The relationship between N-acetyl-L-cysteine and apoptosis and eryptosis

1.17.1 N-acetyl-L-cysteine and glutathione

N-acetyl-L-cysteine ($HSCH_2CH(NHCOCH_3)CO_2H$; NAC) is a natural derivative of the amino acid L-cysteine (L-Cys) and is commonly used as an antioxidant (Dodd et al., 2008) and mucolytic agent (Sadowska, 2012). The former is due

to its role as a precursor of reduced glutathione (GSH) (Bavarsad Shahripour et al., 2014), while the latter is attributed to its function in destroying the disulfide bonds of high molecular weight glycoproteins in mucus (Balsamo et al., 2010).

NAC is the precursor of L-Cys, and many tissues in the body are capable of removing the acetyl group of NAC to produce cysteine (Dilger and Baker, 2007). L-Cys and glutamate are firstly catalyzed by γ-glutamylcysteine synthetase (γ-GCS) to produce γ-Glutamylcysteine, and then synthesize GSH with L-Glycine catalyzed by glutathione synthase (GS) (**Figure 6A**). Among the amino acids required for GSH synthesis in cells, cysteine concentration is the lowest (Aruoma et al., 1989), thus the production of γ-glutamylcysteine by L-Cys and glutamate is the rate-limiting part of GSH synthesis (Bavarsad Shahripour et al., 2014).

1.17.2 Glutathione

GSH (γ-L-glutamyl-L-cysteinyl glycine), a sulfhydryl (-SH) tripeptide, is a well-known endogenous antioxidant acting by eliminating hydrogen peroxide and free radicals or by participating in the catalysis of certain antioxidant enzymes. (Liang et al., 2011). GSH is widely distributed in all tissues, especially in the liver of the human body, and is the most plentiful non-protein thiol. Because of its powerful anti- oxidative stress function, it plays a key role in regulating cell proliferation and apoptosis (Lu, 2013). In addition, GSH also serves other functions, such as regulation of protein functions, regulation of immune responses and detoxification of xenobiotics, etc. (Lu, 2009).

1.17.3 The regeneration of glutathione and the pentose phosphate pathway

GSH exists in two forms, GSH (reduced glutathione) and GSSG (oxidized glutathione disulfide) (Kaplowitz et al., 1985), and GSH also exerts its antioxidant effect through its conversion to GSSG catalyzed by glutathione reductase (GR), a process that requires the consumption of nicotinamide

adenine dinucleotide phosphate hydrogen (NADPH), which is produced in the pentose phosphate pathway (PPP) (Gaucher et al., 2018, Lu, 2013), a prevalent glucose metabolic pathway that is divided into an oxidative phase and a non-oxidative phase (**Figure 6B**).

During the oxidative phase of PPP, Glucose-6-phosphate (G6P) is firstly dehydrogenated to 6-phosphoglucono-δ-lactone catalyzed by Glucose-6-phosphate dehydrogenase (G6PDH), accompanied by 1 NADPH generation, then hydrolyzed to 6-phosphogluconate catalyzed by 6-phosphogluconolactonase (6PGL), and finally oxidized and decarboxylated by 6-phosphogluconate dehydrogenase (6PGDH), to ribulose-5-phosphate (Ru5P), this step is also the key step for GSH to play its antioxidant role (conversion of GSSG to 2 GSH), and the regeneration of GSH is catalyzed by glutathione reductase (GR) (**Figure 6B**). The generation of NADPH is one of the main tasks of the PPP, and PPP also plays a cellular defense role by generating NADPH to neutralize ROS (Ghashghaeinia et al., 2019) (**Figure 6C**). As for the non-oxidative PPP, Ru5P is metabolized into other forms (mainly into glyceraldehyde-3-phosphate (G3P) and fructose-6- phosphate (F6P)) and eventually enters the glycolytic process (Patra and Hay, 2014).

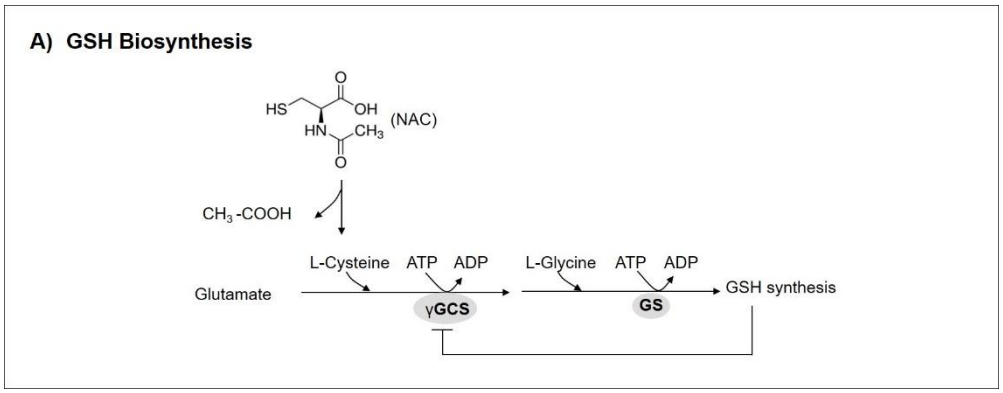


Figure 6: To be continued.

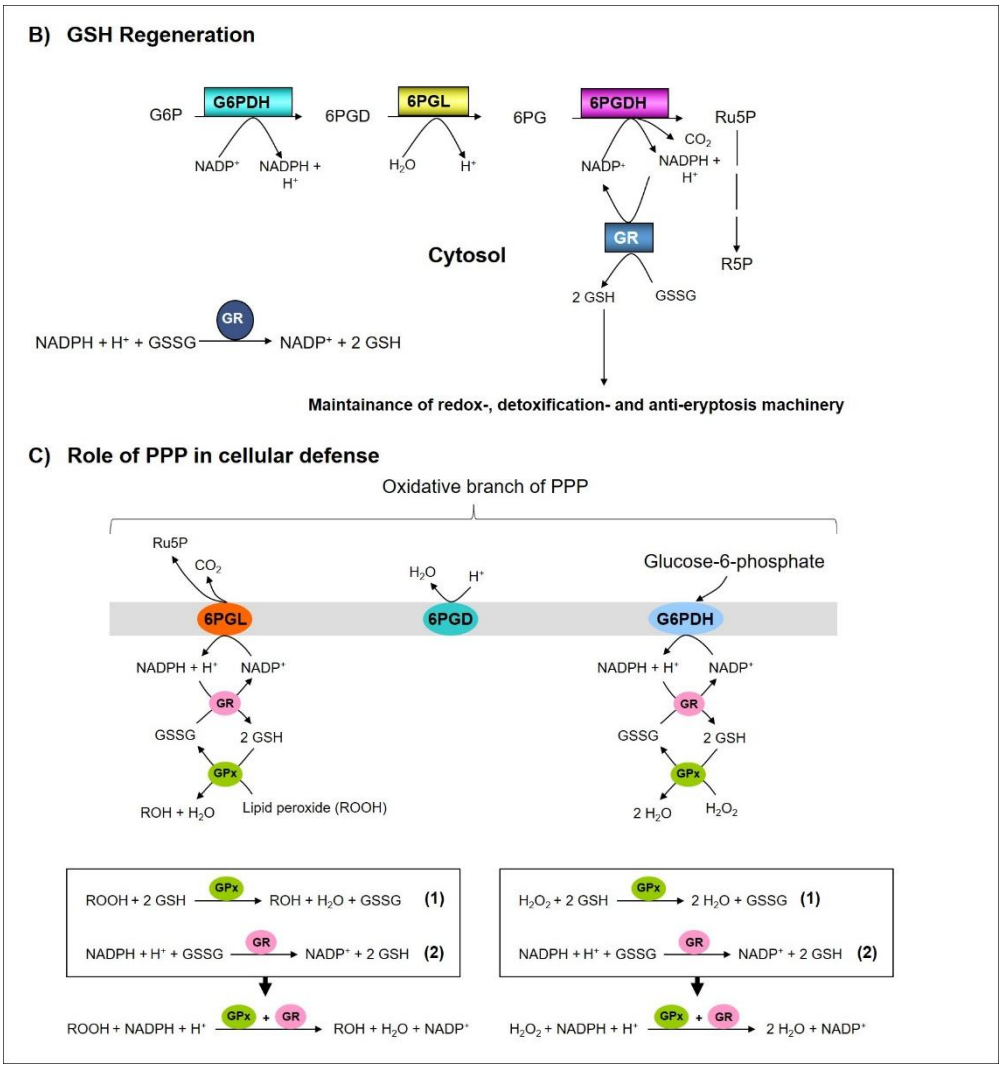


Figure 6: GSH biosynthesis and role of the pentose phosphate pathway (PPP) in cellular defense in both human erythrocytes and nucleated cells. A) Synthesis of GSH from three amino acids, L-cysteine, glutamic acid, and L-glycine, catalyzed by γ-glutamylcysteine synthetase (γGCS) and glutathione synthase (GS). B) In the oxidative branch of PPP (ox-PPP), glucose-6-phosphate (G6P) is converted to ribulose-5-phosphate (Ru5P) accompanied by the generation of two NADPH molecules. C) Linkage of GSH cycle to the NADPH producing PPP provides redox balance and cellular defense mechanism against oxidative stress by GR- and GPx-dependent detoxification of reactive oxygen species, e.g. ROOHs and H_2O_2. Figure 6 has been adapted from (Ghashghaeinia et al., 2016) and (Ghashghaeinia et al., 2019). 6PGD: 6-Phosphoglucono-δ-lactone, 6PG: 6-Phosphogluconate, R5P: ribose-5-phosphate, GR: glutathione reductase, GPx: glutathione peroxidase, ROOHs: lipid peroxides, H_2O_2: hydrogen peroxide.

1.17.4 Different types of GSH depletion. Does desipramine affect intracellular GSH and GSSG concentrations?

Both non-enzymatic and enzymatic reactions act as a source for the generation of superoxide anion ($O_2^{\bullet -}$). For instance, under physiological

conditions autoxidation of approx. 3% of the total body hemoglobin (Hb) leads to generation of $O_2^{\bullet-}$: $HbFe^{2+}$-O_2 (oxy-Hb) → $HbFe^{3+}$ (met-Hb) + $O_2^{\bullet-}$ (Misra and Fridovich, 1972). Analogous to Hb, $O_2^{\bullet-}$ is formed from autoxidation of hemoprotein myoglobin (Mb): $MbFe^{2+}$-O_2 (oxy-Mb) → $MbFe^{3+}$ (met-Mb) + $O_2^{\bullet-}$ (Gotoh and Shikama, 1976). During the mitochondrial respiratory chain about 2% of molecular oxygen (O_2) is reduced to $O_2^{\bullet-}$ by the electron univalent leak pathway (Chance et al., 1979, Liu et al., 2022). Enzymes such as NADPH oxidase (NOX) and xanthine oxidase (XO) (Cantu-Medellin and Kelley, 2013) also generate ($O_2^{\bullet-}$): NADPH + 2 O_2 + NOX → $NADP^+$ + 2 $O_2^{\bullet-}$ + H^+. Subsequent dismutation or disproportionation of $O_2^{\bullet-}$ by the enzyme superoxide dismutase (SOD) leads to generation of other reactive oxygen species (ROS), i.e. hydrogen peroxide (H_2O_2): 2 $O_2^{\bullet-}$ + 2 H^+ + SOD → O_2 + H_2O_2 (Fridovich, 1975). Catalase (Cat) is capable to destroy H_2O_2: (H_2O_2 + Cat → H_2O + 1 ½ O_2). H_2O_2-caused and glutathione peroxidase (GPx)-catalyzed oxidation of GSH and the subsequent formation of GSSG and H_2O is illustrated below: H_2O_2 + 2 GSH + GPx → GSSG + 2 H_2O (Jones, 1981). GSSG is the oxidized form of GSH. Finally, the NADPH-dependent enzyme GR intervenes to replenish the GSH pool: NADPH + H^+ + GSSG + GR → $NADP^+$ + 2 GSH. It is known that NADPH-dependent GSH-glutaredoxin (Grx) and thioredoxin (Trx) systems function as electron transmitting systems for DNA synthesis. The former system permanently oxidizes GSH to GSSG which in turn is converted to GSH by NADPH-dependent GR. For review see: (Ghashghaeinia et al., 2019).These are two of many examples in which an inverse correlation between intracellular glutathione concentrations, prevails, i.e. $[GSH]_{i\downarrow}$ & $[GSSG]_{i\uparrow}$.

However, this dynamic reciprocal proportionality between $[GSH]_i$ and $[GSSG]_i$ cannot be observed for substances or drugs that conjugate with GSH, at least in our experimental set up with hRBCs (Ghashghaeinia et al., 2016, Ghashghaeinia et al., 2020). The crucial point is: against impending GSH

collapse, the hRBC reacts with its re-synthesis as well as GR-dependent GSSG conversion into GSH (**Figure 6A and B**), which in turn is depleted again by conjugate formation. In the end, the hRBC has neither GSH nor GSSG: $[GSH]_{i\downarrow}$ & $[GSSG]_{i\downarrow}$. Here are some drugs or electrophilic compounds that react with redox-sensitive reactive cysteine residues of proteins and with the cysteine of GSH: dimethyl fumarate (Schmidt et al., 2007), costunolide (Choi et al., 2002), parthenolide (Freund et al., 2020) and Bay 11-7082 (Krishnan et al., 2013). Experiments with hRBCs performed in this study show that Des does not affect $[GSH]_i$ & $[GSSG]_i$. The necessary experiments and measurements were carried out in Italy at the working group of Professor Ranieri Rossi. The whole procedure is previously reported in Nature Protocols (Giustarini et al., 2013).

1.17.5 Interplay between vitamin C, pentose phosphate pathway and glutathione

Vitamin C (L-ascorbic acid / vit C) existing in two redox states, ascorbic acid (AA) and its oxidized form dehydroascorbic acid (DHA), is a required nutrient for humans. Both AA and DHA are carrier mediated transported across cell membranes. AA uptake is Na^+-dependent and regulated by the Na^+-dependent vit C transporter (SVCT) family of proteins (Tsukaguchi et al., 1999). DHA transport, on the other hand, is Na^+-independent and is accomplished by glucose transporters (Kern and Zolot, 1987, Bigley et al., 1983, Mann and Newton, 1975, Ingermann et al., 1988, Mooradian, 1987, Hornung and Biesalski, 2019, Ulloa et al., 2013, Goldenberg and Schweinzer, 1994). Human erythrocytes share glucose transporter 1 (GLUT-1) for rapid uptake of glucose and electrically neutral DHA (Sage and Carruthers, 2014, Montel-Hagen et al., 2008, Wann et al., 2006). It is known that DHA enhances the activity of glucose-6-phosphate dehydrogenase (G6PDH) and increases the level of the reduced form of glutathione (GSH) (Cisternas et al., 2014, Puskas et al., 2000, Pallotta et al., 2014) and **Figure 7**.

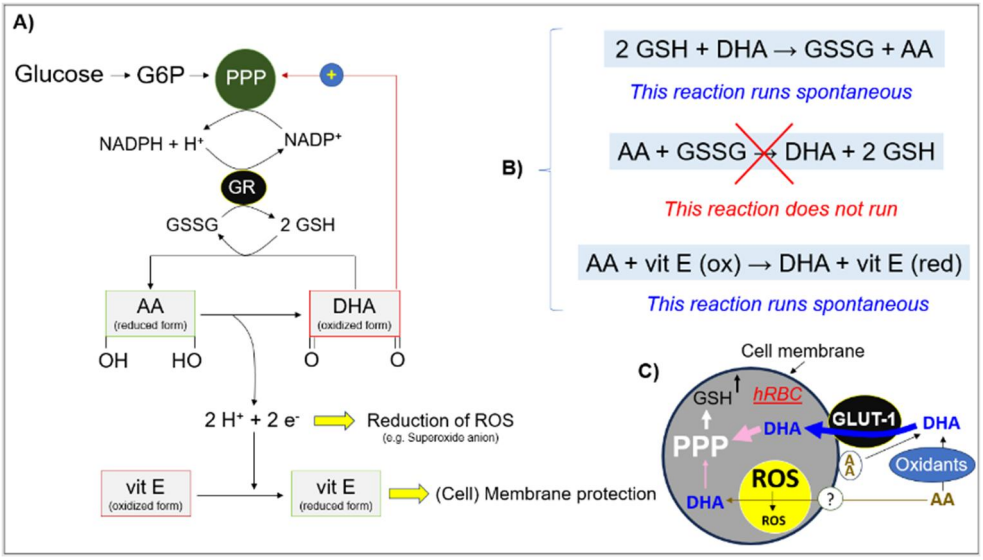


Figure 7: A brief overview of cellular antioxidant cascade in human erythrocytes (hRBC) as well as vitamin C-dependent glutathione (GSH) regeneration. The oxidized forms of these antioxidants, respectively, are: glutathione disulfide (GSSG), dehydroascorbic acid (DHA), and vitamin E (oxidized). A) NADPH molecules produced by cellular glucose metabolism, i.e. by the oxidative branch of the pentose phosphate pathway (PPP) can be used by NADPH-dependent glutathione reductase (GR) to catalyze the reduction of GSSG to GSH (NADPH + H^+ + GSSG + GR → $NADP^+$ + 2 GSH). A and B) From the point of view of redox potential GSH will reduce DHA (2 GSH + DHA → GSSG + AA), but ascorbic acid (AA) is incapable of reducing GSSG to GSH. This also applies to the following reaction: AA will reduce the oxidized form of vitamin E (AA + vit E (oxidized) → DHA + vit E (reduced), but vitamin E (reduced form) is incapable of reducing DHA to AA (Winkler et al., 1994). C) It is known that vitamin C-stimulated PPP leads to increase in GSH level. For more details see: (Cisternas et al., 2014, Puskas et al., 2000, Pallotta et al., 2014).

1.18 The relationship between calcium and eryptosis

1.18.1 Calcium and calcium homeostasis

Of all the mineral elements in the human body, calcium is the most abundant. Maintaining its homeostasis is essential for maintaining many normal physiological functions of the body such as nerve conduction, muscle contraction, and hormone release (Loupy et al., 2012). The normal adult body contains about 1.2 kg of calcium, most of which exists in the form of hydroxyapatite in the bones, while the remaining soluble calcium is mainly stored in the serum and interstitial fluids, and a small portion of soluble calcium is distributed in the skeletal muscle and other tissues (Goldstein, 1990).

Calcium exists in two main forms, diffusible and non-diffusible, through which the body maintains calcium homeostasis by interconverting these two forms (Dreyer, 2011). The normal total (i.e., free and protein-bound) serum Ca^{2+} level is 2.2-2.6 mmol/L. The free concentration of serum Ca^{2+} ranges between 1.12-1.45 mmol/L (Pravina et al., 2013) and is in equilibrium with Ca^{2+} that is bound to serum proteins that generate a Ca^{2+} system (Baird, 2011).

1.18.2 Association of calcium, apoptosis and eryptosis

Ca^{2+} was first associated with cell death by A Fleckenstein et al. in 1974, who suggested that Ca^{2+} overload could lead to myocardial fiber death (Fleckenstein et al., 1974). At normal conditions, there is a 20,000-fold concentration gradient of free Ca^{2+} between extra- and intracellular, and the cell maintains this concentration gradient by Ca^{2+}-ATPase and Na^{+}/Ca^{2+}-Antiporters (Patergnani et al., 2020). In nucleated cells, when Ca^{2+} enters the cell, they are mainly stored in the endoplasmic reticulum (ER) at concentrations between 100 and 800 µM (Raffaello et al., 2016).

When cells are stimulated, various Ca^{2+} channels on the cell membrane, especially transient receptor potential channels (TRPC), are over-activated, leading to massive Ca^{2+} in-flow (Venkatachalam and Montell, 2007). And Ca^{2+}, as a major second messenger of the cell, has been proven to induce apoptosis through a variety of pathways. Ca^{2+} can induce apoptosis by stimulating the release of apoptosis-promoting factors from mitochondria, it can also induce apoptosis by directly participating in the regulation of related enzymes in the cytoplasm or induce apoptosis by combining with Ca^{2+} effectors in the ER (Hajnóczky et al., 2003, Mattson and Chan, 2003). Erythrocytes lack organelles and have lower $[Ca^{2+}]_i$ than nucleated cells. As described in chapter 1.6, Ca^{2+} has a crucial role in regulating eryptosis. The increase in $[Ca^{2+}]_i$ can activate Ca^{2+}-sensitive K^{+} channels and induce PS exposure, ultimately causing eryptosis.

1.19 Aim of project

Depression is the most common psychological disorder, bringing a huge economic and family burden to society every year. Des is a commonly used antidepressant drug, but overdose medication may lead to serious consequences and even death of patients, and there is no effective treatment for Des overdose. Compared to other antidepressants, TCAs, e.g. Des have a higher mortality rate due to overdose and thus have a higher risk of suicide (Kapur et al., 1992). Erythrocytes are the most abundant cells in the body. Thus, the present study was conducted to: a) determine a possible effect of Des on human erythrocytes survival, and b) investigate whether this effect of Des can be reversed by NAC and NO donor SNP, either alone or in combination.

2 Materials and methods

2.1 Materials

2.1.1 Consumables

Table 1: List of consumables

Consumables	Company
Gloves	Hartmann, Heidenheim an der Brenz, Germany
SafeSeal-Reaction tubes (1.5 ml, 2 ml)	Greiner Bio-One, Frickenhausen, Germany
Tips (10 µl, 200 µl, 1,000 µl)	TipOne, Ocala, USA
Eppendorf tubes (1.5 ml, 2 ml)	Eppendorf, Hamburg, Germany
Pipette (10 µl, 100 µl, 1000 µl)	BRAND, Wertheim, Germany
Steritop-GP, 0.22µm, polyethersulfone	Millipore, Darmstadt, Germany
5 mL Polystyrene round bottom tube	Corning, New York, USA
1.3 ml round bottom	Greiner Bio-One, Frickenhausen, Germany
Serological pipette (5 ml, 10 ml, 25 ml)	Corning, New York, USA
Pipette controller	BRAND, Wertheim, Germany
Tubes (15 ml, 50 ml)	Greiner Bio-One, Frickenhausen, Germany
Weighing paper	Macherey-Nagel, Düren, Germany
Disposable Weighing Trays	Carl Roth, Karlsruhe, Germany
Syringe (1 ml)	BD, Franklin Lakes, USA
Needle	BD, Franklin Lakes, USA
Aluminum Foil	Optihome, Bayreuth, Germany
Cell culture plates (96 wells)	Greiner Bio-One, Frickenhausen, Germany

2.1.2 Reagents, buffers, and medium

Table 2: List of reagents

Reagents	Company	Serial number
Desipramine hydrochloride	Sigma-Aldrich, St. Louis, MO, USA	D3900
N-acetyl-L-cysteine	Sigma-Aldrich, St. Louis, MO, USA	A8199
Annexin-V-FLUOS	Sigma-Aldrich, St. Louis, MO, USA	11828681001
BD FACSFlow™	BD, Heidelberg, Germany	342003
BD FACSClean	BD, Heidelberg, Germany	340345
BD FACSRinse	BD, Heidelberg, Germany	340346
Ampuwa® water for injections	Fresenius, Bad Homburg vor der Höhe, Germany	1636071
Descosept Sensitive	Dr. Schumacher, Beiseförth, Germany	
NaCl	Merck, Darmstadt, Germany	1.06404.1000
KCl	Merck, Darmstadt, Germany	1049360250
$MgSO_4.7H_2O$	Carl Roth, Karlsruhe, Germany	8283.2
HEPES	Carl Roth, Karlsruhe, Germany	9105.2
D-(+)-Glucose	Carl Roth, Karlsruhe, Germany	X997.1
$CaCl_2$	Merck, Darmstadt, Germany	1023780500
NaOH	Applichem, Darmstadt, Germany	A68290500
Sodium nitroprusside	Sigma-Aldrich, St. Louis, MO, USA	71778
3 mM KCl	Xylem Analytics, Oberbayern, Germany	
pH 10.01 technical buffer	Xylem Analytics, Oberbayern, Germany	
pH 7.00 technical buffer	Xylem Analytics, Oberbayern, Germany	
Dimethyl Sulfoxide	Merck, Darmstadt, Germany	D8418
EDTA (Free acid)	Merck, Darmstadt, Germany	E6758
EGTA (Free acid)	Merck, Darmstadt, Germany	03777
N-Ethylmaleimide	Merck, Darmstadt, Germany	E3876

Table 3: List of buffers and solutions

Buffer/Solution	Components	g	mM
Annexin wash buffer (pH 7.4)	NaCl	7.305	125
	KCl	-	-
	$MgSO_4$	-	-
	Hepes	2.3831	10
	Glucose	-	-
	$CaCl_2$	0.5549	5
	NaOH	0.18	-
	ddH_2O (ml)	989.6	-
	Total volume (ml)	1000	-
Ringer solution (pH 7.4)	NaCl	7.305	125
	KCl	0.3727	5
	$MgSO_4$	0.2957	1.2
	Hepes	7.6735	32.2
	Glucose	0.901	5
	$CaCl_2$	0.1109	1
	NaOH	0.577	-
	ddH_2O (ml)	982.7	-
	Total volume (ml)	1000	-
Ringer solution without Ca^{2+} plus EGTA (pH 7.4)	EGTA	0.38	1
Ringer solution without Ca^{2+} plus EDTA (pH 7.4)	EDTA	0.292	1
Stock solutions			
50 mM Des: 100 mg Des/6.6 ml ddH_2O (Ampuwa) at 37°C in dark.			
1 mM SNP: 17 mg SNP/57 ml Ringer solution at room temperature in dark.			
0.5 M NAC: 100 mg NAC/1.225 ml ddH_2O (Ampuwa) at 37°C in dark.			
Annexin-V-FLUOS antibody dilution: 40 µl Annexin-V-FLUOS/1.2 ml Annexin wash buffer			
310 mM NEM: 310 mg NEM/8 ml ddH_2O (Ampuwa) at 37°C in dark.			
3.23 M NaOH: 1.8 g NaOH/15 ml ddH_2O (Ampuwa).			

Ringer solution without Ca^{2+} plus EGTA: 0.71 ml from 3.23 M NaOH was given to adjust pH to 7.4

Ringer solution without Ca^{2+} plus EDTA: 1.42 ml from 3.23 M NaOH was given to adjust pH to 7.4

2.1.3 Equipments

Table 4: List of equipments

Equipment	Company
Herasafe® hs/HSP safety cabinets	Heraeus, Hanau, Germany
Refrigerator 4°C	Liebherr, Ehingen, Germany
Refrigerator -20°C	Liebherr, Ehingen, Germany
Refrigerator -80°C	SANYO, Osaka, Japan
BD FACSCanto™	BD, Heidelberg, Germany
BD LSRFortesa™ cell analyzer	BD, Heidelberg, Germany
Cell culture incubator	Binder, Tuttlingen, Germany
Vortex-Genie 2	Scientific Industries, New York, USA
Hettich Rotanta 460R	Hettich, Kirchlengern, Germany
Dishwasher	Miele, Gütersloh, Germany
Dry Block heater	Labnet, Edison, USA
Drying and heating ovens	Memmert, Schwabach, Germany
Autoclave	Varioklav, Oberschleißheim, Germany
pH meter	WTW Inolab, München, Germany
Analytical weight	Sartorius, Gottingen, Germany
Glass equipment	Schott, Mainz, Germany
Vacuum pump	KNF, Breisgau, Germany
Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Stirrer with heater	Carl Roth, Karlsruhe, Germany
Power wave XS2	Biotek, Rheinbrohl, Germany
Agilent 1100	Agilent, Santa Clara, USA
Jasco V-530	Jasco, Tokyo, Japan

2.2 Ethics Statement

All volunteer studies were performed in agreement with the latest revision of the declaration of Helsinki. 32 volunteers' blood was obtained following the ethical vote (184/2003 V) of the University of Tuebingen and the volunteers' written consent.

2.3 Methods

2.3.1 Sterilization and disinfection

Since all materials required for the experiment need to be sterilized, materials like pipette tips and Eppendorf tubes were autoclaved by steam sterilization (autoclaving) method at 121 °C for 20 minutes. All cell culture solutions like

Ringer solution and annexin wash buffer (Ax-WB) were filtered using 0.22 μm Millipore steritop to get general bacteria, fungi, and other microorganisms-free solutions.

2.3.2 Erythrocytes

The high-purity red blood cell suspensions used in this study were obtained from healthy volunteers, extracted and purified by the University of Tuebingen Blood Donation Center, and finally provided by the University of Tuebingen Blood Bank, with less than 0.1% white blood cell (WBC) or platelet contamination (Lang et al., 2005c). The high-purity red blood segments taken from Blood Bank can be stored at 4°C for up to one week.

2.3.3 Treatment of erythrocytes with different concentrations of desipramine

The safety cabinet was first completely disinfected with alcohol and ventilated for 5 minutes. The blood segment was taken out of the 4°C refrigerators and placed in the cabinet. The middle of the blood segment was gently pressed by the index finger, and the two terminals of the blood segment were inserted at an angle of 45° by two needles. Too much force cannot be used during the puncture process to prevent the blood segment from being punctured. Then the blood was extracted with a syringe gently and transferred to a prepared 2 ml tube. After all the preparations were done, 2 ml Ringer solution was first transferred to six 5 ml polystyrene round-bottom tubes. Then 12 μl red blood cells were added to each tube, i.e. erythrocytes were used at 0.6% hematocrit (Hct). By vortexing the tube gently, the erythrocytes were evenly resuspended. Since Des stock solution should be made fresh each time and protected from light, 50 mM Des stock solution was made in the dark at the same time and then diluted to the required concentration (0.5 mM, 5 mM, 10 mM, 25 mM). The negative control group was added with 4 μl of water, and the experimental group was added with 4 μl of different concentrations of Des solutions. Then all the samples were vortexed again gently to homogenize the

Des with the erythrocytes. After all the caps have been loosened, the tubes were transferred to the incubator, and the hRBCs were incubated at 37 °C, 5% CO_2, and 90% relative humidity (all incubation conditions in this project are the same) for 24 and 48 h before assaying **(Figure 8)**.

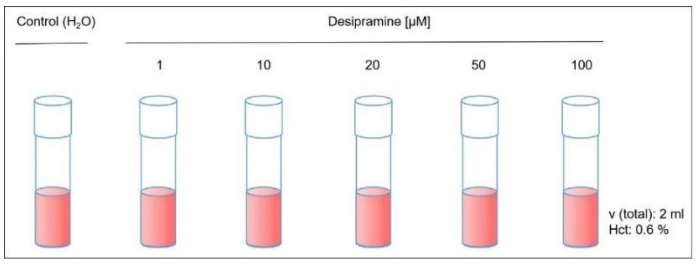


Figure 8: Treatment of human erythrocytes with increasing concentrations of desipramine. Erythrocytes were treated with H_2O (Negative control), 1, 10, 20, 50, and 100 µM of desipramine.

2.3.4 Treatment of erythrocytes with different concentrations of desipramine and sodium nitroprusside

All preparations were done as above and 1 mM SNP stock solution was made in the dark. At the same time, four 50 ml tubes were prepared and 20 ml of Ringer solution was added to each tube. After all the preparations were done, the blood was extracted and then transferred to the prepared 50 ml tubes of 120 µl each. During the transfer, the pipette tip should be placed under the liquid surface to gently transfer the blood to prevent the blood from mixing with the Ringer solution. Then 10 µl, 20 µl, and 30 µl of SNP were added to the three prepared 50 ml tubes respectively and the tubes were gently inverted ten times to mix the samples thoroughly. Then, all the caps were loosened and the tubes were moved to the incubator and incubated for 2 h. During the incubation, the tube should be inverted ten times every half hour to ensure adequate contact between the erythrocytes and the drugs. Meanwhile, 50 mM Des stock solution was made in a water bath at 37 °C and then diluted to the concentration of 25 mM. The entire operation needs to be protected from light. Twelve new flow tubes are prepared and marked. After resuspending the liquid by inverting, 6 ml was moved from each 50 ml tube and added to each three new flow tubes of 2 ml each. Then 4 µl of H_2O, 25

mM Des, or 50 mM Des were added to different flow tubes by the group. At last, all flow tubes were vortexed gently for 10 seconds to mix the liquid homogeneously and then moved to the incubator and incubated for 24 h before assaying **(Figure 9)**.

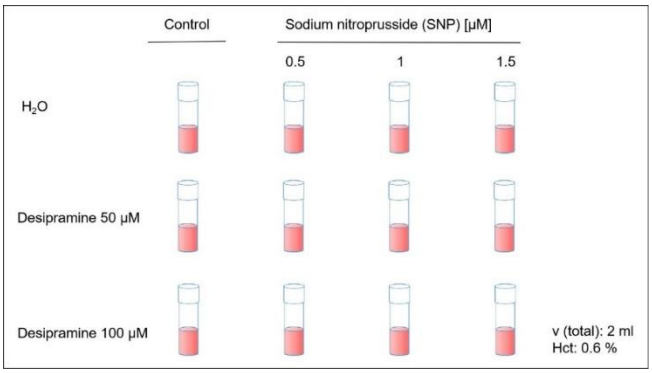


Figure 9: Treatment of human erythrocytes (hRBCs) with different concentrations of desipramine and sodium nitroprusside. In the upper panel, hRBCs were treated with H_2O (Negative control), 0.5, 1, and 1.5 µM SNP individually; In the middle panel, hRBCs were treated with 50 µM desipramine alone and in combination with 0.5, 1 and 1.5 µM SNP separately; In the lower panel, hRBCs were treated with 100 µM desipramine alone and in combination with 0.5, 1 and 1.5 µM SNP respectively.

2.3.5 Treatment of erythrocytes with different concentrations of desipramine and N-acetyl-L-cysteine

All preparations were done as described above. 0.5 M NAC stock solution was first made and diluted to 0.05 M and 0.25 M in the dark. Twelve 5 ml polystyrene round-bottom tubes were prepared and 2 ml Ringer solution was added to each tube by a pipette. Then 12 µl of red blood cells were transferred to each polystyrene round-bottom tube by a pipette gently. After mixing by vortex, 4 µl of H_2O, 0.05 M, and 0.25 M NAC stock solution were transferred to the hRBCs suspension by the group, and all the samples were vortexed for 10 seconds gently again to homogenize the erythrocytes with the NAC solution. After mixing, all tubes were moved to the incubator and incubated for 2 h. To ensure adequate contact between the erythrocytes and the NAC, the tubes should be inverted ten times every half hour. Meanwhile, the Des stock solution was prepared as described. After incubation, 4 µl of H_2O, 25 mM Des, and 50 mM Des were transferred to different flow tubes by

the group and all tubes were vortexed gently for 10 seconds to ensure adequate contact between the erythrocytes and the drugs. All the samples were then moved to the incubator and incubated again for 24 h before measurement **(Figure 10)**.

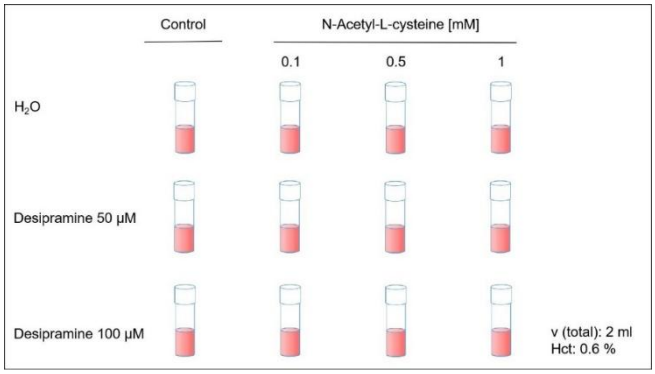


Figure 10: Treatment of human erythrocytes (hRBCs) with different concentrations of desipramine and N-acetyl-L-cysteine. In the upper panel, hRBCs were treated with H_2O (Negative control), 0.1, 0.5 and 1 mM N-acetyl-L-cysteine individually; In the middle panel, hRBCs were treated with 50 µM desipramine alone and in combination with 0.1, 0.5 and 1 mM N-acetyl-L-cysteine separately; In the lower panel, hRBCs were treated with 100 µM desipramine alone and in combination with 0.1, 0.5 and 1 mM N-acetyl-L-cysteine respectively.

2.3.6 Treatment of erythrocytes with different concentrations of desipramine, sodium nitroprusside, and N-acetyl-L-cysteine

All preparation procedures were done and Des, SNP, and NAC stock solutions were made as above. Twenty-four 50 ml test tubes were prepared and labeled, with 13 ml of Ringer solution and 78 µl blood added to each. First, 6.5, 13, and 19.5 µl of SNP stock solution were added to each sample in groups, and the tubes were then repeatedly inverted to ensure adequate mixing. After mixing, all samples were incubated in an incubator for 1 h. Secondly, after 1 h incubation, 26 µl of different concentrations of NAC were added to each sample according to the group, then all samples were transferred to the incubator for another 1 h of incubation. Then 2 ml of each sample were transferred to new 5 ml polystyrene round-bottom tubes in groups. In the end, 4 µl of Des stock solution at different concentrations were added to the samples in groups and then transferred to the incubator for 24 h, after which erythrocytes size and eryptosis were determined **(Figure 11)**.

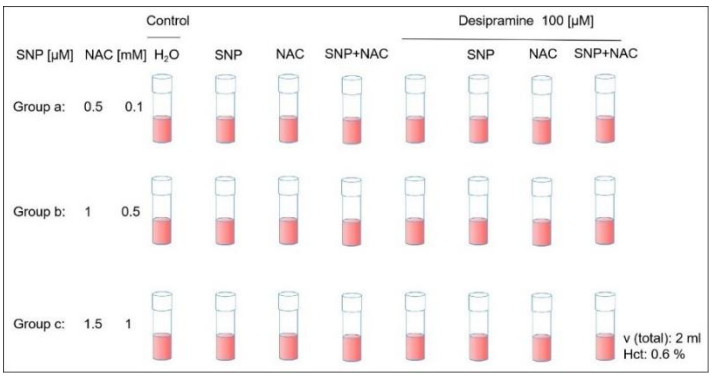


Figure 11: Treatment of human erythrocytes with different concentrations of desipramine, sodium nitroprusside, and N-acetyl-L-cysteine. Erythrocytes were treated with the lowest (0.5µM, 0.1mM), middle (1µM, 0.5mM) or highest (1.5µM, 1mM) SNP, NAC or their combination in the presence or absence of desipramine, H_2O-treated erythrocytes were used as the negative control.

2.3.7 Treatment of erythrocytes with different concentrations of desipramine in Ringer solution with or without calcium±EDTA or EGTA

All procedures were the same as 2.3.3, except the Ringer solution without Ca^{2+}, Ringer solution without Ca^{2+} with ethylene glycol tetraacetic acid (EGTA) (1 mM), and Ringer solution without Ca^{2+} with ethylenediaminetetraacetic acid (EDTA) (1 mM) was used throughout. Erythrocyte size and eryptosis were determined in the end **(Figure 12)**.

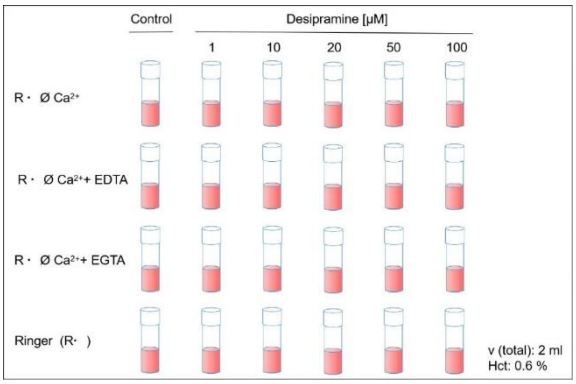


Figure 12: Treatment of human erythrocytes (hRBCs) with different concentrations of desipramine in Ringer solution with or without calcium (Ca^{2+}) ±EDTA or EGTA. In the upper panel, hRBCs were treated with H_2O (Control), 1, 10, 20, 50, and 100 µM desipramine in Ringer solution without Ca^{2+}; In the second panel, hRBCs were treated under the same conditions in Ringer solution without Ca^{2+} plus EDTA; In the third panel, hRBCs were treated with the same conditions in Ringer solution without Ca^{2+} plus EGTA; In the lower panel, hRBCs were treated with the same conditions in Ringer solution.

2.3.8 Measurement of GSH and GSSG of erythrocytes under the treatment of desipramine

All preparation procedures were done as described above. 50 mM Des stock solution was made and then diluted to the concentration required (0.5 mM, 5 mM, 10 mM, 25 mM). Six 50 ml tubes were prepared, 30 ml of Ringer solution and 180 µl blood was transferred to each tube. Then 60 µl H_2O and different concentrations of Des were added to the respective tubes, and the tubes were inverted 10 times to resuspend the blood. After mixing, all the tubes were moved to the incubator and incubated for 24 h. On the second day, 310 mM NEM stock solution was made in the dark at 37 °C in advance. After incubation, 56 µl 310 mM NEM stock solution was added to each tube and all the tubes were inverted for 2 min to ensure adequate contact between the erythrocytes and the NEM, then all the samples were centrifuged at 1000 ×g for 10 min at 10 °C. After centrifugation, the supernatant was discarded and the pellet was collected.

For the measurement of Hb determination, 10 µl of erythrocytes were diluted to a concentration of 1:200 for hemolysis (Di Iorio, 1981). For the measurement of GSH and GSSG, 120 µl erythrocytes were treated with an equal volume of trichloroacetic acid and the clear supernatant was collected. Then 50 µl supernatant was loaded onto HPLC and measured by Agilent series 1100 and the GS-NEM conjugate was displayed at 265 nm wavelength (Giustarini et al., 2011). GSSG was measured by the GSH recycling method at the spectrophotometer (Giustarini et al., 2013)

2.3.9 Measurement of GSH and GSSG of erythrocytes under the treatment of N-acetyl-L-cysteine

All preparation procedures were as described above and the 0.5 M NAC stock solution was made and diluted to 0.05 M and 0.25 M in advance. Four 50 ml tubes were prepared and 30 ml of Ringer solution was added to each tube, then 180 µl of blood was transferred to each 50 ml tube. 60 µl H_2O and

different concentrations of NAC were added to the respective tubes, and the tubes were inverted 10 times to ensure adequate contact between the erythrocytes and the NAC. After mixing, all the samples were moved to the incubator and incubated for 24 h. After incubation, 466 µl of 310 mM NEM stock solution was added to each tube and the tubes were inverted for 2 min, then all the samples were centrifuged at 1000 ×g for 10 min at 10 °C. Subsequently, cell pellets were used for GSH, and GSSG measurement as described above.

2.3.10 Reversed treatment of erythrocytes, i.e. inducer (desipramine) first and inhibitor (sodium nitroprusside) second

All preparation procedures were done and Des, SNP stock solutions were made as described in chapter 2.3.4. Six 5 ml polystyrene round-bottom tubes were prepared and labeled, with 2 ml of Ringer solution and 12 µl blood added to each tube. 4 µl of Des stock solution at different concentrations were added to the respective samples to get the required concentration (50 µM, 100 µM), and then moved to the incubator for 2 h. After incubation, 2 µl of SNP stock solution was added according to the group to get the required concentration (1 µM) and mixed. Then, all the samples were incubated for 24 h. In the end, erythrocyte size and eryptosis were determined as described below.

2.3.11 Measurement of erythrocytes hemolysis

After incubation of hRBCs with different concentrations of Des for 24 h as above, all the blood samples were taken out from the incubator and vortexed on a shaker. Then 600 µl of blood samples were transferred to the related tubes according to the group and centrifuged at 4° C, 2000 rpm for 4 min. The supernatants were harvested and transferred to a cell culture plate (96 wells) of 150 µl each with 3 wells per sample. The pellet of the negative control group was resuspended in 600 µl H_2O and incubated at room temperature for 10 min and then a serial dilution (100%, 50%, 25%, 12.5%,

6.25%, 3.125%, 1.56%) was prepared and used as standard. For the measurement of hemolysis, all the samples were detected at 405 nm photometrically and the average of the three measurements should be calculated (Ghashghaeinia et al., 2017).

2.3.12 Measurement of erythrocytes size with flow cytometry

Erythrocytes size was measured as described previously (Ghashghaeinia et al., 2017). 24 new 1.3 ml round bottoms were prepared and labeled, 2 tubes for each sample. Then the blood samples incubated for 24 h were taken out from the incubator and vortexed on a shaker. The corresponding cultured samples were added to the corresponding 1.3 ml round bottoms tubes of 200 µl each according to the labeled number.

2.3.13 Measurement of eryptosis with flow cytometry

Eryptosis was measured as described previously (Ghashghaeinia et al., 2017). 24 new 1.3 ml round bottoms were prepared and marked, 2 tubes for each sample and 500 µl Ax-WB were added to each tube. After all the preparations were done, the erythrocyte samples incubated for 24 h were taken out from the incubator and vortexed on a shaker ten times. Then the corresponding cultured samples were added to the corresponding 1.3 ml round bottoms tubes of 100 µl each according to the labeled number and then were transferred to a 4 °C refrigerator and incubated for 30 minutes. After incubation, all the blood samples were centrifuged at 4 °C, 3600 rpm for 5 min. Then the supernatant was discarded and the pellet was resuspended in the remaining Ax-WB (~50 µl) on a vortex. At the same time, the annexin binding buffer was made at a ratio of 1: 33 in the Ax-WB. Then 48 µl annexin binding buffer was added to each sample and vortexed for 10 seconds on a shaker and then all the samples were incubated at room temperature for 20 min. In the end, 200 µl Ax-WB was added to each sample. The whole process should be protected from light. Annexin-V binding was detected in the fluorescence

channel FL-1 (488 nm excitation, 530 nm emission). Samples were analyzed in two technical replicates and data averaged (Lang et al., 2005c).

2.4 Statistics

The values of the continuous variables in this experiment conformed to a Gaussian distribution. A one-way ANOVA and Dunnett's test were used to analyze the values (comparisons between the experimental and control groups). At least three independent experiments with different samples were performed for each data set, expressed as arithmetic mean ± SEM. Statistical significance was considered when the P value was less than 0.05. In this paper, the P-value is expressed as follows, $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***), and $P<0.0001$ (****).

3 Results

3.1 Desipramine can induce human erythrocytes to undergo eryptosis

In this experiment, we first investigated whether Des can induce eryptosis. When erythrocytes undergo eryptosis, PS is transferred from intracellular to extracellular, and the exposed PS is recognized by macrophages. Eryptotic erythrocytes are later on removed from the circulation. We used Annexin-V-FLUOS to detect eryptosis since Annexin-V-FLUOS is a phospholipid-binding protein that can recognize and bind to the exposed PS and thus detect eryptosis.

Here, human blood erythrocytes were treated with H_2O or 1, 10, 20, 50, 100 μM of Des, and Annexin-V binding was detected by flow cytometry after 24 (**Figure 13A**) and 48 h (**Figure 13B**). The eryptosis in each group obeyed normal distribution and the results of one-way analysis of variance (ANOVA) showed that compared with the control group, a significant increase in Annexin-V binding was observed after 24 h in 50 and 100 μM of Des-treated hRBCs, and the eryptosis of hRBCs treated with 50 and 100 μM of Des increased significantly after 48 h. The eryptosis was positively correlated with Des concentration in all cases. Taken together the results indicated that both 50 and 100 μM experimental doses of Des could induce eryptosis in erythrocytes and were dose-dependent (**Figure 13C**).

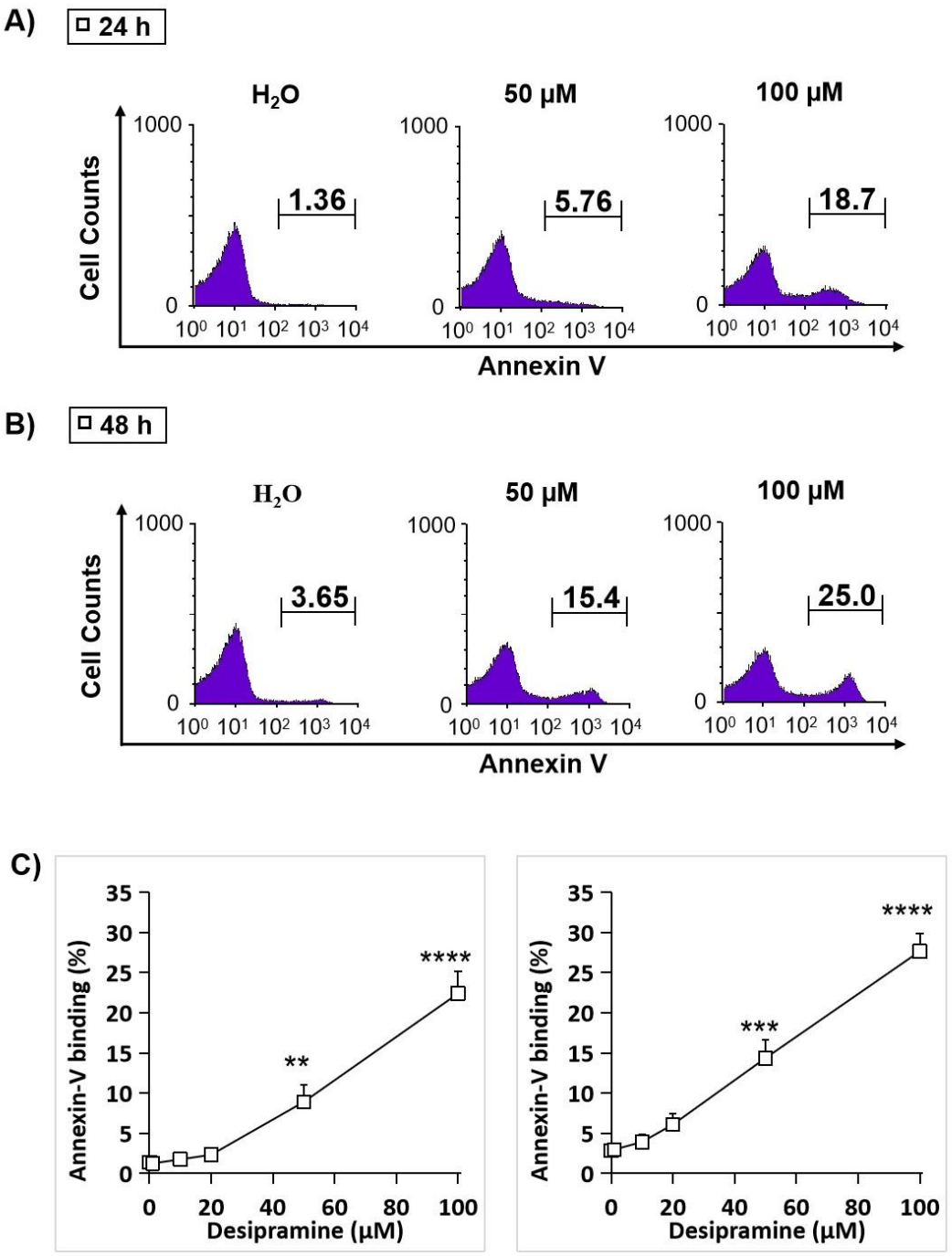


Figure 13: Induction of eryptosis by desipramine in human erythrocytes. A-B) Original histograms of eryptosis (annexin-V binding) in the absence (left, control) or presence of desipramine (50 µM middle) or (100 µM right) for 24 and 48 hours, respectively. C) Arithmetic mean ± standard error of the mean of the percentage of erythrocytes annexin-V binding in the absence (H_2O) and presence of desipramine (1, 10, 20, 50, 100 µM) for 24 and 48 hours (n = 4). P<0.05 (*), P<0.01 (**), P<0.001 (***), P<0.0001 (****).

3.2 Effect of desipramine on the cell size of human erythrocytes

In the next experiment, we also measured whether cell size changes in Des-treated erythrocytes. As mentioned above, cell shrinkage is a hallmark of eryptosis, but we found that this statement is not entirely true.

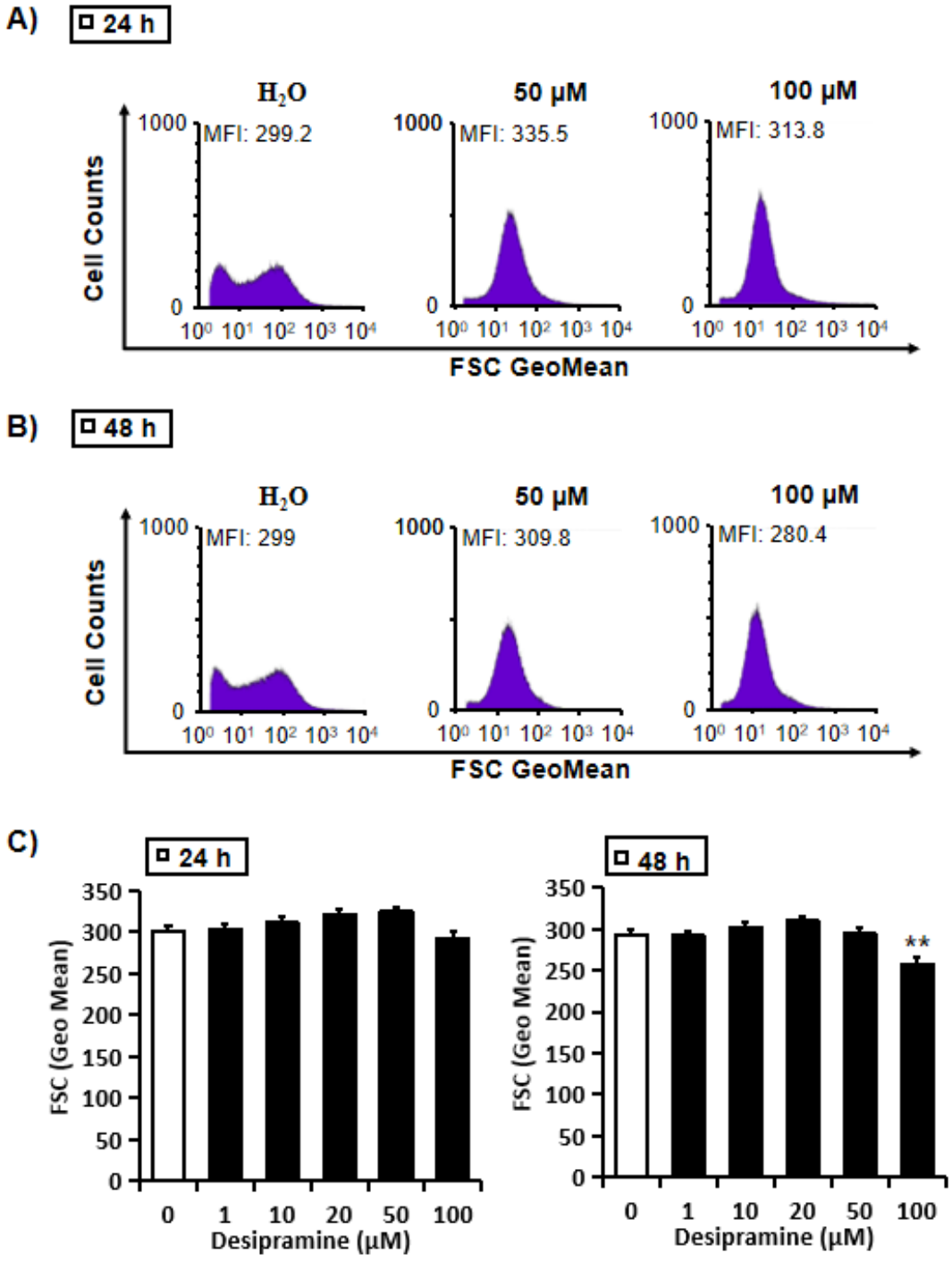


Figure 14: Alteration of cell size by desipramine in human erythrocytes. A-B) Original histograms of erythrocyte size in the absence (left, control) or presence of desipramine (50 µM

middle) or (100 µM right) for 24 and 48 hours, respectively. C) Arithmetic mean ± SEM (n = 4) of the size of the erythrocytes in the absence (H_2O) and presence of desipramine (1, 10, 20, 50, 100 µM) for 24 (left) and 48 hours (right).

Interestingly, cell volume changes were not significantly affected except for the highest Des concentration (100 µM) after 48 h incubation (**Figure 14**). In conclusion, Des-induced eryptosis (**Figure 13**) is not necessarily associated with cell shrinkage.

3.3 Effect of desipramine on the hemolysis of human erythrocytes

Eryptosis has an inextricable relationship with hemolysis (change in the integrity of the erythrocyte cell membrane). As we learned from the results in section 3.1. that Des can induce eryptosis, based on this, we measured the hemolysis of erythrocytes when erythrocytes are induced to undergo eryptosis by Des.

We found that after 24 and 48 h of incubation in hRBCs with Des, the hemolysis of erythrocytes at 10 µM tended to decrease slightly. However, at concentration >10 µM hemolysis of erythrocytes increased (**Figure 15**). There was a statistically significant difference at 100 µM Des as compared with the control group ($P< 0.01$) after 24 h, and at 50 µM and 100 µM after 48 h of incubation, Thus, at concentration >50 µM Des induced significant hemolysis albeit this effect is much weaker as compared with its pro-eryptotic effect.

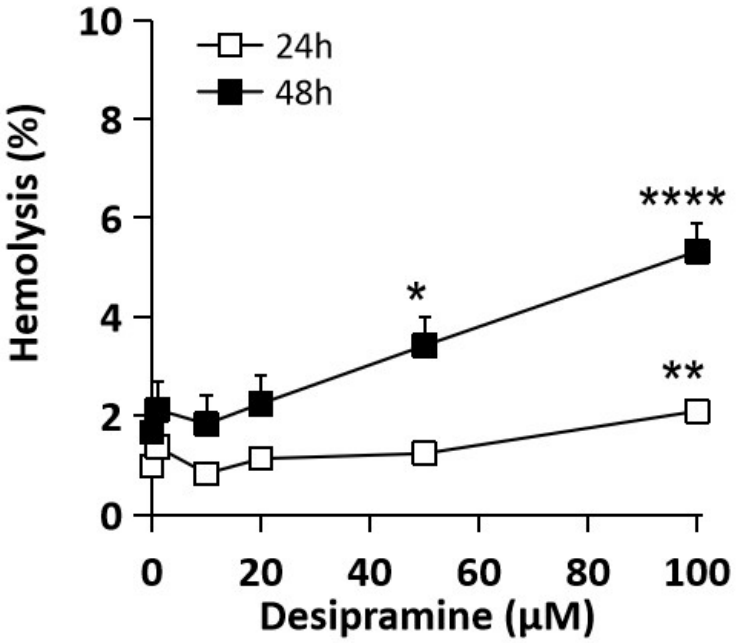


Figure 15: Induction of hemolysis by desipramine in human erythrocytes. Arithmetic means ± SEM (n = 4) of hemolysis in the absence (H_2O) and presence of desipramine (1, 10, 20, 50, 100 µM) (left to right) after 24 and 48 hours of incubation.

3.4 Sodium nitroprusside has an inhibitory effect on desipramine-induced eryptosis

As shown in the previous chapters, Des can be considered as an eryptosis-inducing drug. In the next experiments, we therefore explored whether this induction of eryptosis would be affected by NO donor SNP. We pretreated hRBCs with three different concentrations of SNP (0.5, 1 and 1.5 µM) for 2 h, and then incubated the erythrocytes with Des at a concentration of 100 µM for 24 h before measuring the annexin-V binding of the erythrocytes.

At a Des concentration of 50 µM, the annexin-V binding of erythrocytes was significant reduced by pretreatment with all concentrations of SNP. At a Des concentration of 100 µM, samples pretreated with high concentrations of SNP were significantly reduced as compared with Des-treated control group (**Figure 16**). We concluded from the data that NO donor SNP can inhibit Des-induced eryptosis.

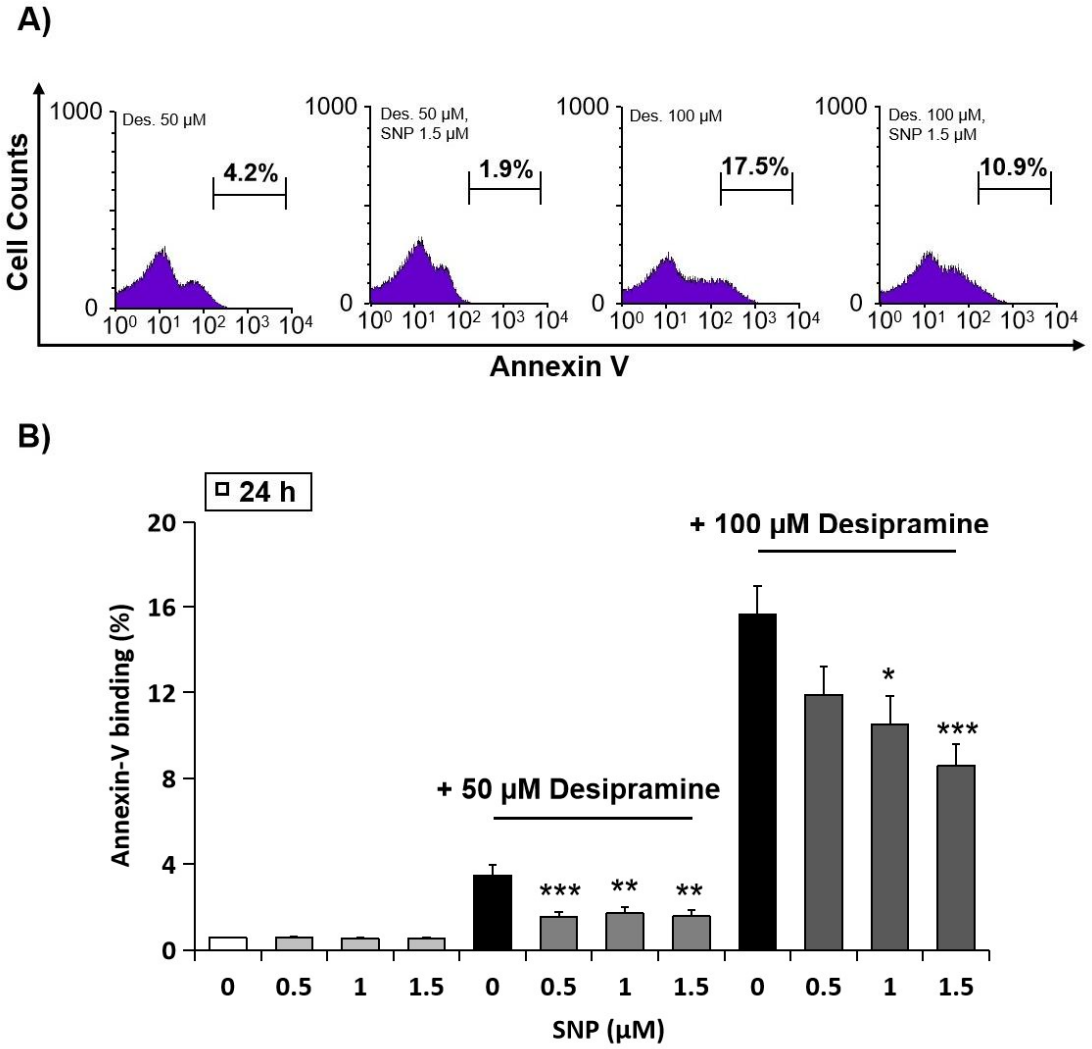


Figure 16: Effect of sodium nitroprusside on desipramine-induced eryptosis. A) Original histograms of erythrocytes annexin-V binding in the presence or absence of desipramine (50 µM or 100 µM) with SNP concentration of 0.5, 1 and1.5 µM. H_2O was used as control. B) Arithmetic mean ± SEM (n = 7) of the percentage of erythrocytes annexin-V binding in the H_2O (white column, control), SNP (light gray column), desipramine (black column) and the combination of SNP and desipramine (dark gray column) for 24 h.

3.5 N-acetyl-L-cysteine has an inhibitory effect on desipramine-induced eryptosis

Our next experiments further investigated whether Des-induced eryptosis could be affected by incubation in the presence of NAC. We pretreated hRBCs with three different concentrations of NAC (0.1, 0.5 and 1 mM) for 2 h, and then incubated the erythrocytes with two different concentrations of Des

(50 and 100 µM) for 24 h before measuring the annexin-V binding of the erythrocytes.

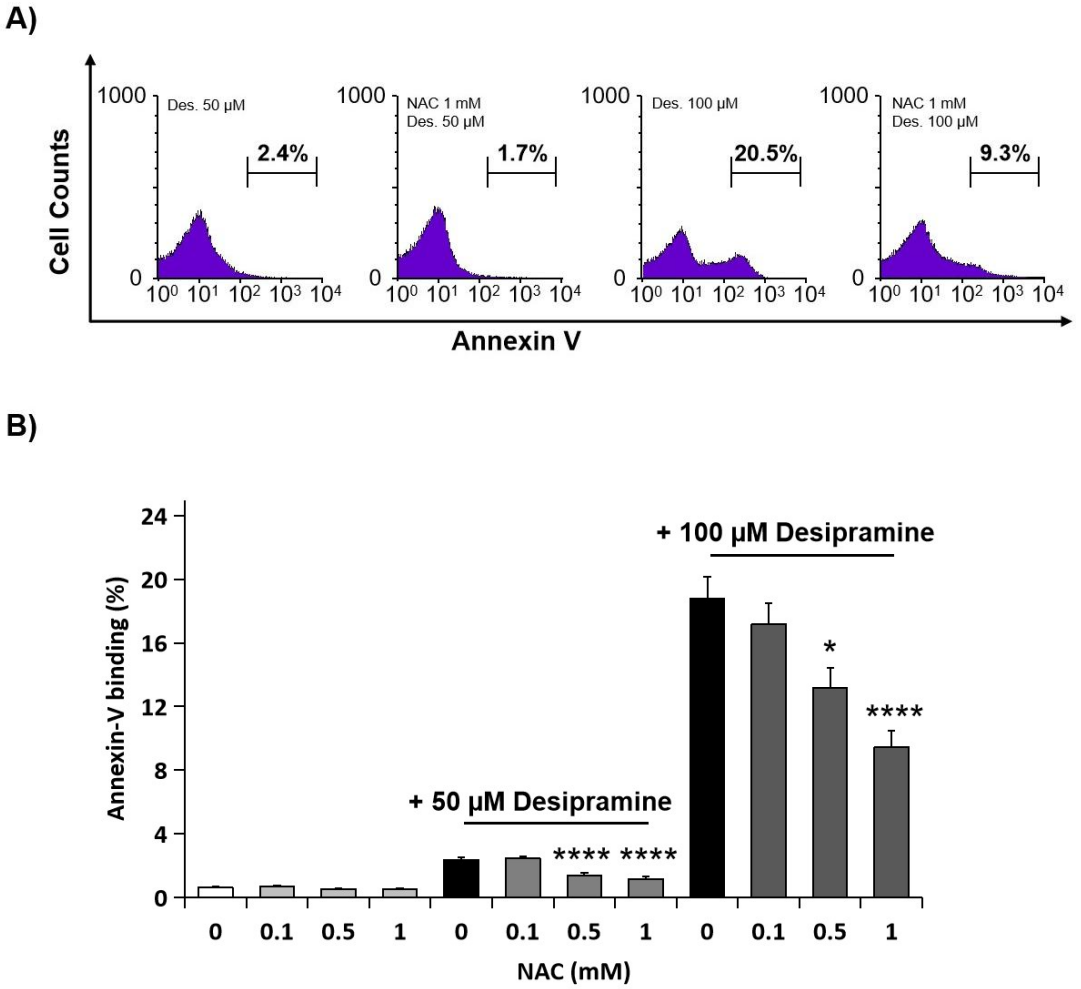


Figure 17: Effect of N-acetyl-L-cysteine on desipramine-induced eryptosis. A) Original histograms of erythrocytes annexin-V binding in the presence or absence of desipramine (50 µM or 100 µM) with NAC concentrations of 0.1, 0.5 and 1 mM. H_2O was used as solvent control. B) Arithmetic mean ± SEM (n = 4) of the percentage of erythrocytes annexin-V binding in the H_2O (white column, control), NAC (light gray column), desipramine (black column) and the combination of NAC and desipramine (dark gray column) for 24 h.

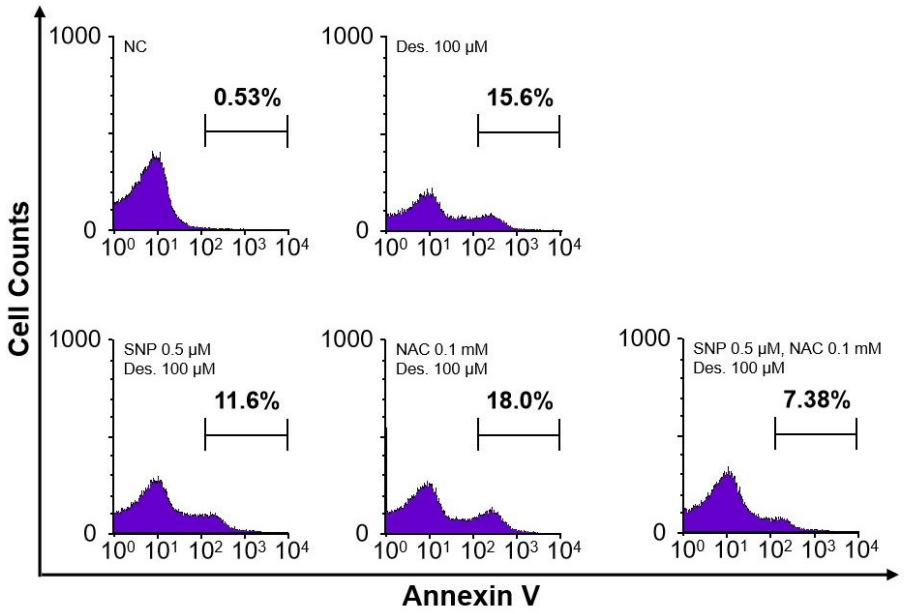
Annexin-V binding in erythrocytes pretreated with medium and high concentrations of NAC under two different concentrations of Des was significantly reduced compared with the Des-treated control group (**Figure 17**). From the experimental result, we can learn that NAC has an inhibitory effect on Des-induced eryptosis.

3.6 The combination of sodium nitroprusside and N-acetyl-L-cysteine has a better inhibitory effect on desipramine-induced eryptosis than their individual use

As both SNP and NAC have inhibitory effects on Des-induced eryptosis, we sought to further explore whether the combination of these two drugs would produce a better inhibitory effect. We pretreated hRBCs with three different concentrations of SNP (0.5, 1 and 1.5 µM) and NAC (0.1, 0.5 and 1 mM) for 2 h, and then incubated the erythrocytes with Des at a concentration of 100 µM for 24 h before measuring the annexin-V binding of the erythrocytes.

There was a significant decrease of the Annexin-V binding rate in erythrocytes pretreated with the combination of low (**Figure 18A and B**), medium (**Figure 18C and D**) and high (**Figure 18E and F**) concentrations of SNP and NAC compared to erythrocytes pretreated with SNP or NAC alone followed by application of Des. We could learn from these experiments that combined treatment with SNP and NAC had a better inhibitory effect on Des-induced eryptosis. This was especially true for moderate concentrations of SNP and NAC which had a better inhibitory effect on Des-induced eryptosis only when the inhibitors were combined.

A)



B)

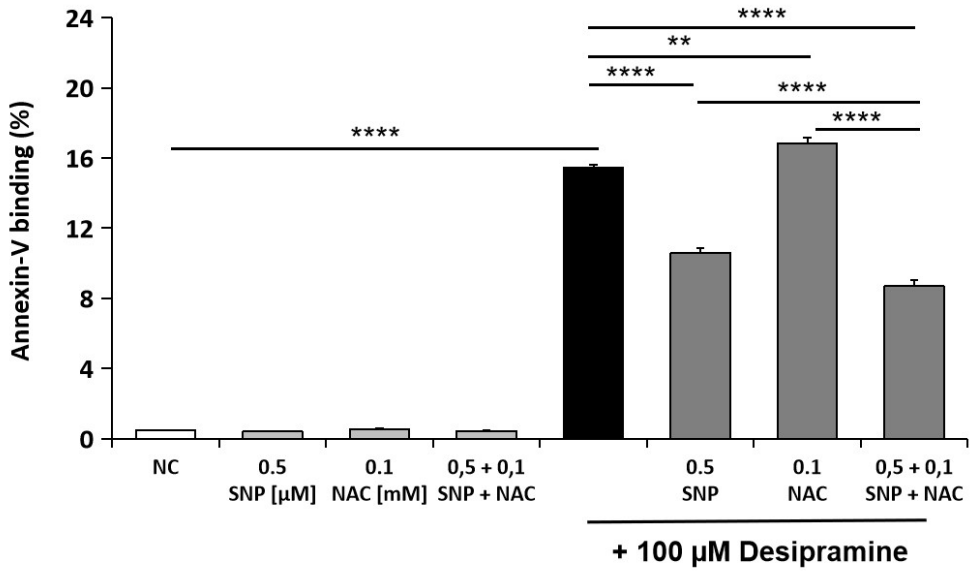
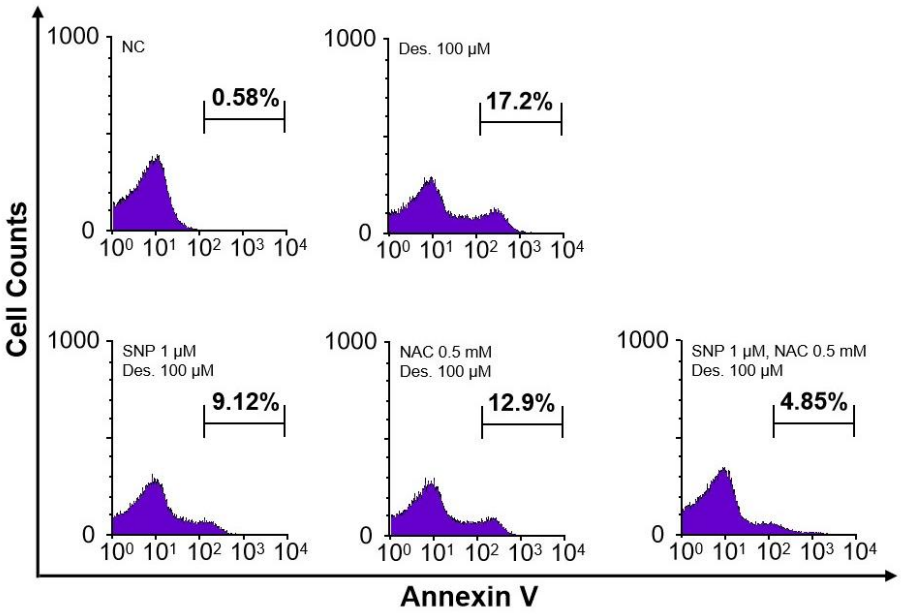


Figure 18: To be continued.

C)



D)

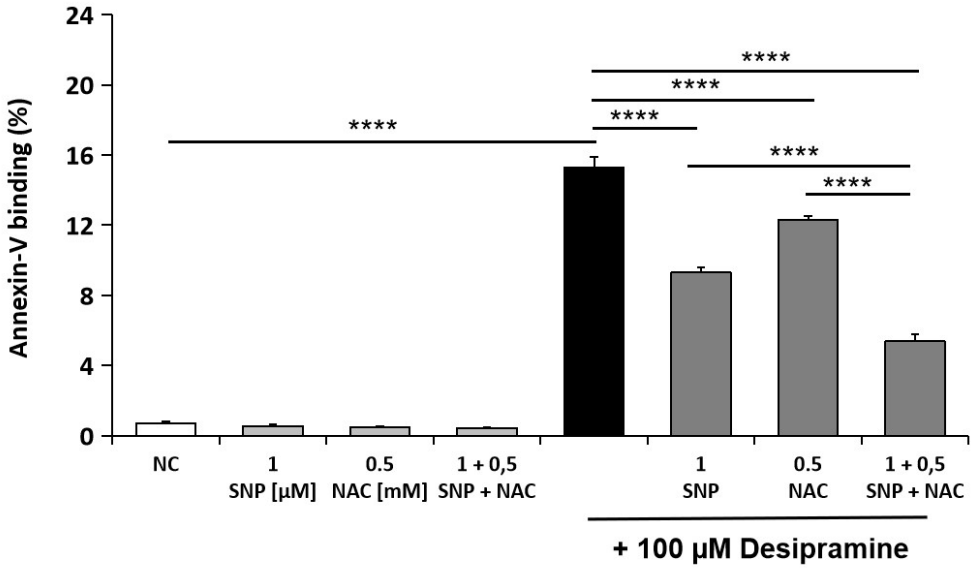
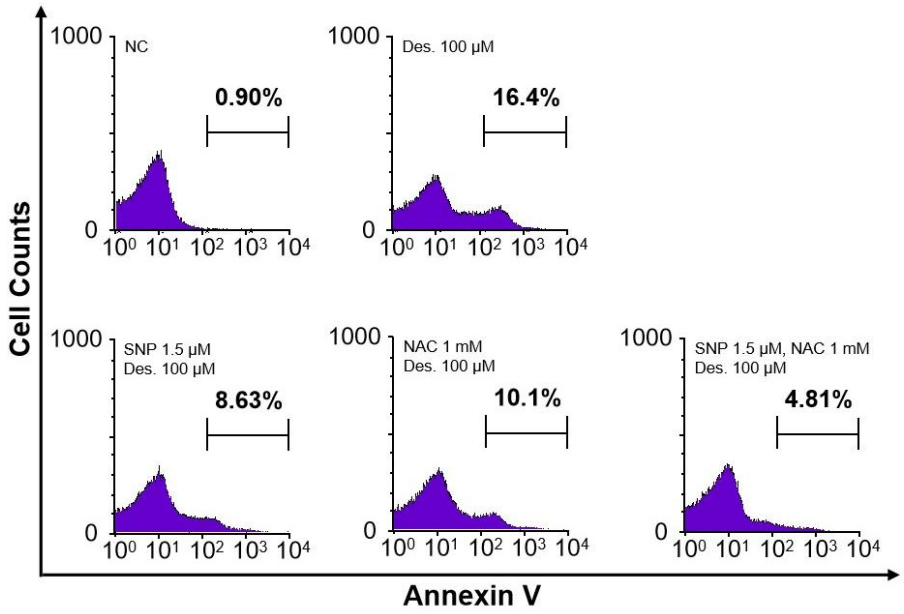


Figure 18: To be continued.

E)



F)

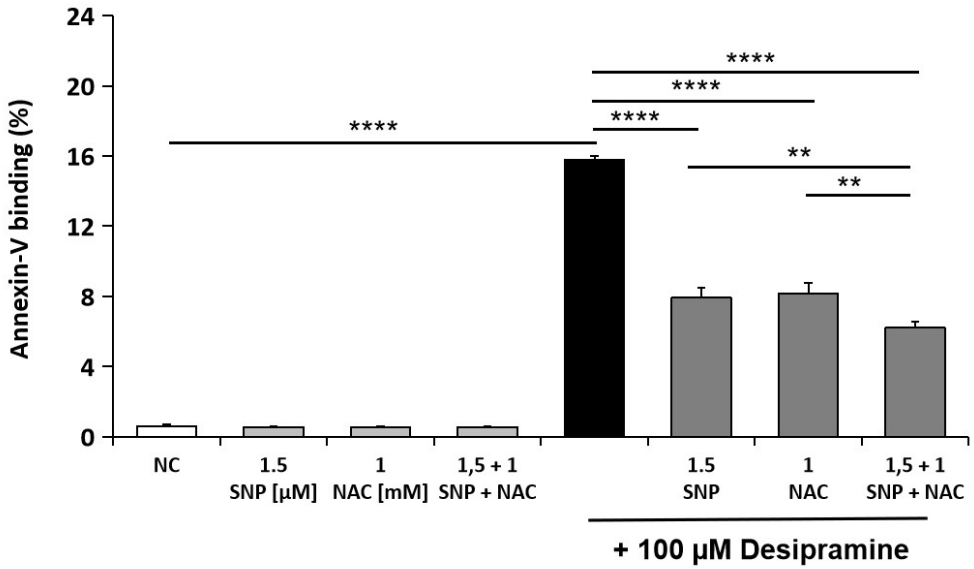


Figure 18: Concentration-dependent effect of N-acetyl-L-cysteine and sodium nitroprusside pretreatment on desipramine-induced eryptosis. A, C, E) Original histograms of erythrocytes annexin-V binding in the presence or absence of SNP (0.5, 1 and 1.5 µM), NAC (0.1, 0.5, 1 mM) and desipramine (100 µM). H_2O was used as control. B, D, F) Arithmetic mean ± SEM (n = 3) of the percentage of erythrocytes annexin-V binding in the H_2O (white column, control), SNP, NAC and the combination of both (light gray column), SNP NAC and the combination of both pretreatments on desipramine-induced eryptosis (dark gray column) and desipramine (black column) for 24 h.

3.7 Ca^{2+} depletion enhances desipramine-induced eryptosis

It is known that sustained increase of $[Ca^{2+}]_i$ is directly associated with eryptosis. Annexin-V binding requires calcium (Ca^{2+}). Prior to analysis, as noted in materials and methods, hRBCs were washed and incubated in 5 mM calcium-containing Ax-WB. Numerous studies by the Florian Lang group clearly showed that neither washing with nor incubation of hRBCs for 20 minutes in 5 mM Ca^{2+}-containing Ax-WB affected the eryptosis measurements. Most impressive were Ca^{2+} ionophore ionomycin-induced cell shrinkage and eryptosis whose effects were completely abolished in the absence of extracellular calcium, despite washing and incubation of erythrocytes in 5 mM Ca^{2+}-containing Ax-WB (Ghashghaeinia et al., 2017, Lang et al., 2003a).

In the next experiment, we intended to explore whether Ca^{2+} depletion influences Des-induced eryptosis. We measured the erythrocyte Annexin-V binding rate using four different Ringer Solutions (Ringer solution, Ringer solution without Ca^{2+}, Ringer solution without Ca^{2+} + EGTA, and Ringer solution without Ca^{2+} + EDTA) at the same five different concentrations of Des (1, 10, 20, 50, 100 µM) treated for 24 h. H_2O was used as solvent control.

Surprisingly, at 10 µM of Des, there was a significant increase in Annexin-V binding of erythrocytes cultured in Ringer solution without Ca^{2+} as compared with erythrocytes cultured in Ringer solution. At concentrations of 20 and 50 µM Des, this effect was even more pronounced. There was a significant increase in Annexin-v binding of erythrocytes cultured in Ringer solution without Ca^{2+}, Ringer solution without Ca^{2+} + EGTA, and Ringer solution without Ca^{2+} + EDTA as compared with those cells cultured in Ringer solution (**Figure 19**). From the results, we learned that Ca^{2+} depletion does not inhibit, but on the contrary, have a synergistic effect on Des-induced eryptosis.

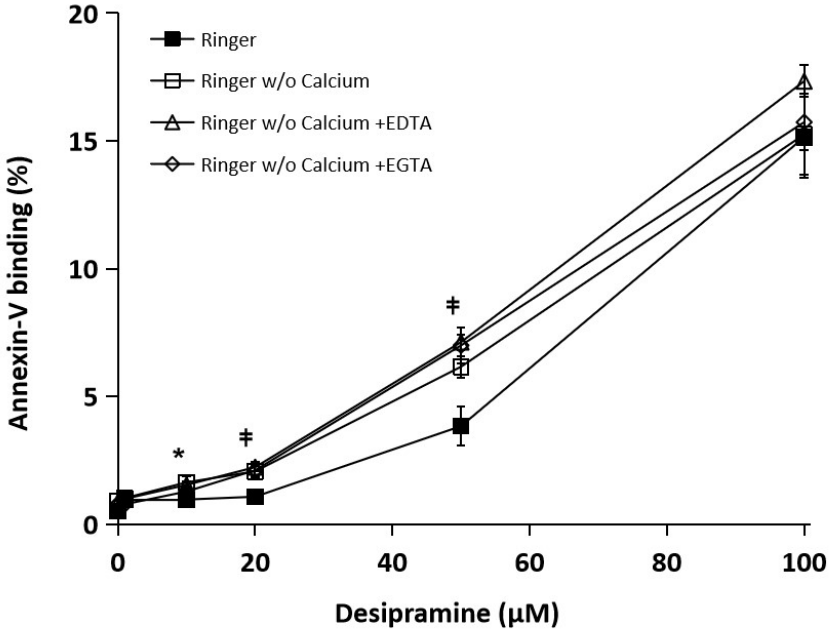


Figure 19: Effect of Ca^{2+} depletion on desipramine-induced eryptosis. Arithmetic means ± SEM of the Annexin-V binding in human erythrocytes treated with H_2O and five concentrations (1, 10, 20, 50, 100 µM) of desipramine (n=4) in four kinds of Ringer solution (Ringer solution, Ringer solution without Ca^{2+}, Ringer solution without Ca^{2+} + EGTA, and Ringer solution without Ca^{2+} + EDTA) for 24 h. H_2O was used as a solvent control. *$P < 0.05$, ǂ$P < 0.01$.

3.8 Under physiological condition neither desipramine nor exogenous addition of N-acetyl-L-cysteine (NAC) influence glutathione (GSH) level in human erythrocytes

The fact that NAC as precursor of cysteine significantly inhibited Des-induced eryptosis led us to consider a possible Des-induced intracellular GSH change/decrease in erythrocytes (**Figure 17, 18**). For this purpose, large amounts of hRBCs (180 µl RBCs / 30 ml Ringer solution) were incubated in the presence or absence of Des (1-100 µM) for 24 h and subsequently GSH and GSSG contents as well as GSH/GSSG-ratio were determined. The result was surprising as we could not detect any Des-induced change in intracellular glutathione concentration. The logical consequence would be that under physiological conditions, i.e. 5 mM glucose-containing Ringer solution neither Des nor its many possible metabolites such as 2-OH-Des are capable to

affect the glutathione level of intact hRBCs. We were even more surprised as under the same conditions, the addition of NAC (0.1-1 mM) had no effect on the GSH concentration (**Figure 20**). However, plausible explanations emerged from the literature search. Several research studies indicate that exogenous addition of NAC and its biological/physiological effects may not necessarily be associated with an increase in intracellular GSH concentration (Jones et al., 1995, Patten et al., 2021, Steenvoorden and van Henegouwen, 1998, Echeverri-Ruiz et al., 2018). Moreover, incorporation of glycine into GSH of intact hRBCs proceeds rapidly in the absence of glucose and is inhibited by glucose (York et al., 1982). In addition, simultaneous dietary supplementation of elderly subjects with two amino acid precursors of GSH – glycine and cysteine – stimulates and boosts GSH synthesis in erythrocytes (Sekhar et al., 2011). All this leads us to the following statement: under physiological conditions, the addition of solely one of the three GSH components has no influence on GSH de novo biosynthesis.

The GSH/GSSG-ratios we obtained (i.e. about 500-600 for Des and 590-680 for NAC) are nearer to the physiological ones measured in fresh bloods (i.e. a GSH/GSSG-ratio of approx. 800). As mentioned in section: materials and methods, these experiments and measurements were carried out in Italy at the working group of Professor Rossi. The whole procedure is previously reported in Nature Protocols (Giustarini et al., 2013). These GSH/GSSG-ratios also speak for the excellent storage conditions of the erythrocytes concentrates we received in Tübingen from Transfusion Medicine.

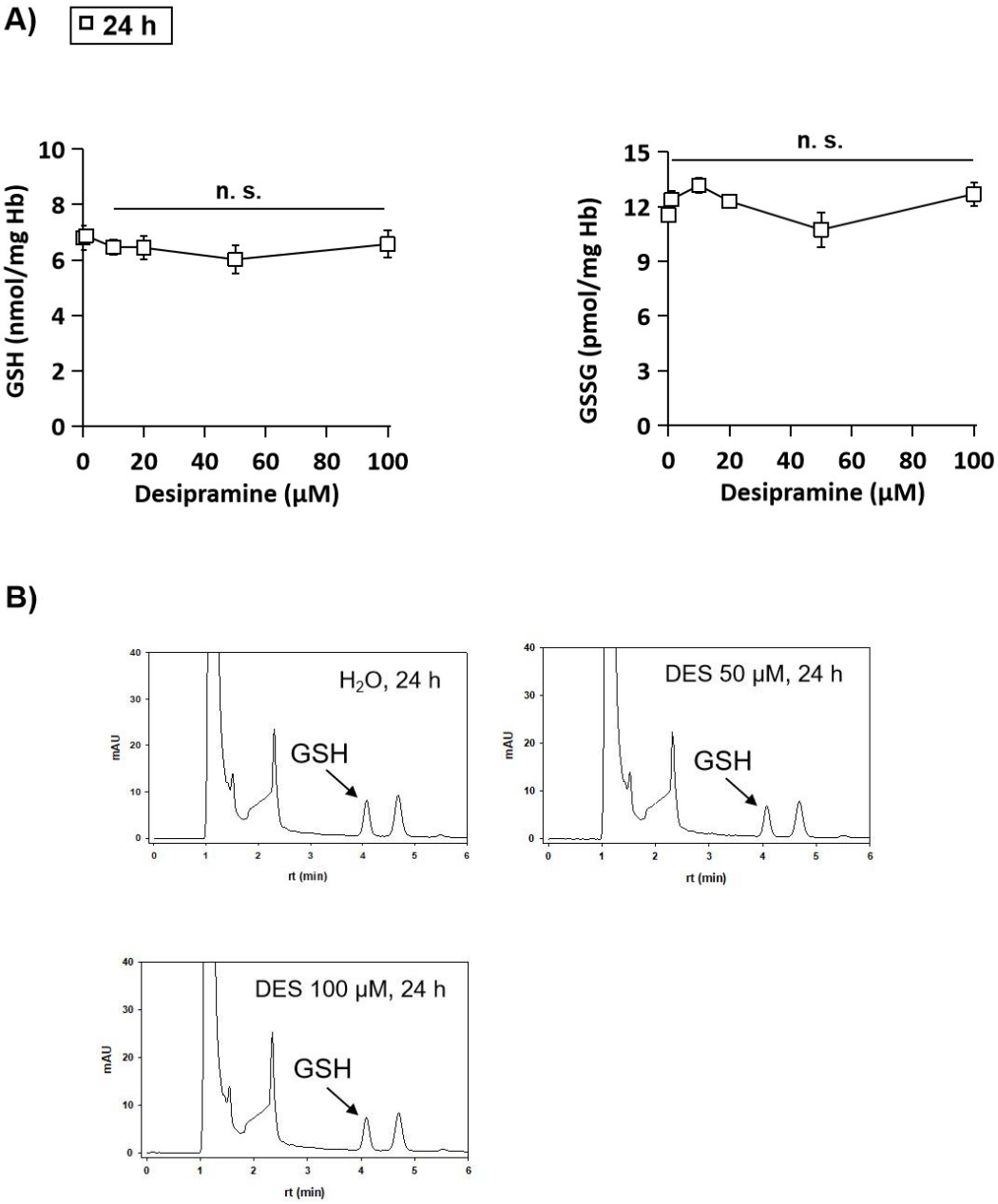


Figure 20: To be continued.

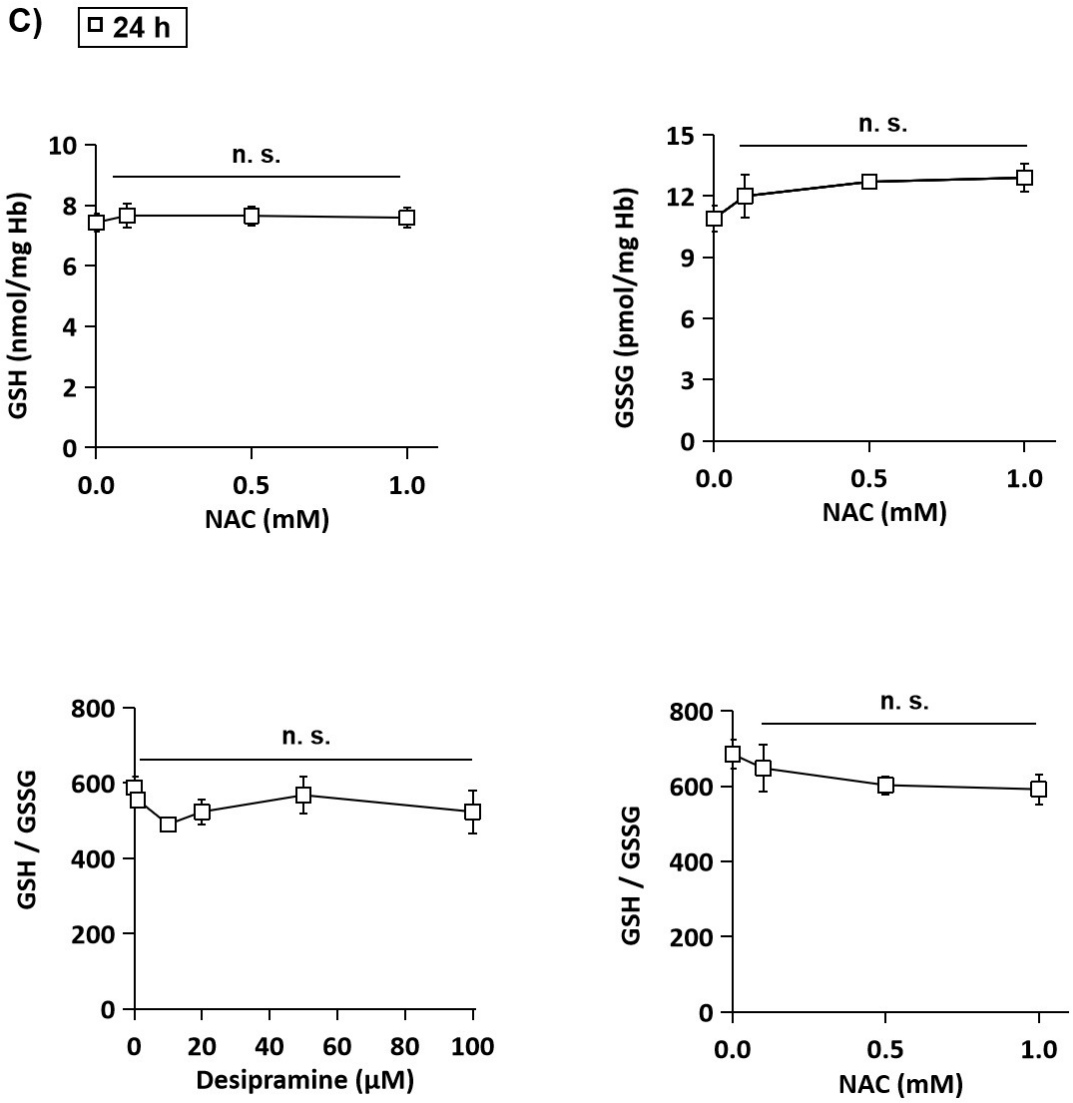


Figure 20: Effect of desipramine and N-acetyl-L-cysteine on the GSH, GSSG levels and GSH/GSSG-ratios of human erythrocytes. A and C) Arithmetic means ± SEM of the GSH, GSSG levels and GSH/GSSG-ratios in human erythrocytes (hRBCs) treated with H_2O and five concentrations (1, 10, 20, 50, 100 µM) of desipramine (n=4) or three concentrations (0.1, 0.5, 1 mM) of NAC (n=3) for 24 h. B) Original chromatograms of GSH in hRBCs after 24 h of desipramine treatment. The area under the curve of the peak indicated by the arrow in the graph is the detected level of GSH.

3.9 Inhibitory effect of sodium nitroprusside on desipramine-induced eryptosis is independent of the sequence of treatment

Previously we have shown that Des-induced eryptosis can be inhibited in the presence of SNP pretreatment. In the next experiment we thus tried to explore whether Des-induced eryptosis can be intervened by post application of SNP.

We pretreated the erythrocytes with two concentrations of Des (50, 100 µM) for 2 h, followed by further treatment with 1 µM of SNP for the entire incubation time of 24 h. Again, H_2O was used as solvent control. After incubation for 24 h, we measured the Annex-V binding of erythrocytes (**Figure 21**).

There was a significant decrease of Annexin-V binding rate in erythrocytes pretreated with Des followed by the application of SNP as compared with erythrocytes treated with Des alone (**Figure 21**). We can learn from the experiments that the SNP-treatment can inhibit Des-induced eryptosis in erythrocytes even when erythrocytes have already been intoxicated by Des.

A)

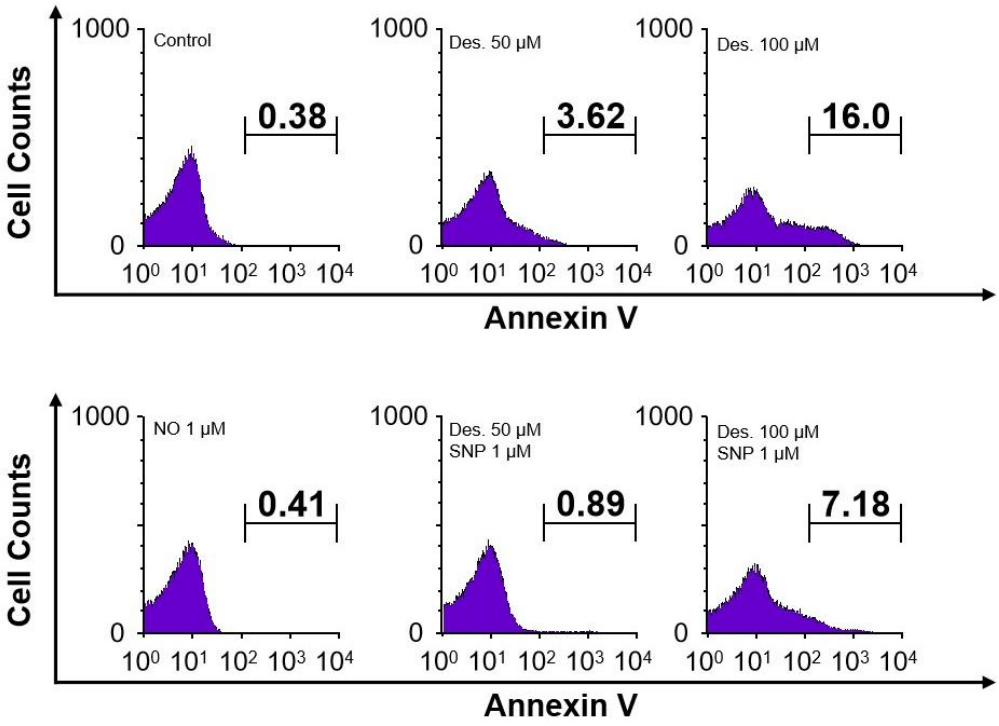


Figure 21: To be continued.

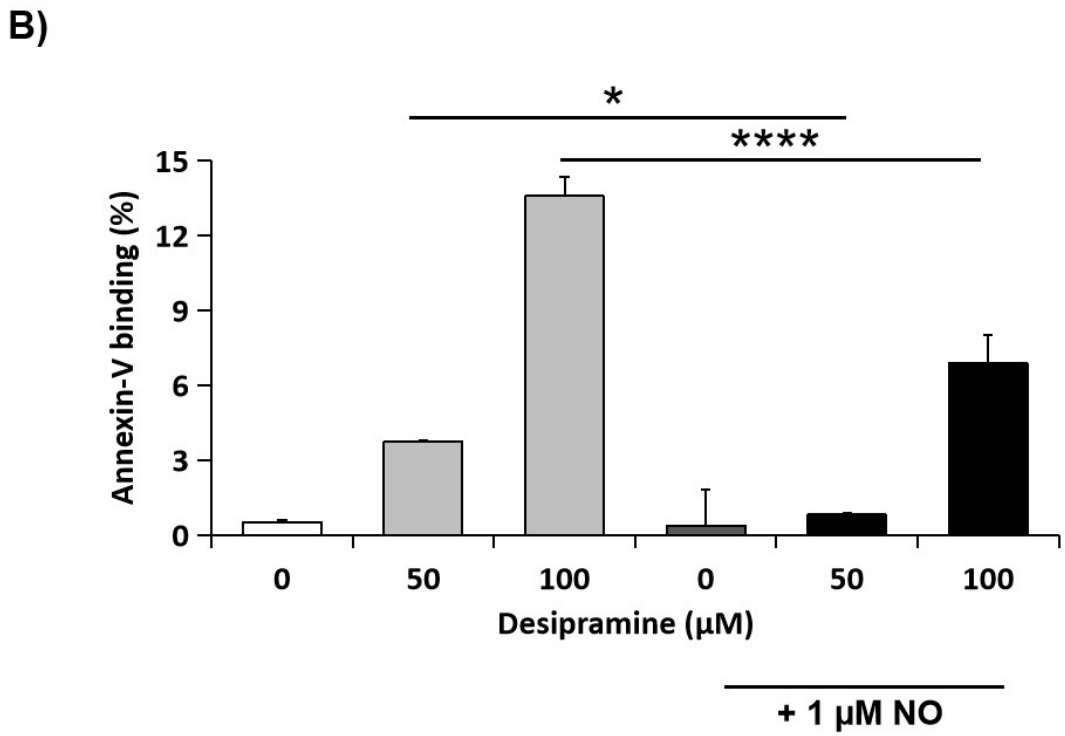


Figure 21: Inhibitory effect of sodium nitroprusside on desipramine-induced eryptosis. A) Original histograms of erythrocytes annexin-V binding in the presence or absence of desipramine (50 µM or 100 µM) with post-treatment of SNP (1 µM). H_2O was used as a control. B) Arithmetic mean ± SEM (n = 3) of the percentage of erythrocytes annexin-V binding in the H_2O (white column, control), desipramine (light gray column), SNP (dark gray column) and the pretreat of desipramine then followed by application of SNP (black column) for 24 h.

4 Discussion

To maintain the normal physiological functions of various tissues and organs of the body, the cell size needs to be maintained within a fixed range. However, cell size will change when intracellular and extracellular osmotic pressure changes. To keep this change in a physiological balance, shrunken and swollen cells may regain their original volume by regulatory volume increase (RVI) and regulatory volume decrease (RVD), respectively (Hoffmann et al., 2009). Interestingly, cell volume changes were not significantly affected except for the highest Des concentration (100 µM) after 48 h incubation (**Figure 14**).

To our knowledge, there are no studies related to the induction of eryptosis by Des. One important function of Des is its inhibitory effect on ASMase, the key enzyme that catalyzes sphingomyelin (SM) degradation to ceramide (CER) (Kölzer et al., 2004, Liu and Anderson, 1995). CER is then further deacylated by alkaline ceramidase 2 (ACER2) to sphingosine (SPH), which is phosphorylated by SPH kinase 1 to form SPH-1-P (S-1-P). However, in the SM-CER-SPH-S-1-P pathway, the two metabolites CER and SPH induce eryptosis (Maceyka et al., 2002). On the other hand, S-1-P binds to the corresponding ligands to regulate cell migration (e.g. T cells), proliferation and differentiation (Kariya et al., 2005). Normally, erythrocytes do not possess ASMase (Hofmann et al., 2000). However, when patients have inflammatory diseases (e.g. Wilson Disease and Multiple Sclerosis), erythrocytes can receive ASMase released by platelets, T cells, and EC (Awojoodu et al., 2014, Kempe et al., 2007, Kornhuber et al., 2015), which in turn induces eryptosis via the SM-CER-SPH-S-1-P pathway. Thus, Des may inhibit eryptosis by inhibiting ASMase (**Figure 22**).

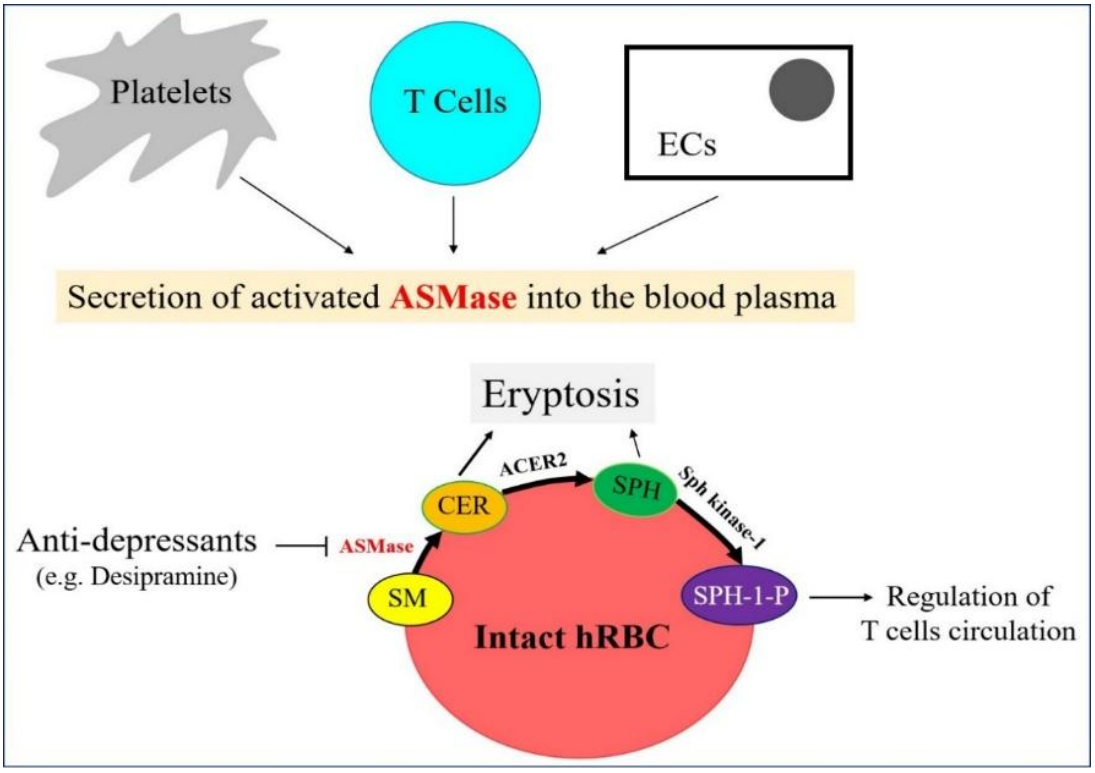


Figure 22: Sphingomyelin metabolism in human erythrocytes. When patients have inflammatory diseases, human erythrocytes (hRBCs) will undergo eryptosis induced by ASMase that released by other cells, which can inhibit by desipramine. ECs: endothelial cells.

In our study, we found that Des can instead induce eryptosis. The eryptosis rate is concentration-dependent and ranged from 1.4% to 22.4% after 24 h of treatment with gradually increasing concentrations of Des. After 48 h, the eryptosis rate still increased ranging from 2.84% to 27.6% after 48 h of treatment under the same conditions (**Figure 13**). To further investigate the mechanism of eryptosis induced by Des, we first measured the hemolysis of erythrocytes in the presence of Des.

Some drugs e.g. nanoparticles concentration-dependently rupture the membrane of erythrocytes, thus triggering hemolysis and the release of erythrocyte contents e.g. Hb into the blood plasma (Dreischer et al., 2022). In our research, we found that the hemolysis rate of erythrocytes ranged from 0.98% to 2.09% after 24 h of treatment with gradually increasing concentrations of Des, while the hemolysis rate ranged from 1.67% to 5.3% after 48 h of treatment under the same conditions. Taking into consideration

the eryptosis rate of erythrocytes induced by the highest concentration of Des (100 µM) after 24 and 48 h of incubation, i.e. 22.4% after 24 h and 27.6% after 48 h, the eryptosis: hemolysis ratio was 10.7 and 5.2, respectively. This suggests that Des does not severely impair the erythrocyte membrane. Fišar et al. showed the enrichment of imipramine and Des in the plasma membrane of erythrocytes (Fišar et al., 1996). Des localization/distribution is not only restricted to the cell membrane. It is known that Des influences the functions of subcellular compartments, e.g. lysosomes (Kölzer et al., 2004, Kuzu et al., 2017, Allemailem et al., 2021), mitochondria (Luo et al., 2014, Corda et al., 2001, Levitsky et al., 2020, Abdel-Razaq et al., 2011, Chan et al., 2020, Hroudova and Fisar, 2010, Hroudová and Fišar, 2011), endoplasmic reticulum (Ma et al., 2011, Mao et al., 2019) and nucleus (Pallis et al., 2001), for more details see **Figure 3** (page 16).

Since the eryptosis hemolysis ratio of Des incubation for 24 h was much higher than that of 48 h, the 24 h incubation time was chosen in the subsequent experiments.

NO is an important effector molecule in the human body and can be dose-dependently involved in mediating apoptosis (Bao et al., 2007, Taylor et al., 2003, Haendeler et al., 1997). As the biological precursor of NO, L-arginine is first hydroxylated and then oxidized under the catalyst of NOS with the substrate of NADPH and O_2 to produce NO and L-citrulline (Alderton et al., 2001, Stuehr, 2004). In the EC, NOS can be influenced by many vasoactive molecules, e.g. acetylcholine (Ach) binds to the corresponding receptor, leading to stimulation of NO production by stimulating NOS (Furchgott, 1999, Wilson et al., 2016). However, Des has an inhibitory effect on the nicotinic AchR (Shytle et al., 2002, Rana et al., 1993), and thereby impairs the NO synthesis process. Of note, hRBCs possess eNOS and arginine (Kleinbongard et al., 2006, Ikemoto et al., 1989). Of note, hRBCs possess functional AchR (Bennekou, 1993, Huestis and McConnell, 1974).

Interestingly, hRBCs possess AchE (Rosenberry and Scoggin, 1984). In the present study we could clearly show that exogeneous addition of NO donor SNP significantly inhibited Des-induced eryptosis (**Figure 16**). How and to what extent AchR is implicated in Des-induced eryptosis needs to be investigated in future studies.

It has been reported that some TCAs such as Des and amitriptyline can induce apoptosis by stimulating ROS production and damaging mitochondria through oxidative stress (Lee et al., 2015). NAC, as a precursor of GSH, may counteract oxidative stress due to its well-known antioxidant effect (Bavarsad Shahripour et al., 2014). Therefore, we speculated whether NAC could inhibit Des-induced eryptosis. Our results show that the eryptosis rate of hRBCs pretreated with Des was significantly decreased by the intervention of moderate and high concentrations of NAC (**Figure 17**). However, the GSH levels of erythrocytes cultured with Des and NAC alone were not significantly altered (**Figure 20**). This suggests that Des-induced eryptosis was not triggered by GSH depletion. Therefore, we are convinced that in our experimental setup, (i.e., under physiological conditions as present of 5 mM glucose) NAC effects are due to its thiol disulfide exchange activities rather than the commonly accepted NAC-mediated GSH synthesis (for more details see section 3.8). We also found a significant decrease in the rate of eryptosis after Des pretreatment followed by dual treatment (SNP+NAC) as compared with the mono treatment. Co-treatment of the middle concentrations of SNP (1 µM) and NAC (0.5 mM) exhibited the most remarkable effect on suppressing Des-induced eryptosis (**Figure 18 C and D and Figure 23**), although their individual inhibitory mean values were smaller than the individual inhibitory mean values of the highest concentrations of SNP (1.5 µM) and NAC (1 mM) (**Figure 18 E and F**). The weakest inhibitory effect on Des-induced eryptosis, although highly significant, were obtained with the lowest concentrations of SNP (0.5 µM) and NAC (0.1 mM) (**Figure 18 A and B**). Our results indicate:

regardless of the inhibitors concentrations used, their co-treatment exerted (at least) an additive inhibitory effect (**Figure 18 A-E**).

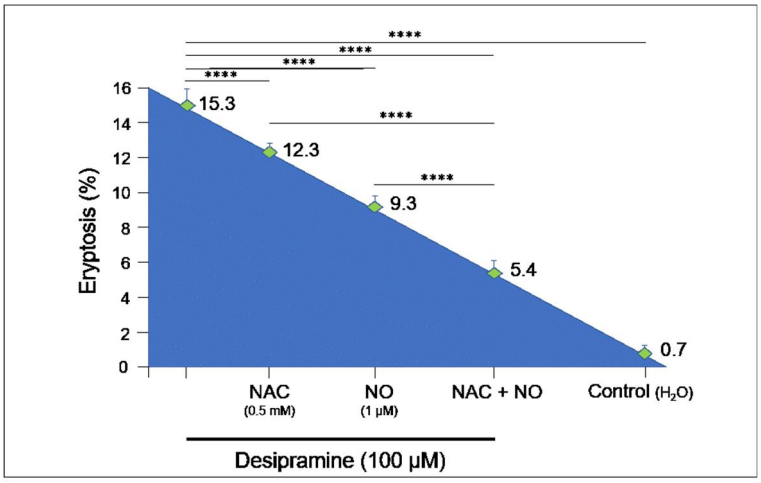


Figure 23: Co-treatment of human erythrocytes with SNP and NAC has additive inhibitory effect on desipramine-induced eryptosis. For a better and faster overview, I have presented Figure 18 C and D in this new form (see page 57 and section 3.6).

We have already mentioned above that Ca^{2+} is a key trigger and regulator of apoptosis and eryptosis. When apoptosis occurs in nucleated cells, the activation of Ca^{2+} channels in the cell membrane and the endoplasmic reticulum leads to an increase of $[Ca^{2+}]_i$, which causes several reactions finally leading to apoptosis (Hajnóczky et al., 2003). However, when eryptosis occurs in organelle-free erythrocytes, the increase of $[Ca^{2+}]_i$ is induced by the activation of Ca^{2+} channels in the cell membrane, which triggers a Ca^{2+} inward flow (Foller et al., 2009). This leads us to wonder whether Des-induced eryptosis is Ca^{2+}-dependent. Interestingly, we found that erythrocytes cultured in Ca^{2+}-free Ringer solution even had a higher rate of eryptosis after Des intervention (**Figure 19**). This suggests that the induction of eryptosis by Des is not Ca^{2+}-dependent. Des-induced eryptosis is more severe in the presence of Ca^{2+} depletion. This may be related to the possibility that the enzymes associated with the metabolism of Des in erythrocytes are Ca^{2+}-dependent. It is worth noting that this should be considered when Des is applied in patients suffering from hypocalcemia.

Reverse treatment: in our final experiment, we first treated hRBCs with the highest concentration of Des (100 µM) to mimic the situation after a clinical drug overdose, and then cultured erythrocytes with SNP to check its possible inhibitory effect on Des-induced eryptosis. In fact, SNP was able to significantly inhibit Des-induced eryptosis by 49.4% (13,57 vs. 6,87%) (**Figure 21**). In conclusion, SNP is able to neutralize an ongoing Des-induced eryptosis.

5 Summary

5.1 Summary in English

Depression is currently the most common psychiatric disorder, and its prevalence is increasing annually. One of the primary targets of tricyclic antidepressants (TCAs) among them also desipramine (Des) are serotonin and norepinephrine transporters (SERT and NET). Des interaction with NET preferentially increase norepinephrine (NE) transmission by inhibiting NE reuptake, thus relieving depressive symptoms. Overdose of Des causes systemic intoxication. Until today there is no effective treatment for this condition and many patients die yearly due to drug abuse. Therefore, further studies on Des may provide new therapeutic possibilities to combat drug abuse. Erythrocytes represent the largest cell population in our body and permanently interact with other organs. Thus, drugs changing the biological activities of erythrocytes, directly influence the whole organism. Therefore, we aimed to investigate a) the relationship between Des and eryptosis, and b) shed light on the underlying mechanisms. Experiments such as annexin V binding, hemolysis and glutathione measurement were performed to determine eryptosis, cell membrane integrity and redox status of erythrocytes after treatment with Des. Interestingly, calcium depletion led to the enhancement of Des-induced eryptosis. We also checked the possible inhibitory effects of both inhibitors the NO donor sodium nitroprusside (SNP) and N-acetyl-L-cysteine (NAC) on Des-induced eryptosis. Indeed, single treatment of erythrocytes with SNP and NAC could significantly inhibit Des-induced eryptosis and their co-treatment had the highest inhibitory effect. The most important in vitro findings of this study are: SNP and NAC are able to inhibit an ongoing Des-induced eryptosis.

5.2 Summary in German

Depressionen sind derzeit die häufigste psychiatrische Störung, und ihre Prävalenz nimmt jährlich zu. Einer der primären Angriffspunkte von trizyklischen Antidepressiva (TCAs), darunter auch Desipramin (Des), sind Serotonin- und Noradrenalin-Transporter (SERT und NET). Die Interaktion von Des mit dem NET erhöht vorzugsweise die Verfügbarkeit von Noradrenalin (NE) durch Hemmung der NE-Wiederaufnahme, wodurch depressive Symptome gelindert werden. Eine Überdosierung von Des führt jedoch zu einer Reihe von schwerwiegenden Folgen wie z.B. systemischen Intoxikation. Bis heute gibt es keine wirksame Behandlung für diesen Zustand. viele Patienten sterben jedes Jahr an den Folgen des Medikamenten-missbrauchs. Daher könnten weitere Studien über Des neue therapeutische Möglichkeiten zur Bekämpfung des Medikamenten-missbrauchs bieten. Die Erythrozyten stellen die größte Zellpopulation in unserem Körper dar und stehen in ständiger Wechselwirkung mit anderen Organen. Daher Medikamente, die die biologischen Aktivitäten der Erythrozyten verändern, üben einen direkten Einfluss auf den gesamten Organismus aus. Unser Ziel war es daher, a) den Zusammenhang zwischen Des und Eryptose zu untersuchen, und b) die zugrunde liegenden Mechanismen aufzuklären. Experimente wie Annexin-V-Bindungen, Hämolyse und Glutathion-Messung wurden durchgeführt um Eryptose, Zellmembran-Integrität und Redox-Status der Erythrozyten nach Behandlung mit Des zu ermitteln. Interessanterweise führte Calciumdepletion zur Verstärkung der Des-induzierten Eryptose. Wir überprüften auch die mögliche hemmende Wirkung der beiden Inhibitoren des NO-Donors Natriumnitroprussid (SNP) und N-Acetyl-L-Cystein (NAC) auf die Des-induzierte Eryptose. Tatsächlich konnte die Einzelbehandlung der Erythrozyten mit SNP bzw. NAC die Des-induzierte Eryptose signifikant hemmen und eine gemeinsame Behandlung zeigte die stärkste hemmende Wirkung. Die wichtigsten in-vitro-Ergebnisse dieser Studie sind: SNP und

NAC sind in der Lage, eine laufende bzw. stattgefundene Des-induzierte Eryptose zu unterbinden.

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7 Declaration of contribution

This work was carried out at the Institute of Physiology of the Medical Faculty at the University of Tübingen under the supervision of Prof. Dr. rer. nat. Thomas Wieder and Dr. Mehrdad Ghashghaeinia.

The study was designed by Dr. Mehrdad Ghashghaeinia.

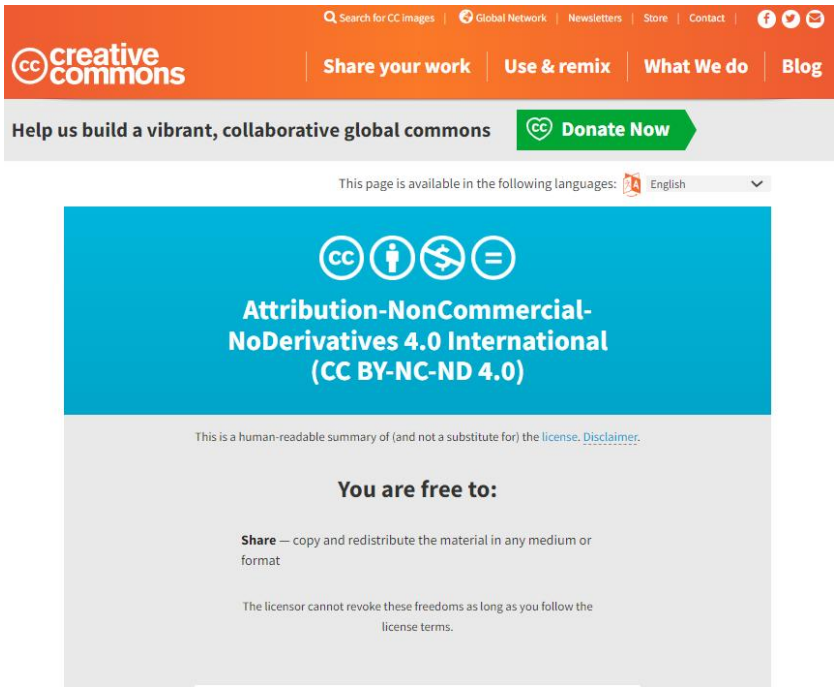
I declare that I and Dr. Mehrdad Ghashghaeinia performed eryptosis and FACS experiments together, Dr. Mehrdad Ghashghaeinia, Dr. Daniela Giustarini and Prof. Dr. Ranieri Rossi performed glutathione measurements in Italy.

Dr. Daniela Giustarini and Prof. Dr. Ranieri Rossi made Figure 20 B in Italy. All the rest of the excels data were made by me, Dr. Mehrdad Ghashghaeinia and Dr. Martin Köberle. All data were analyzed and discussed by me, Dr. Mehrdad Ghashghaeinia, Dr. Martin Köberle, Dr. Daniela Giustarini, Prof. Dr. Florian Lang, Prof. Dr. Ranieri Rossi and Prof. Dr. rer. nat. Thomas Wieder. Figure 1, 2, 4, 5 and 22 was made by me and Dr. Ghashghaeinia.

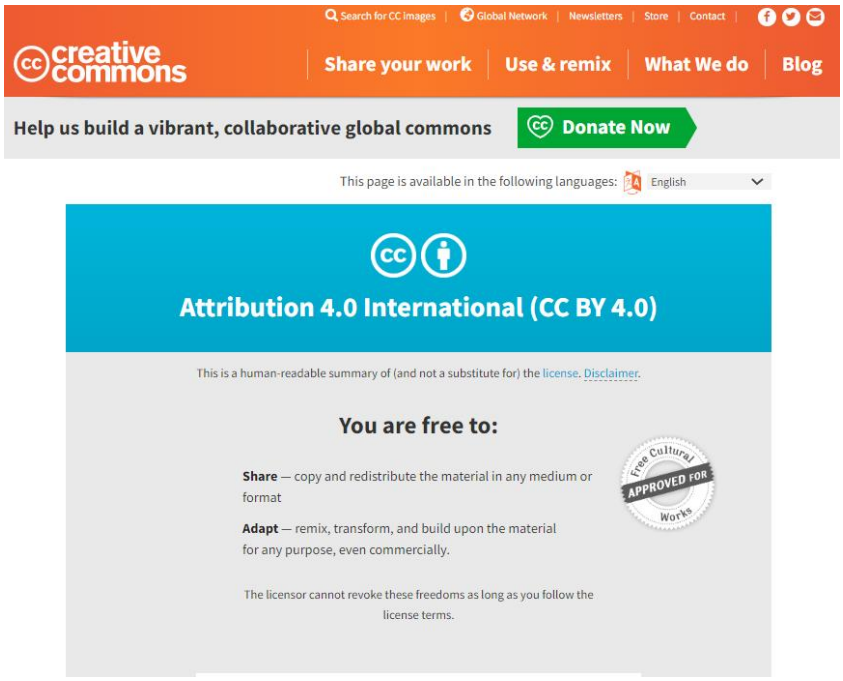
The MD thesis: "The inhibitory effect of sodium nitroprusside and N-acetyl-L-cysteine on desipramine-induced eryptosis" was written by me independently and any additional sources of information have been duly cited.

8 Permission of quoting Figure 6

8.1 Permission of quoting Figure 6A and 6C



8.2 Permission of quoting Figure 6B



9 Publication

Based on our findings in this study we have published the following manuscript in the Journal: Cell Cycle (impact factor: 5.2).
Entitled:
“Desipramine induces eryptosis in human erythrocytes, an effect blunted by nitric oxide donor sodium nitroprusside and N-acetyl-L-cysteine but enhanced by calcium depletion”

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