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The role of hydrogen sulfide and its mechanism of effect in the regulation of vascular tone and in the cell-based therapies of myocardial infarction

PhD thesis

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Abbreviations

AA: arachidonic acid

AC: adenylyl cyclase

ACA: anterior cerebral artery

ACEI: angiotensin-converting-enzyme inhibitor

ADRF: adipose-tissue relaxing factor

AE1: anion exchanger 1

AngII: angiotensine II

AOAA: aminooxyacetic acid

APOE: apolipoprotein E

ASC: adipose-derived stem cells

ATTM: ammonium tetrathiomolybdate

BDNF: brain-derived neurotrophic factor

BK_{Ca}: large conductance calcium-activated potassium channels

BMI: body mass index

BMSC: bone marrow stem cells

Calcein-AM: Calcein acetoxymethylester

Ca^{2+}-CaM: calcium-calmodulin complex

cAMP: cyclic adenosine monophosphate

CAT: cysteine aminotransferase

CBS: cystathionine-β-synthase

CBS-KO: cystathionine-β-synthase-knock out

cGMP: cyclic guanosine monophosphate

CGRP calcitonin gene-related peptide

CNS: central nervous system

CO: carbon monoxide

COPD: chronic obstructive pulmonary disease

COX: cyclooxygenase

COX-1: cyclooxygenase-1

CSE: cystathionine gamma-lyase

CSE-KO: cystathionine gamma-lyase -knock out

CVD: cardiovascular disease

CYP450: cytochrome-P450-enzymes

DALY: disability adjusted life years

DAS: diallyl sulfide

DADS: diallyl-disulfide

DAG: diacylglycerol

DATS: diallyl trisulfide

DMEM: Dulbecco's modified eagle medium

DIDS: 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid

EC: endothelial cell

EDHF: endothelium-derived hyperpolarizing factor

eNOS: endothelial nitric oxide synthase

ERK 1/2: extracellular signal-regulated kinase ½

ESC: embryonic stem cells

ET-1: endothelin-1

EthD: ethidium homodimer

FMD: flow mediated vasodilation

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GC: gas chromatography

GPx: glutathione peroxidase

hASC: human adipose-derived stem cells

Hb: hemoglobin

hBMSC: human bone marrow derived stem cell

HBVSMC: human brain vascular smooth muscle cell

HCO_3^-: bicarbonate

HIF-1α: hypoxia-inducible factor 1-alpha

HNO: nitroxyl

HO-1: heme oxygenase-1

HPLC: high performance liquid chromatography

HSNO: thionitrous acid

HUVEC: human umbilical vein endothelial cells

H_2S: hydrogen sulfide

IP_3R: Inositol trisphosphate receptor

iPSC: induced pluripotent stem cells

ITC: isothiocyanate

K_{ATP}: ATP-sensitive potassium channel

Keap-1: Kelch-like ECH-associated protein 1

K_{IR}: inward rectifier potassium channel

K_V: voltage-gated potassium channel

LDH: lactate dehydrogenase

LDL: low density lipoprotein

lncRNA: long noncoding RNA

LTP: long term potentiation

LVEF: left ventricular ejection fraction

L-DOPA: levodopa

MALAT1: metastasis associated lung adenocarcinoma transcript 1

MAPC: multipotent adult progenitor cells

MAPK: mitogen-activated protein kinase

MEK-1: mitogen-activated protein kinase kinase

metHb: methemoglobin

miR-497: microRNA-497

MLC: myosin light chain

MLCK: myosin light-chain kinase

MSC: mesenchymal stem cell

NADH: nicotinamide adenine dinucleotide

NADPH: nicotinamide adenine dinucleotide phosphate

NaHS: sodium hydrosulfide

Na_2S: sodium disulfide

Na_V: voltage-gated sodium channel

NCX: sodium-calcium exchanger

NF-κB: nuclear factor-κB

NMDA: N-methyl-D-aspartate

Nrf-2: nuclear factor erythroid 2-related factor 2

NO: nitric oxide

NOS: nitric oxide synthase

NSAID: non-steroidal anti-inflammatoric drug

O_2^-: superoxide anion

Oct-4: octamer-binding transcription factor 4

$ONOO^-$: peroxinitrite

oxyHb: oxyhemoglobin

$PaCO_2$: partial pressure of carbon dioxide

PAG: propargylglycine

PARP-1: Poly (ADP-ribose) polimerase-1

PBS: phosphate buffered saline

PDE: phosphodiestherase

PIP2: phosphatidylinositol 4,5-bisphosphate

PI3K: phosphoinositide 3-kinase

PKA: protein kinase A

PKG: protein kinase G

PLC: phospholipase C

PLP: pyridoxal phosphate

PPARγ: peroxisome proliferator-activated receptor γ

PPG: propargylglycine

ppm: parts per million

PTEN: phosphatase and tensin homolog protein

PYK2: proline-rich tyrosine kinase 2

RhoA: ras homolog family member A

ROK: Rho-associated kinase

ROS: reactive oxygen species

RSS: reactive sulfur species

SAC: S-allyl-cysteine

SAM: S-adenosyl methionine

sGC: soluble guanylyl cyclase

SHR: spontaneously hypertensive rat

SMC: S-methylcysteine

SSEA: stage-specific embryonal antigen

$SSNO^-$: nitrosopersulfide

STAP: stimulus-triggered acquisition of pluripotency

SUR-1: sulfonylurea receptor-1

SUR-2: sulfonylurea receptor-2

TRP: transient receptor potential cation channel

TRPA1: transient receptor potential ankyrin 1

TRPV4: transient receptor potential vanilloid 4 receptor

TxA2: thromboxane A2

VEGF: vascular endothelial growth factor

VEGFR-2: vascular endothelial growth factor receptor 2

VSELs: very small embryonic-like stem cells

3-MST: 3-mercaptopyruvate sulfurtransferase

3-MST-KO: -3-mercaptopyruvate sulfurtransferase knock out

1. Introduction

Globally, cardiovascular diseases (CVDs) represent an enormous medical burden; they are responsible for more than 11 million new cases and 3.9 million deaths per year in Europe, making them the leading cause of death from non-communicable diseases in the world (Fig. 1.) [1-3].

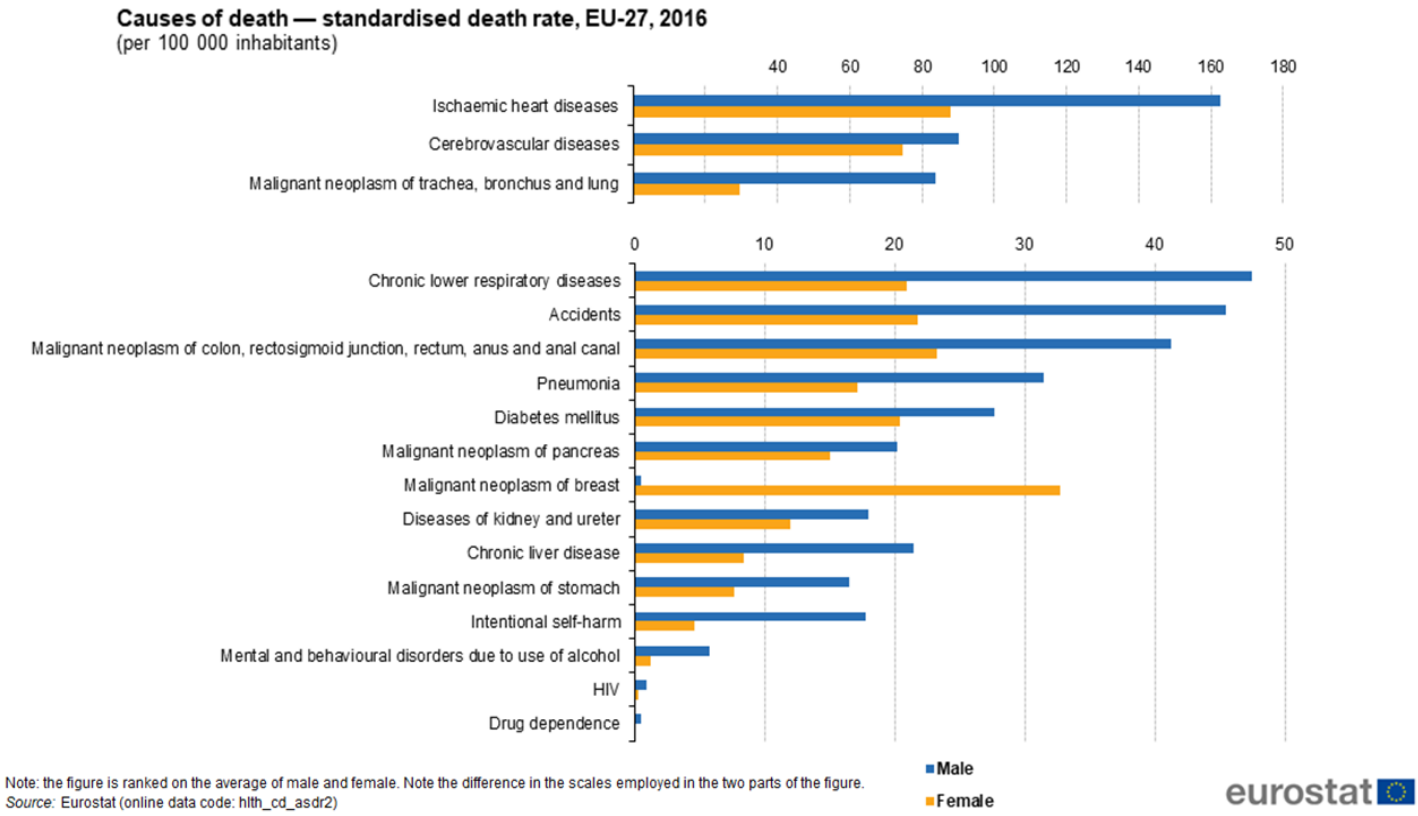

Figure 1. The mortality of cardiovascular disorders in the European Union, according to Eurostat [4].

Even though,the CVD-related mortality decreased in the last four decades, the numbers are still alarming. The mortality rate of CVDs and the degree of their decline are quite different between countries – currently the Central-Eastern European and Baltic countries have the highest cardiovascular mortality [5-7]. As a good tendency, the mortality significantly declined amongst the lower socio-economic groups, which may be due to the positive change in their health-related behavior and also represent their better

access to healthcare [6]. These diseases deserve attention also from the aspect of economical impact and because of their effect on the patients' quality of life [1, 8]. Among CVDs, ischemic heart disease, stroke, peripheral vascular disease and atrial fibrillation are the most prevalent and most important diseases – the incidence vary between the high and middle-income countries, but in general, CVDs affect more individuals in high-income countries than in the middle-income regions [8]. The prevalence of risk factors, morbidity and mortality tends to be higher in the Central and Eastern European region than in other locations of the continent [1].

CVDs cover all the congenital and acquired morbidities that are affecting the circulatory system (heart and vasculature). Main forms are: (1) atherosclerosis and its general complications (coronary-, cerebral-, and peripheral artery disease); (2) myocardial infarction and stroke; (3) heart failure; (4) cardiac valvulopathies and arrhythmias; (5) rheumatic heart disease; (6) congenital heart disease; (7) deep vein thrombosis and pulmonary embolism [3]. In the case of acquired diseases, the correct management of risk factors are of outstanding importance [9]. The main risk factors are unhealthy diet, smoking, the lack of physical activity, alcohol consumption, raised blood pressure and cholesterol, overweight and diabetes [1, 8]. The relative contribution of these risk factors are constantly changing: lately the fruit consumption (as an important aspect of healthy diet) is increased, while smoking rates are unfortunately stable or rising after a decline [1]. Alcohol consumption and blood cholesterol levels decreased, while the prevalence of diabetes increased [1].

These above-mentioned facts clearly demonstrate that CVDs are affecting the human population worldwide, causing serious health damage or even premature death. This brief summary also underlines the medical and socio-economical relevance of extensive cardiovascular research.

1.1. The emerging importance of hydrogen sulfide in living organisms

For centuries in the past, hydrogen sulfide (H_2S) was solely known by its diverse toxic effects. However, this gaseous molecule is not only representing a real environmental risk and occupational hazard these days still [10], but it is also clear that H_2S has an important physiological role in living organisms.

H_2S penetrates to the surface from deeper layers of Earth, where organic matter is decomposed in a low-oxygen environment, and the gases of magma interact with hydrogen and water, e.g. in volcanoes, sulfur springs or geothermal fields. Because of the industrial use of geothermal energy, the related H_2S poisoning is a considerable danger in distinct geothermal areas, as in New Zealand (e. g. Rotorua), Iceland, and Italy (district of Mt. Amiata) [10-13]. The result of studies about the health effect of H_2S on the residents of these geothermal areas are conflicting [13]. Besides these natural sources, H_2S can also be found in natural well-water [10].

H_2S had a distinct role in the evolution of life on Earth (see Fig. 2.) [14].

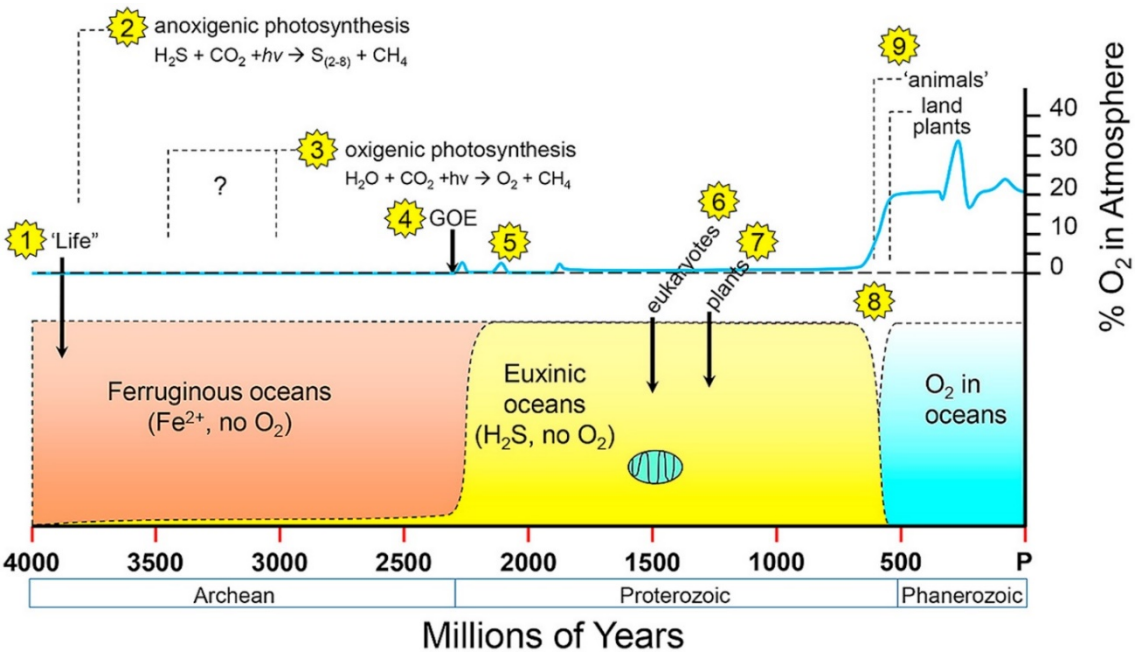

Figure 2. Role of H_2S in the evolution of eukaryotic life [15].

Life began in an anoxic, reducing oceanic environment (named "ferruginous oceans"), where sulfur metabolizing organisms developed [15]. It was followed by the

occurrence of "anoxygenic photosynthesis", by which these organisms used H_2S and CO_2 to produce elemental sulfur and methane – this process is still present in purple and green sulfur bacteria [15]. It is also suspected that the antioxidant defense mechanisms were formed primarily not against reactive oxygen species (ROS), but – considering the fact of the initially sulfur-dependent organisms - reactive sulfur species (RSS) [14, 15]. RSS molecules are formed from H_2S, as thiyl radical ($HS^{\cdot}$), hydrogen persulfide (H_2S_2) and persulfide radical ($HS_2^{\cdot-}$) [15]. Later on, oxygenic photosynthesis also evolved in cyanobacteria, and it was followed by the increase of atmospheric oxygen level, causing oxidation of sulfur to sulfate, which was reduced in the sea, resulting in anoxic and sulfidic environment ("euxinic oceans"), in line with the appearance of the first eukaryotic organisms [15]. Cyanobacteria were then incorporated by eukaryotic cells, as an important step towards the formation of modern plants [14]. According to the actual knowledge, a number of prehistoric mass extinction periods developed as a consequence of the toxic effect of H_2S gas in the atmosphere, produced by the sulfur bacteria during periods of low oxygen content in the air [16]. Finally, after transient fluctuations of the atmospheric oxygen level, the air and also the seas became permanently oxidized at today's level, and O_2 became the main source of energy instead f H_2S [15]. It is suspected that at this point the defense mechanisms of the living organisms were simply adapted to cope with ROS, instead of RSS [14]. Taken together, it is suspected that the mechanisms of sulfur metabolism and signaling in the living organisms are a legacy from these ancient times, when H_2S was the most important source of energy for the cells instead of oxygen [14, 15].

Interestingly, sea sulfur bacteria are also able to produce H_2S, or even H_2 by decomposition of hydrogen sulfide. Certain layers of the Black Sea accumulated high amount of sulfur molecules by degradation of organic compounds in an anoxic way [17].

Industrial sources of H_2S and typical locations for toxic events are the geothermal power plants, tanneries, pulp/paper mills [10]. Furthermore, workers in the petroleum industry are affected by the risk of poisoning due to incidental increase of H_2S in the air during the exploitation or the refinery process [18]. As a special aspect of environmental risk, the cases of suicide by H_2S inhalation (free sulfide originating from mixed household chemicals) are increasing in Japan and also in the United States as well [10].

Toxic effects of H_2S are mainly depending on the inhaled concentration and/or the duration of exposure: symptoms are widespread from headache in mild poisoning to acute life-threatening conditions as respiratory failure or coma/death in more severe cases [10]. Although humans can detect even a very low concentration of H_2S (0.0005-0.3 ppm) in the air by its distinct smell, the onset of olfactory paralysis makes H_2S undetectable by smelling after a few minutes, regardless to the inhaled concentration [10]. Acute exposure means inhalation of more than 300 ppm H_2S, which is followed by the so-called "knockdown" phenomenon, which is a sudden loss of consciousness, that can even be followed by death [10]. In the case of post-acute exposure, when a victim inhales more than 100 ppm H_2S for longer time, breathing disturbances, pulmonary edema, and death can occur as well [10]. Chronic exposure means long term inhalation of less than 1 ppm H_2S; the toxic effect in this case is not obvious; according to clinical data nausea, visual complications, respiratory irritation and headaches are possible effects [10].

Today it is known that H_2S affects several biological function in living organisms (including bacteria, fungi, plants, animal organisms and the human body as well), and in mammals the endogenously produced H_2S is described as the third gasotransmitter molecule after nitric oxide (NO) and carbon monoxide (CO) [12, 19]. All of the three compounds are endogenously produced through distinct enzymatic pathways, act as a signaling molecule, are able to penetrate the cell membranes, and have dose-dependent effects [20]. The role of endogenous H_2S in the function of nearly every mammalian organ was already extensively studied – the results revealed that it is accountable for pathophysiological processes either below the normal amount or above the physiological level [12].

Regarding its physical and chemical properties, H_2S is a weak acid, at pH 7.4 and 37 °C the P_{Ka1}=6.8 and the P_{Ka2}>12. Therefore 80% of molecules are in a dissociated form as HS^- in the extracellular fluid [12]. In the intracellular compartments, the differences between local pH values determine the dominant molecular form: in the mitochondrial matrix at pH=8 mostly HS^- can be found. However, in the lysosomes (pH=5) the bulk of H_2S is in the non-dissociated form. Due to its lipofilic character, the non-dissociated molecule gets easily across biological membranes, while the penetration of HS^- ion requires transport mechanisms [10].

1.1.1. Production and excretion of hydrogen sulfide in the human body

Endogenous hydrogen sulfide is produced by enzymatic and non-enzymatic processes. The enzymatic production involves four different pathways (Fig. 3.). Two enzymes are part of the reverse transsulfuration pathway, these are the cystathionine-β-synthase (CBS) and the cystathionine-γ-liase (CSE). The third pathway consists of 3-mercaptopyruvate sulfurtransferase (3-MST) and cysteine aminotransferase (CAT). The cofactor of CBS and CSE is the pyridoxal phosphate (PLP). CSE is localized to the cytosol, while CBS and 3-MST are present in the cytosol and in the mitochondria as well [21, 22]. A special property of CBS enzyme is that this is the only PLP-dependent enzyme which contains a heme group, and its positive allosteric regulator is S-adenosyl methionine (SAM) [21]. In the recently discovered fourth pathway the D-amino acid oxidase and the 3-MST produce H_2S from D-cysteine in the mitochondria [23]. The occurrence and physiological importance of these enzymes differ between the mammalian organs and tissues: CBS is the main form in the central nervous system (CNS), while CSE is the predominant enzyme in the cardiovascular system [12]. 3-MST has the highest activity in the adrenal glands, and it is suspected to have a role in the H_2S-production of red blood cells and in the endothel as well [23, 24]. The fourth, D-cysteine pathway produces H_2S in the cerebellum and in the kidneys [23].

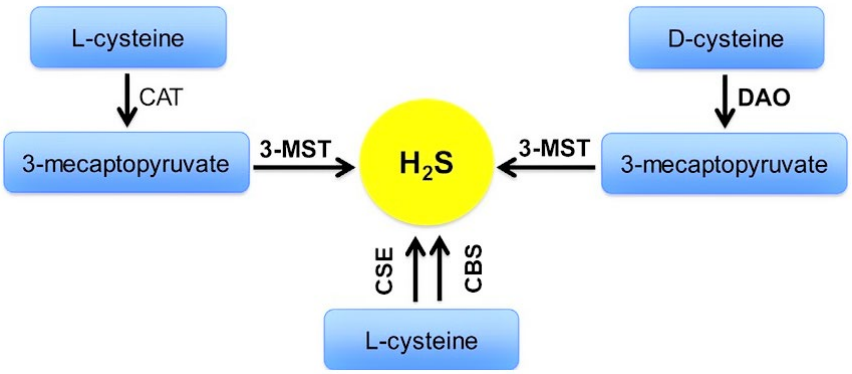

Figure 3. Enzymatic pathways of endogenous H_2S production (modified after Dugbartey et al.) [25]

The presence of sulfur atoms is essential for the activity of endogenous H_2S-producing enzymes. In the human body these originate solely from food – generally through intake of sulfur-containing proteins [26]. Sulfane sulfur stores (bound sulfane sulfur and acid-labile sulfur) are often referred to as endogenous sulfur stores. These

molecules contain a divalent sulfur atom, which is bound to another sulfur atom, resulting in a reactive and labile compound [27]. Persulfides, polysulfides, thiosulfates, thiosulfinates, polythionates and elementar sulfur also belong to this pool. Sulfane sulfurs are redox partners of H_2S, and always coexist with each other in biological samples: the oxidation of H_2S forms sulfane sulfur species, while the reduction of the latter produces H_2S [27]. Sulfane sulfur donors are L-cysteine, thiaxolidine derivatives, N-acetylcysteine, garlic-derived allyl sulfide, as diallyl-disulfide (DADS) and diallyl trisulfide (DATS), isothiocyanates and lipoic acid [27].

The group of sulfur-containing amino acids contains four compounds: methionine and cysteine are components of proteins, while homocysteine and taurine are not [28]. Taurine contains sulfonic acid, which has a role in the H_2S production of gut bacteria. Furthermore it increases the level of CBS and CSE enzymes, and thereby, enhances the amount of endogenous H_2S [19]. Certain bacteria are able to generate H_2S from inorganic and organic sulfate intake by different enzymatic mechanisms; these pathways are producing significant amount of H_2S in the oral cavity and also in the colon [26, 29]. Sulfate-reducing bacteria can be found universally in the human intestinal mucosa, and these bacteria are able to use sulfate as a terminal electron acceptor molecule during respiration, parallel with H_2S generation [30].

In *ex vivo* experiments, human erythrocytes were also capable of producing H_2S through sulfur reduction in the presence of glucose [31]. In red blood cell lysates, H_2S production was measured after glutathione, nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) were added to the solution [31].

H_2S is oxidized mainly in the mitochondria, where it is degraded to thiosulfate, then sulfate, which is eventually excreted through the kidneys by the urine. The sulfide-quinone reductase, persulfide dioxygenase and the thiosulfate sulfurtransferase enzymes take part in these mitochondrial processes [12]. Minor amount of H_2S is exhaled through breathing or excreted with the feces without any chemical modification [23]. Besides these mechanisms, the methemoglobin (metHb) of red blood cells is able to bind H_2S, resulting in the formation of a common intermedier product, metHb-SH complex, which is then slowly metabolized to inorganic polysulfides, $HS_2O_3^-$ and oxyhemoglobin

(oxyHb) [32]. Besides the decomposition of H_2S, this process supports hemoglobin (Hb) to remain in its reduced form, and fulfil its main biological function, oxygen binding [32].

1.1.2. Concentration of hydrogen sulfide in the human body and the measurement of sulfide level

The first studies about the endogenous concentration of H_2S suggested that its plasma level is around the micromolar range [33]. In virtue of our current knowledge and the available measuring methods, this level is considered to be unlikely high, and in reality the concentration is assumed to be significantly less, in the nanomolar range [20]. Furthermore, plasma samples lack the typical odor of H_2S, while in micromolar concentration it should be easy to smell it [33, 34]. It is likely that the intracellular concentration of H_2S is not equal in every location: similarly to calcium signaling, the presence of intracellular "hotspots" are suspected, where the local concentration of H_2S is significantly higher than the average cellular level [35]. Similarly, the H_2S-production of resident bacteria in the intestinal tract results in higher local concentrations than measured in the blood plasma. Interestingly, in a germ-free mouse model, which lack the intestinal bacteria, the serum H_2S level and CSE-activity was lower than in normal controls, while the cysteine level was higher [36]. According to these results, the gut bacteria play a role in the control of systemic H_2S bioavailability and metabolism [36].

The applicable measuring methods to define the H_2S concentration of biological samples are diverse. The currently used techniques can be divided into 4 groups, and all of them have their specific drawbacks, therefore neither of them is universally suitable for all type of studies [20, 37, 38]:

(1) Colorimetric methods as the methylene blue assay (general disadvantages: low sensitivity, overestimation of H_2S concentration, frequent interference in biological samples);

(2) *Chromatographic methods* as high performance liquid chromatography (HPLC) or gas chromatography (GC) (general disadvantages: special equipment and special care of samples are needed);

(3) *Electrode measurements*, as ion-selective electrodes or polarographic H_2S sensors (general disadvantages: difficult calibration and disturbed measurements by subsequent Ag_2S formation);

(4) Different types of *fluorescent probes* as the 7-azido-4-methylcoumarin (general disadvantages: limited sensitivity, perturbed by autofluorescence, nonspecific reactions with other thiols).

It is also possible to detect persulfidation in the biological samples by different methods, although their sensitivity and specificity are not yet optimal [37, 39].

1.1.3. Mechanism of action

Hydrogen sulfide exerts biological effects via multiple ways in the living organisms. Considering its mechanisms of action, three groups can be differentiated: (1) *production of polysulfides/persulfides*; (2) *interaction with reactive oxgen and nitrogen species (ROS, NOS)*; (3) *interaction with metalloproteins*. Persulfidation (or S-sulfhydration) is suspected to be the most significant from these above mentioned groups [40].

1.1.3.1. Production of polysulfides/persulfides

Persulfidation is a posttranslational modification, analogous to nitrosylation - a thiol group is added to distinct cysteine residues of the target proteins, which are usually enzymes, transcription factors or ion channels [39]. Besides the similarities, the greatest difference between the two modification is while nitrosylation reduces protein activity, persulfidation enhances the function in general [41]. Noteworthy, persulfidation is able to change the activation of target proteins only if the affected cysteine residue is in the key domain of the protein, otherwise the modification will not alter the function, this is called 'ineffective S-sulfhydration' [39]. In certain proteins, the balance between the amount of nitrosylation and persulfidation are responsible for the dynamic homeostasis of the cells [39]. Persulfidation is a reversible process, the thioredoxin system and exogenous reducing agents like dithiothreitol are able to reverse the modification and subsequently modify the effect of hydrogen sulfide [21]. According to former studies, the

25% of the total enzyme content of liver tissue is persistently under persulfidation [21]. It is supposed that because of the reversibility, the persulfidated proteins serve as circulating endogenous hydrogen-sulfide pools also [21]. Persulfidated proteins are more resistant to oxidative stress, therefore their function are preserved by this modification [42]. Although persulfides are common modification in the cells, their production and degradation are under complex regulation, which is only party deciphered yet [43].

Depending on the function of target proteins, persulfidation modifies their behavior either immediately, or in a slower manner. After persulfidation of ion channels, as the ATP-sensitive potassium channel (K_{ATP}), the relaxation of smooth muscle cells occurs in seconds, while alterations in gene expression causes detectable changes in cell function after hours or days [21]. One of the first recognized and most important cellular targets of H_2S are the K_{ATP} channels that are activated by the persulfidation of two NH_2 terminal cysteine residues on the sulfonylurea-receptor 2 (SUR2) domains of vascular smooth muscle cells [44]. Besides this, numerous other targets of persulfidation were identified (Table 1.); these are potassium channels like the voltage-gated potassium channel (K_V), KCQN channels, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH), inositol trisphosphate receptor (IP_3R), mitogen-activated protein kinase kinase (MEK-1), poly (ADP-ribose) polimerase-1 (PARP-1), cytochrome-P450-enzymes (CYP450), nuclear factor-κB (NF-κB), phosphatase and tensin homolog protein (PTEN), Kelch-like ECH-associated protein 1 (Keap-1), proline-rich tyrosine kinase 2 (PYK2), transient receptor potential vanilloid 4 receptor (TRPV4), peroxisome proliferator-activated receptor γ (PPARγ) [21, 39, 42, 44].

Table 1. Target proteins for persulfidation by H_2S.

	Target of persulfidation	Ref.
Enzymes	MEK-1	[45]
	PTEN	[46]
	CYP450	[47]
	PYK2	[42]
	GAPDH	[21]
	cytochrome *c*-oxidase	[21]
	eNOS	[48]
Ion channels	K_{ATP}	[49]
	K_V	[50]
	KCQN	[51]
	TRPA1	[52]
Receptors	IP_3R	[21]
	TRPV4	[39]
	PPARγ	[39]
Transcription factors	Keap-1 (Nrf-2)	[21]
	NF-κB	[21]
	SP-1	[53]
	SIRT-1	[54]

1.1.3.3. Interaction with metalloproteins

Hydrogen sulfide has biologically important interactions with metals and metalloproteins, as the heme proteins, where H_2S or HS^- can directly bind to the Fe^{III} heme center, or – with lower affinity – to Fe^{II} heme proteins [55] (Table 2.). Because of the reversibility of H_2S-Fe^{III} complexes, certain Fe^{III} heme proteins are able to transport or store H_2S [55]. The effect of H_2S on the function of cytochrome c oxidase is also based on the interaction of H_2S with the mitochondrial heme proteins [56]. At lower concentrations, H_2S provides electrons for respiration by the reduction of Fe^{III} to Fe^{II} [55], however, at higher concentrations, cellular respiration is blocked by inhibition of Complex IV. This is an important aspect of H_2S toxicity [35, 57].

Table 2. Interaction of H_2S with metalloproteins

	Target of redox modification or H_2S binding	Ref.
Type of metalloprotein	hemoglobin	[58]
	myoglobin	[58]
	sGC	[48]
	cytochrome *c*-oxidase	[56]

Iron-sulfur complexes are formed by the interaction of H_2S and biologically or toxicologically important metals, such as Cu^+, Fe^{2+}, Zn^{2+}, Cd^{2+}, Hg^+, Hg^{2+}, Ch_3Hg^+, Pb^{2+} [55]. Similarly to H_2S, persulfides and hydropersulfides are also able to interact with metals [55]. Metal-persulfide complexes can be formed with various metals (Cu, Ir, Pt, Ti, W, Fe, Ru, Zn, Mo), from which the Mo-SSR complexes are of great biological importance, because of several molybdenium-based redox enzymes as the xanthine oxidase or the aldehyde oxidase [55]. Hydropersulfides also play a role against heavy metal toxicity: for example, their interaction with methylmercury (Ch_3Hg^+) result in the formation of the nontoxic Ch_3Hg-S- Ch_3Hg compound [55].

1.1.3.4. NO-H_2S interaction

It was proven by several studies that NO and H_2S mutually regulate the endogenous production and effect of certain molecules [20]. Numerous observations supports that they are potentiating the effect of each other in vasorelaxation [59], however other studies suggest that these two molecules are attenuating the vascular effects of each other. The interaction between the two molecules examplifies the direct interplay between the two gasotransmitters, which results in production of nitrosopersulfide molecules with autonomous biological actions [20]. It was proven *in vitro* that the reaction of H_2S with low molecular weigth protein nitrosothiols results in the formation of the smallest known nitrosothiol compound, thionitrous acid (HSNO) [60]. Many observations support the role of NO in H_2S-production and also the effect of H_2S on the whole NO- sGC-cGMP axis [61]. Although S-nitrosylation was considered as a stable post-translational modification,

it is suspected that S-nitrosothiols generally react with other thiols to form disulfides, which are responsible for nitrosative signaling in cells [62].

1.1.4. Possibilities for manipulation of the endogenous hydrogen sulfide level

1.1.4.1. Hydrogen sulfide donors

Beyond the use of pure H_2S gas in experiments, numerous compounds are known that liberate H_2S (Table 3.). From the aspect of timing, fast- and slow-release compounds are distinguished. *Fast-releasing compounds* are the sulfide salts, sodium hydrosulfide (NaHS), sodium disulfide (Na_2S) [63]. *Slow releasing compounds* are Lawesson's reagent, GYY4137 (and the further constructed derivatives of this latter molecule), and numerous newly constructed compounds, that are expected to be adequate for clinical studies. Although Lawesson's reagent was used for a long time in organic chemistry as a sulfurization reagent, its biological activity was recognized later [64]. Today it is considered as a H_2S-donor molecule, liberating H_2S from its aqueous solution through hydrolysis, however its application is limited by numerous factors such as poor water solubility, unknown by-products and kinetics [64].

GYY4137 was the first identified slow-releasing H_2S donor, which was formerly used in the rubber industry, without any knowledge about its possible biological actions [64]. The release of H_2S from GYY4137 is pH- and temperature-dependent: it had the lowest rate of H_2S liberation at +4 °C, while the greatest sulfide release was observed at pH 3.0 [64]. The long-term effect of GYY4137 was proven by an in vivo study on spontaneous hypertensive rats (SHR), where the hypotension was still observed after 7 days from the end of a 14 days long treatment [64]. Further slow-release H_2S donors had been constructed from the GYY4137 molecule. These are known as phosphonothioate-based donors or "GYY analogs", which have slightly different physico-chemical properties (water-solubility, pH-dependency, H_2S-producing activity, cell membrane permeability). It needs further validation, whether the biological activity of these molecules are due to the released H_2S, or the donor molecules, or their by-products [65].

SG-1002 is a synthetic H_2S prodrug, consisting of α-sulfur sodium polysulfonate with trace amounts of sodium sulfate [42]. Based on the data currently available, this

compound has favorable effect on cardiac function, lowers oxidative stress and also upregulates eNOS signaling, therefore attenuates endoplasmic reticulum (ER) stress [42]. The first clinical trial on sulfide-based therapy was started with this orally administered prodrug in 2014 [66]. After the completed 1st phase, the therapy was declared safe in patients with heart failure [67]. As a result, H_2S level and NO bioavailability were sufficiently restored in the volunteers [42, 67].

The *pH-controllable H_2S donors* are derivatives of GYY4137. In normal cellular circumstances, H_2S release is slow and sustained, however under acidic conditions H_2S release is increased – this phenomenon suggests promising therapeutic availability of these donors in ischemic/reperfusion injury [42]. Ammonium tetrathiomolybdate (ATTM) is not only pH-dependent, but also a temperature-regulated and thiol-dependent slow H_2S donor, which also exerts cytoprotective and anticancer activity [42, 68]. *Enzyme-dependent sulfide donors* are a special type of prodrugs, that require esterase catalyzed lactonization to produce H_2S [42, 69]. These compunds were designed in order to construct hybrid prodrugs through conjunction with non-steroidal anti-inflammatory drugs [69]. *Reactive oxygen-species activated donors* as peroxyTCM-1 are able to release sulfide under oxidative stress, when certain ROS molecules (hydrogen peroxide/H_2O_2, superoxide/O_2^-, peroxinitrite/$ONOO^-$) are present [42, 66]. It is supposed that these prodrugs have the ability to protect cells from the harmful effects of ROS molecules where it is the most needed [66].

Sulfide donors can be divided to different groups from another viewpoints as well. Certain specially constructed donors are able to release H_2S only in distinct cellular compartments, as the mitochondrial targeted AP39 and AP123 [42]. As another group, hybrid drugs have biological activity in more ways than through H_2S release. ZYZ803 is a H_2S-NO common donor, which is able to release both gasotransmitter molecules [42, 70].

A relevant limitation of studies with the use of H_2S-donor molecules is the uncertain and uncontrolled free H_2S-level in the experimental setup [65]. It is suspected that the fast-releasing donors provide an unbalanced H_2S-release because shortly after their delivery sulfide level can reach toxic concentration, then decrease rapidly [42, 64]. In a study, where Na_2S was diluted in pH 7.4 buffer, H_2S reached its maximum concentration in the gas-phase above the solution after 1 minute of administration [44].

In another study, NaHS reached its maximum H_2S release after 5 to 8 seconds, then fell shortly after, whereas H_2S generation from GYY4137 reached its highest level after 15 min, and sustained a plateau phase for 75 min in an aqueous solution [64]. The quick loss of H_2S is primarily due to its volatilization: in a study H_2S, formed from Na_2S, had halflife as short as 4-5 minutes examined in cell culture wells, myograph baths or Langendorff systems [71]. Another way for the disappearance of free H_2S after administration is the suspected bonding and accumulation in the sulfur stores of the tissues [72]. Impurities are also common in sulfide salt formulations. Therefore, these facts make the observed effects less reliable from physiological stand-point [65].

Hydrogen sulfide is also a compound actively examined from the aspect of its utilization in drug development. Sulfur containing medicines are known and used from a long time ago. In ancient times organosulfur compounds (i.e. molecules that include a sulfur-carbon bond) were used as an antiseptic ointment [19]. Today sulfur-containing substances are applied in a variety of medications, from which antihypertensive agents, analgetics, anti-inflammatory drugs and anti-tumor medications are the most commonly used [19]. The fact that the greater part of H_2S donors and prodrugs are not suitable for oral administration makes the drug development even more challenging [42].

Certain angiotensin-converting-enzyme inhibitors (ACEI) - which are antihypertensive medications - already contain sulfur moieties. These are the captopril and S-zofenoprilat. In the latter case it was proven that it has additional vascular effect due to the H_2S originating from the active form of the drug molecule . Besides the liberation of H_2S, S-zofenoprilat restored the impaired vascular response to Ach of spontaneous hypertensive (SHR) rats, and increased CSE expression in these animals as well; these effects were independent from its blood pressure lowering action (namely ACE inhibition) [73]. According to studies with non-steroidal anti-inflammatoric drugs (NSAIDs) which were already combined with H_2S donors, the occurrence of peptic ulcers was decreased by reduced prostaglandine production through inhibition of cyclooxygenase-1 (COX-1). Today S-diclofenac, S-mesalamine and S-naproxen preparations are under development [19].

Table 3: H_2S donor compounds

	Name	Ref.
Fast-release donors	NaHS	[63]
	Na_2S	[63]
Slow-release donors	Lawesson's-reagent	[64]
	GYY4137	[64]
	SG-1002	[42]
	ATTM	[68]
	peroxyTCM-1	[42]
Natural H_2S donors	DADS	[74]
	DATS	[74]
	ITCs	[75]
Hybrid molecules	ZYZ803	[70]
	S-zofenoprilat	[73]
	S-diclofenac	[19]
	S-mesalamine	[19]
	S-naproxen	[19]

Numerous natural materials are known from which H_2S can liberate; from these, garlic *(Allium sativum)* should be named in the first place. Animal studies and human clinical trials have also proven its anticarcinogenic, antioxidant, antimicrobial, anti-atherogenic and blood pressure-lowering effect [76, 77]. Behind these effects, there are four major groups of biologically active compounds: sulfur-containing molecules (alliin, allicin, allyl-substituted polysulfides, etc), enzymes, amino acids, trace minerals [74-76]. Diallyl sulfide (DAS), diallyl trisulfide (DATS) and diallyl disulfide (DATS) are allyl-substituted polysulfides [74]. In a study on angiotensine II (AngII) treated mice, DATS reduced vascular remodeling including cell proliferation, migration, phenotype switch, vessel wall thickening [78]. Further members of the *Alliaceae* family are also known for their beneficial cardiovascular effects and these plants contain the upper mentioned alliin derivatives also [75]. Another natural H_2S sources are the isothiocyanate (ITC) compounds, which are common in plants from the Brassicaceae (Cruciferae) family, as the broccoli *(Brassica oleracea convar. botrytis)* [75]. The most investigated ITC

molecule is sulforaphane, which is primarily known for its antitumor effects, but also has favorable effects in the vascular system and in nephropathy [19]. Both organosulfur compounds and ITCs are suspected to exert their activity through H_2S, by modulating the Nrf2/Keap1/ARE pathway, which is a regulator of cytoprotective responses to oxidative and electrophilic stress [75].

1.1.4.2. Inhibitors of hydrogen sulfide production

Propargylglycine (PPG or PAG) is inhibitor of the CSE enzyme, it connects by a covalent bond to its cofactor, PLP. PAG and aminooxyacetic acid (AOAA) are the most widely used inhibitors, however they can also affect other PLP-containing enzymes, decreasing their specificity [64, 72]. There are numerous attempts to discover novel, selective inhibitors of the endogenous H_2S-producing enzymes. In one of these studies 1900 compounds were tested for CBS inhibition, from which 12 appeared to inhibit the enzyme effectively, and finally two compounds (1,4-naphtoquinone and tangeritin) were proven to be selective to CBS [38].

1.1.4.3. Transgenic animals

The cystathionine-β-synthase-knock out (CBS-KO) mouse model was the first available transgenic animal related to H_2S-research [12]. Unfortunately these animals perished at a few week of age because of their complex developmental disabilities (including growth retardation and chronic renal dysfunction), therefore they were not optimal for experimental purposes [12, 79]. Due to these circumstances, today heterozygous animals are preferred instead of homozygotes [80]. Congenital deficiency of CBS results in hyperhomocysteinemia or homocystinuria in humans [79]. Cystathionine gamma-lyase -knock out (CSE-KO) animals lack any serious phenotypic aberrations, but age-related hypertension, hyperhomocysteinaemia and decreased endothelial relaxation are characteristic [21, 81]. 3-mercaptopyruvate sulfurtransferase knock out (3-MST-KO) animals were developed as well, these animals were described with anxiety-like behavior and altered neurotransmitter levels [82, 83].

1.1.5. Physiological and pathophysiological effects of hydrogen sulfide

Hydrogen sulfide has widespread effects in the mammals and also in the human body, affecting the function of practically all organs and tissue types [12].

Hydrogen sulfide has a role in the regulation of gastrointestinal motility, promotes mucosal defense, microcirculation and repair [84, 85]. In animal models of colitis, the H_2S-releasing NSAIDs caused less gastric injury, and were even more effective therapeutically than the regular NSAIDs [85]. (The three known gasotransmitter molecule are working in a complex system in the gastrointestinal tract [86].)

Hydrogen sulfide is endogenously produced in the kidney - decreased level of endogenous H_2S was proven in different kidney disease models, and H_2S donor treatments effectively attenuated both the histological and functional alterations [87]. In murine studies, H_2S treatment reduced the morphological and functional signs of diabetic or hyperhomocystinaemia-induced nephropathy. Although no clinical study was performed yet, H_2S is considered as a potential therapeutic tool against diabetic nephropathy [88].

According to animal studies, hydrogen sulfide regulates local and systemic glucose metabolism, has anti-apoptotic effect on the pancreatic islet β-cells, suppresses insulin secretion and also regulates the insulin sensitivity in the responsive tissues [89]. The endogenous plasma level of H_2S was found to be lower in diabetic patients, than in normal controls [89]. H_2S also regulates the activity of circadian clock genes in the skeletal muscle [89]. Thyroid hormone and growth hormone are also regulated by H_2S [20].

The role of H_2S in the modulation of immune functions (the regulation of both innate and adaptive immunity) was proven by several studies [29, 90]. H_2S has effect also under physiological and pathophysiological circumstances. Low levels of endogenous H_2S is suspected to play a part in the development or worsening of autoimmune diseases, while exogenous H_2S treatment may support the normal function of immune cells [29].

Hydrogen sulfide has mucolytic, antioxidant, anti-inflammatory, potent antiviral and antibacterial effects in the respiratory system, while changes in the H_2S production are suspected to be related to certain acute or chronic respiratory diseases [91, 92]. Sulfide-containing thermal water effectively ameliorates the symptoms of rhinitis, asthma

or chronic obstructive pulmonary disease (COPD) – it is suspected that these results are based on the effect of inhaled H_2S on lung epithelial-immune crosstalk [91].

Endogenous production of H_2S is proven in different cell types of the skin, and H_2S has beneficial effects in numerous dermatological disease and promotes the healing of different types of wounds [93].

In the central nervous system, H_2S exerts anti-inflammatory, antioxidant, anti-apoptotic and neuroprotective effects [94]. From the synthetizing enzymes, CBS , CSE, 3-MST and the D-cysteine pathways were all identified in the brain [23, 95, 96]. In Parkinson's disease, levodopa (L-DOPA) increased the dopamine and gluthatione level in the liquor, if it was conjugated with H_2S-donors [19]. Furthermore it is proven that the high amount of H_2S or polysulfides in the liquor are resulting in "sulfide stress", where the amount of 3-MST and H_2S increases ., memory disturbances develop at toxic H_2S concentrations, however exogenous H_2S treatment promoted hippocampal long term potentiation (LTP), inasmuch as the persulfidation and activation of transient receptor potential ankyrin 1 (TRPA1) channels resulted in the activation of N-methyl-D-aspartate (NMDA)-receptors [94]. In neurodegenerative disorders as Parkinson's disease, Alzheimer-disease or Huntington disease the CSE enzyme and also the endogenous H_2S level was decreased [19]. On the contrary, in the case of ethylmalonyl encephalopathy the accumulation of H_2S in the brain tissues was proven [19].

1.1.5.1. Cardiovascular system

CSE, CBS and 3-MST are present in the cardiovascular system as H_2S producing enzymes. The cardiovascular effects of H_2S are quite complex and widespread; studies were performed in numerous animal models and study setup including *in vitro*, *ex vivo* and *in vivo* examinations as well. The main effects are summarized in Fig.4.

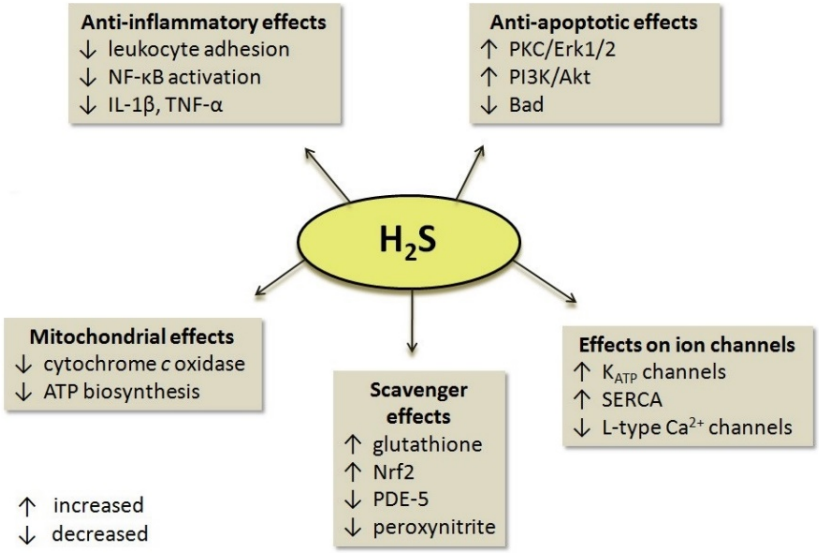

Figure 4: Cardiovascular effects of H_2S [34].

1.1.5.1.1. Vascular tone

Vasodilatation and blood pressure lowering due to hydrogen sulfide are well known effects, as these were already proven mostly by *ex vivo* experiments on vascular segments [40]. H_2S has effects on vascular tone involving signaling mechanisms in the endothelial cells, vascular smooth muscle cells, perivascular adipocytes and free nerve endings as well [40]. The main suspected mechanisms behind the effects are ion channel activation, interaction with NO- cyclic guanosine monophosphate (cGMP) signaling, inhibition of mitochondrial complexes I and III, and the role of H_2S as adipose-tissue derived relaxing factor (ADRF) [97, 98].

The main ion channel effect behind vasorelaxation is supposed to be the activation of K_{ATP} channels [49, 99]. H_2S causes persulfidation in the C43 cysteine residue of the Kir6.1 subunit, which promotes the binding of phosphatidylinositol 4,5-bisphosphate (PIP_2), necessary for the activation of the K_{ATP} channel [41]. It is also noteworthy that numerous studies found evidence for the role of further ion channels in the vascular effects of H_2S. These are the Cl^-/HCO_3^- exchanger, BK_{Ca} channels, KCQN, K_v and K_{IR} channels [21, 50, 51, 100, 101]. Transient receptor potential (TRP)-channels are also involved in the vascular effects of H_2S, as TRPV4 or TRPA1 are activated by polysulfides. [52, 102, 103].

The H_2S-NO interaction also has an important role in the vasorelaxant effect. In the first publication about the vasorelaxant effect of H_2S, its synergistic action with NO was proven as well [59]. H_2S was proven to activate multiple points of the NO-cGMP pathway, or by non-selective inhibition of phosphodiestherase activity [40, 48, 104-106]. The H_2S-NO common products may have a pivotal role in the effect of H_2S on vascular tone: nitroxyl (HNO) exerts vasodilatative effect through the activation of the HNO-TRPA1- calcitonin gene-related peptide (CGRP) pathway [107, 108]. Nitrosopersulfide (SSNO-) can be responsible for the vasodilation and the increased cGMP level [21]. In a study on wild type and CSE-KO mice, it was revealed that H_2S increases endothelial nitric oxide synthase (eNOS) activity through S-sulfhydration and inhibition of S-nitrosylation, promoting the phosphorylation and dimerization of the enzyme [109], whereas activation of protein kinase G (PKG) was reported to be performed by polysulfides [110].

In general, hydrogen sulfide acts through endothelium-dependent and independent mechanisms; as an evidence, vasorelaxation and decrease of smooth muscle hyperpolarization due to K^+-channel blockers was observed both in intact and endothelium-denuded vessels [41]. H_2S was described several times as a potential endothelium-derived hyperpolarizing factor (EDHF), which underlies the importance of its endothelium-dependent effects [54, 111]. It is suspected that H_2S, which is synthesized in the endothelium, reaches the vascular smooth muscle through gap junctions, where it exerts vasorelaxant effect through hyperpolarization of K^+-channels [41]. Since in some experimental models endothelium-denudation did not affect the vascular function of H_2S, it is feasible that the EDHF effect has a role in vessels from a distinct vascular territory (e.g. mesenterial arteries), but is absent in other organs or tissues (e.g. the cerebral circulation) [72]. After the blockade of the nitric oxide synthase/cyclooxygenase (NOS/COX) pathway, the deletion of CSE lowered the cholinergic relaxation by 60% on mesenteric vessels, but the decrement was only 25% in the aortic tissue, examined in a rat model [41]. Congruent to these data, the vasorelaxant effect was more pronounced in the territory of peripheral resistance vessels or smaller arteries, than in the large, conductive arteries (e.g. fivefold higher sensitivity to H_2S was observed in rat mesenteric arteries than in the rat aortic segments) [72, 112]. It was supposed that the EDHF effect of H_2S is conveyed through K_{ATP} channels, while in another study the role of charybdotoxin and apamine-sensitive K_{Ca} channels were proven [41, 112]. In a study

compairing the vascular effects of the fast-releasing H_2S-donor NaHS and the slow-releasing donor GYY4137 it was revealed that NaHS caused only a fast and transient relaxation on rat aortic rings, while the effect of GYY4137 was far more prolonged with greater potency, too [64]. The longer contact with the GYY4137 molecule (due to its slower release profile of H_2S) presumably induced accumulation of H_2S in the tissue samples [64]. As an indication of similarities in the mechanism of action of both molecules, the effect of both GYY4137 and NaHS was abolished by K_{ATP} channel inhibitors and reduced after endothelium denudation or pretreatment with inhibitors of he vascular NO pathway [64].

In certain vessel models and experimental setups vasoconstrictive and two-phased effects (vasoconstriction followed by vasodilatation) were also observed [21, 113, 114]. Vasoconstriction was reported at lower concentrations in rat mesenteric arteries, mouse aortic segments and rat gastric arteries, which was followed by vasorelaxation at higher doses of H_2S [21]. On rat aortic segments, this biphasic effect of H_2S was revealed to be a result of the regulation of NO bioavailability by H_2S [114]. It is suggested that the interaction of H_2S and NO result in production of a biologically inactive nitrosothiol molecule [114]. In rat basilar arteries the vasoconstriction was the result of blocked adenylyl cyclase and therefore reduced cyclic adenosine monophosphate (cAMP) level. The efficacy differed between the used H_2S-donors: the slow-releasing GYY4137 had minor effect compared to NaHS [21, 115]. Another interesting finding is that the presence of NO enhanced the vasorelaxation by exogenous H_2S in certain experimental models, but endothelium denudation did not change the effect of H_2S [21]. On isolated mouse coronary arteries, exogenous H_2S treatment resulted in vasoconstriction by the inhibition of NO formation through the blockade of the eNOS enzyme [113]. In a study on perfused rat kidneys, low concentration of NaHS caused a transient drop of perfusion pressure, while at higher doses a biphasic response was observed, which consisted of a vasoconstriction followed by dilatation [64]. Interestingly, perfusion of the kidney in this study with GYY4137 only had a vasodilatator effect, which was examplified as a reduced response to vasoconstrictor drugs [64]. In a study on isolated rat aorta and on HUVEC cells, H_2S downregulated the vascular NOS/NO pathway, supposedly via activation of K_{ATP} channels [116].

The complex effect of H_2S on the vascular system is further supported by a study on human umbilical vessels, in which the H_2S treatment did not change the vascular tone of arteries but generated a dose-dependent contraction in the veins by the blockade of the NO-sGC pathway [117]. After serotonin (5-hydroxytryptamine, 5-HT) precontraction, H_2S exerted vasorelaxation, which was abolished by K-channel blockers [117].

The pathways of the vascular effects of H_2S are summarized in Fig.5**.**

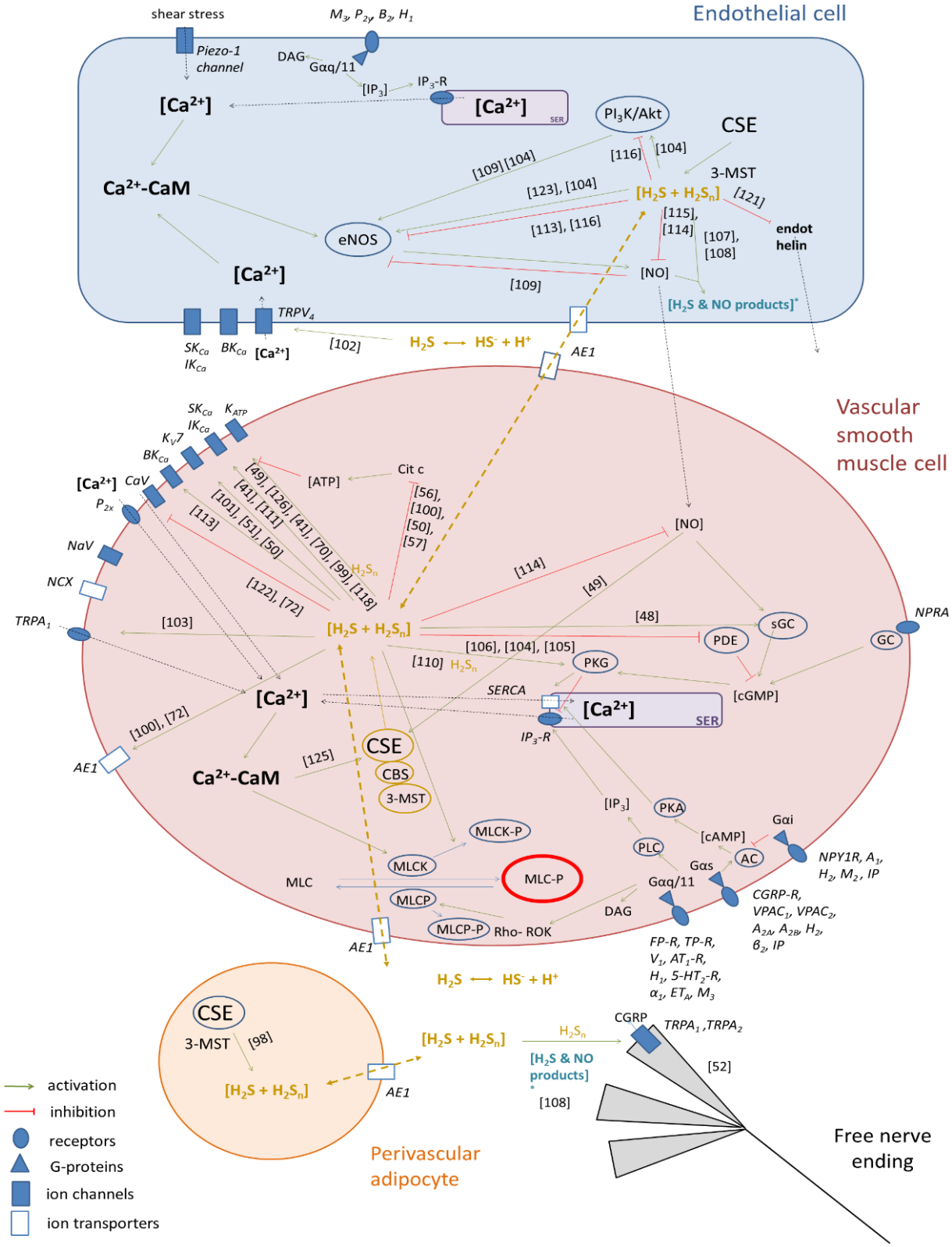

Figure 5. Details of the vascular effects related to H_2S [40].

1.1.5.1.2. Cardiac function and remodeling

The common NO-H_2S donor compound, ZYZ-803 increased H_2S and NO concentrations and also attenuated cardiac dysfunction (ejection fraction, fractional shortening, left ventricular volume and left ventricular internal dimension diastole were examined), improved myocardial injury (histological analysis on local necrosis and fibrosis) in an in vivo murine study of heart failure [70]. ZYZ-803 upregulated the vascular endothelial growth factor (VEGF) -cGMP signalization, and the level of important antioxidants, glutathione peroxidase (GPx) and heme oxygenase 1 (HO-1) [70]. On a Langendorff-perfused rat heart, NaHS caused a negative inotropic and chronotropic effect, while GYY4137 had no effect in this setup, presumably the slower rate of H_2S generation and therefore its constantly low tissue concentration is the explanation for the lack of direct cardiac effects [64].

1.1.5.1.3. Angiogenesis and vascular functions

According to previous studies, low-dose treatment with a H_2S donor increases angiogenesis, while at higher doses this effect was absent [54]. The interaction of H_2S and NO is necessary in angiogenesis, similarly to the vasorelaxation [21, 104]. The role of phosphoinositide 3-kinase (PI3K)/Akt, mitogen-activated protein kinase (MAPK), K_{ATP}, and extracellular signal-regulated kinase 1/2 (ERK 1/2) were proven in the effect of H_2S on vascularization [21, 54, 118].

The connection between H_2S and VEGF has a significant role in the vascular effects. Exogenous H_2S enhances VEGF expression, and endogenous H_2S is required for normal function of VEGF; H_2S also affects the VEGF signaling pathway through the modification of vascular endothelial growth factor receptor 2 (VEGFR2) activation [21, 54].

Hydrogen sulfide has an additional role in tumor angiogenesis and in the regulation of vascular permeability as well [21].

1.1.5.1.4. Atherosclerosis

H_2S has favorable effects on atherosclerosis – it decreases the level of lipid peroxidation and the intracellular lipid accumulation and foamy cell formation (caused by oxidized low density lipoprotein, LDL) through lowering the oxidative stress [21, 54]. H_2S treatment also lowered the extent of monocyte accumulation. CSE/ apolipoprotein E (ApoE) KO mice showed even more pronounced atherosclerosis than the ApoE-KO animals [21]. It is also a relevant risk that through its pro-angiogenic effect H_2S can increase the possibility of rupture on the existing atherosclerotic degenerations [21, 54].

1.1.5.1.5. Vascular complications in diabetes and hypertension

In obese patients, serum sulfide concentration had positive correlation with body mass index (BMI) and body fat mass, however serum sulfide concentration was decreased in patients with impaired fasting glucose levels compared to subjects with normal glucose metabolism [119].

In diabetes, the plasma H_2S level is lower than in the normal control as it was formerly proven in murine and human studies also [120]. Under experimental conditions, the hyperglycaemia-induced oxidative stress and cellular apoptosis could be decreased by exogenous H_2S treatment [54]. H_2S exerts beneficial effects in the vascular wall by direct scavenging effects and also by increasing the level of antioxidant enzymes. Due to these actions, it counteracts the damage of free radicals produced in pathophysiological circumstances such as hypertonia or diabetes [21]. Hyperglycemia is supposed to play a role in the downregulation of CSE and therefore in the decrease of endogenous H_2S synthesis, and the increasing level of endothelin-1 (ET-1) has a role in these processes [121]. Despite the lower serum level of endogenous H_2S, exogenous H_2S-induced vascular relaxation is enhanced in diabetes, due to sensitization or overexpression of K-channels participating in the effect [120].

Insufficient wound healing in diabetes is a consequence of impaired angiogenic potency partly due to reduced endogenous H_2S level [120]. The use of mitochondria-targeted H_2S donors, such as AP123 and AP39 are especially useful in prevention of microvascular complications [54, 122].

In ischemic lesions, the cooperation of H_2S and NO is necessary for cytoprotection: the beneficial effects of H_2S treatment was abolished both in CSE-KO and eNOS phosphomutant mice [123]. H_2S donors increased NO production and hypoxia-inducible factor 1α (HIF1α) expression after limb ischemia. The increased endothelial proliferation and angiogenesis induced by H_2S treatment ameliorated the impaired blood supply of the peri-infarct area in a rat cerebral ischemia model [21]. H_2S supplementation had beneficial effects during the postinfarct remodeling after myocardial ischemia: the dilatation of the left ventricule decreased, the left ventricule function improved, and the blood supply of myocardial tissue increased through angiogenesis [21].

In hypertension the plasma H_2S level is decreased; this phenomenon was proven by a number of animal models and on human samples as well (SHR, Dahl salt-sensitive hypertensive rat, angiotensin-II induced hypertension, preeclamsia) [54]. In animal models (such as the SHR) the CSE-dependent endogenous hydrogen sulfide level was lower than in the control group; deletion of the CSE gene resulted in hypertension and insufficient endothelium-dependent relaxation [124, 125]. In SHR, vascular SUR2B and Kir6.1 expressions were downregulated, which were increased by exogenous H_2S treatment; these observations underline the role of K_{ATP} channels [126]. Conversely, in animal models with hypertension, exogenous H_2S supplementation by GYY4137 lowered blood pressure [21].

In hypertensive patients the plasma H_2S levels were lower than in normotensive controls [54, 127]. As an interesting finding, children with vasovagal syncope had increased plasma H_2S levels compared to controls, and erythrocyte H_2S production positively correlated with the elevated flow mediated vasodilation (FMD) values [128, 129]. According to human *in vivo* microcirculation studies on hypertensive patients, the activity of endogenous H_2S-producing enzymes, and therefore the plasma H_2S level is decreased, the H_2S-mediated vasodilation is absent, however the vascular answer to exogenous H_2S remains intact [61]. These findings suggest that the low bioavailability of endogenous H_2S has a pathophysiological role in hypertension.

1.2. Stem cells and their potential role in the cardiovascular system

1.2.1. Definition and description of stem cells

The term 'stem cells' refers to a special group of undifferentiated cells, which have clonogenic potential, are capable to renew their population, and have the ability to differentiate into multiple cell lineages. Stem cells are functioning both in embryos and adult organisms, and a general rule, their developmental potency is decreasing with their specialization into different cell types [130].

Currently four types of stem cells are used in experiments related to cell-based therapies (Fig. 6.). These are (1) embryonic tissue; (2) fetal tissues; (3) adult stem cells; (4) genetically reprogrammed differentiated somatic cells, for instance the induced pluripotent stem cells (iPSCs).

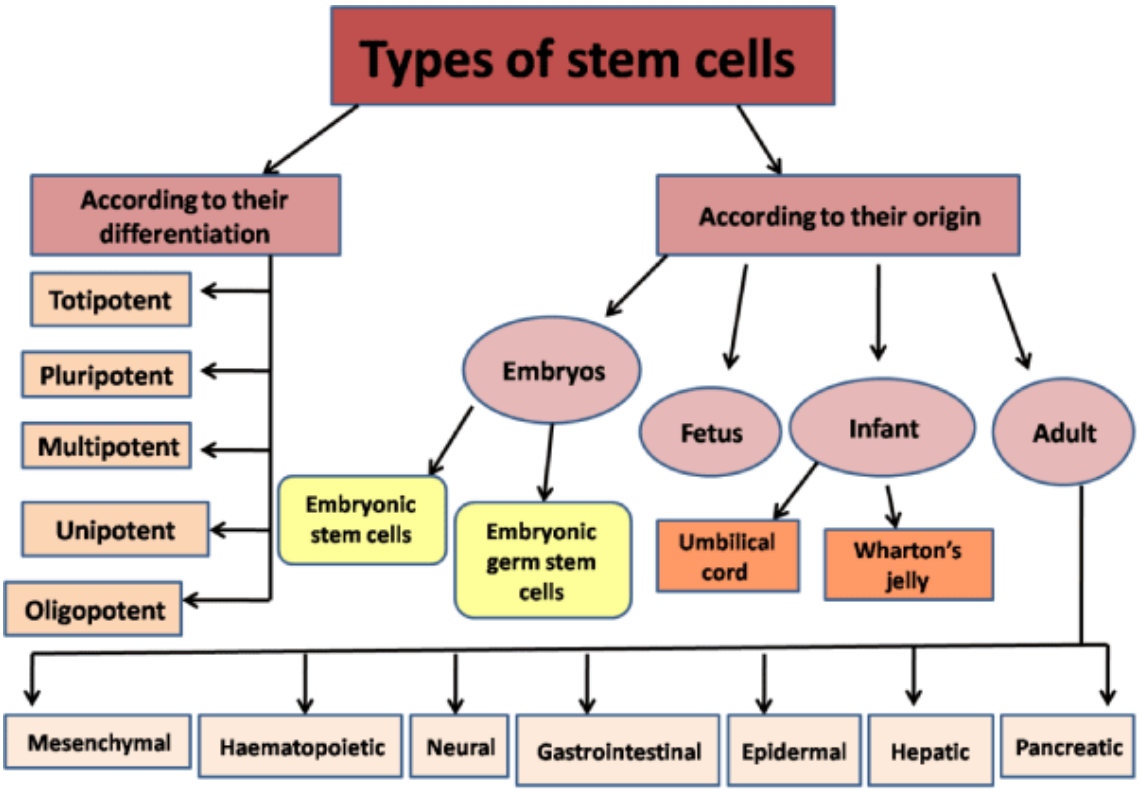

Figure 6. Stem cell types and their differentiating potency [131].

Embyonic stem cells (ESCs) are originating either from the morula (totipontent cells), or from the blastocyst (pluripotent cells). Totipotency means the ability to differentiate to all embryonic cell types, including the placenta, while pluripotent cells are not able to differentiate into cell types that build up the placenta, but they can create any other tissue of the body. These stem cells are identified by expression of the stage-specific embryonal antigen (SSEA), homeobox protein Nanog, and octamer-binding transcription factor 4 (Oct-4) [132].

Fetal cells (and umbilical cord cells) are multipotent, as they only differentiate to limited cell types [132].

iPSCs are pluripotent cells, originating from somatic cells, which were reprogrammed by ectopic expression of certain transcription factors, with or without the use of viral vectors [132]. Initially iPSCs were constructed from autologous fibroblasts, but later on relatively easily accessible other cell types were used, such as peripheral blood cells, keratinocytes, and urine-derived renal epithelial cells were also used in this process [130]. Both the use of ESCs and iPSCs are restricted by several circumstances, for instance their potential to teratoma formation (teratoma-forming assay is performed as a proof of pluripotency *in vitro*) and also ethical concerns [130].

1.2.1.1. Adult stem cells

Adult stem cells are obtained from tissues or body fluids of the adult body [132]. The greatest advantage of this stem cell type is that their use is not affected by ethical or legal issues as with the three other type mentioned above [132]. As a further benefit, they are suitable for autologous administration, and by this, it rules out the possibility of immunological complications [132]. According to certain studies, in special locations like the bone marrow, adult stem cells can be found with pluripotency markers, these are named as the 'very small embryonic-like stem cells' (VSELs) – however, the real existence of these cells are under debate and the data are conflicting, similarly to the case of multipotent adult progenitor cells (MAPC) or stimulus-triggered acquisition of pluripotency (STAP) cells [132, 133]. On the contrary, other adult stem cells are oligopotent, bipotent or unipotent [132]. The main difference between the adult stem cells and the progenitor cells are in their different potency, number of divisions, level of

differentiation in a specific cell line – progenitor cells are not capable of self-renewal and are often omnipotent [133]. In some cases there is no clear distinction between these two categories, and these cells are referred to as stem/progenitor cells (in the nervous system or in the liver tissue) [132].

Adipose-tissue derived stem cells (ASCs) have mesodermal origin, and can be found in the perivascular regions (stromal-vascular fraction) of white adipose tissue [132, 134]. Their physiological role is expansion of the adipose tissue by adipogenesis [132]. ASCs were discovered in 2002, and this cell type have some favorable characteristics that makes this cell type most appropriate for use in cell therapies: it can be easily harvested by minimally invasive methods from the subcutaneous adipose tissue, it is abundant and can be isolated in large quantities [132]. ASCs are generally isolated from white adipose tissue, however according to certain studies, these cells can be also found in the brown adipose tissue or in the mediastinal fat [134]. A special location from where ASCs can be harvested is the cardiac adipose tissue; cells are divided to epicardial and pericardial ASCs, and they support primarily the cardiac regeneration by enhancement of angiogenesis and by differentiation towards cardiovascular cells [134]. The main deposit of white adipose tissue is the subcutaneous fat and the visceral fat – this latter is proven to contain more ASCs [134]. By *in vitro* stimulation ASCs are able to differentiate towards other mesodermal cell types as osteoblasts, chondrocytes, cardiomyocytes, skeletal myocytes, smooth muscle cells, endothelial cells, dermal fibroblasts; however the study results are often conflictuous or data is limited [132, 134]. These cells also have the potential to transdifferentiate into ectodermal and endodermal cell types if treated with specific inductive factors [132]. As a further benefit, the density of stem cells in the adipose tissue is a few hundred times higher, than in the bone marrow [132]. Primarily the clinical use of ASCs mainly consisted of aesthetic, reconstructive interventions [132]. Since the immunomodulatory and immunosuppressive effect of these cells were discovered, they are under examination in order to use them as treatment for inflammatory and autoimmune disorders [132]. Their effectivity was proven in further cell therapies, such as repairing bone tissue defects, treatment of critical limb ischemia, diabetic foot, traumatic spinal cord injuries, pulmonary arterial hypertension, optic nerve injury [132]. Noteworthy, there are conflicting results about the effect of ASC-based therapies in cancer and cardiac diseases [132].

1.2.2. Therapeutic application of stem cells in the cardiovascular system

The main idea behind cell-based therapies or regenerative therapies is the replacement or regeneration of damaged cells and tissues by various cell types [132]. These efforts are of great interest as a potential treatment mainly in degenerative disorders, traumas, tumors, congenital defects and dentistry [130, 132]. The most simple version of cell therapy is the use of exogenously expanded autologous cells obtained by biopsy, even though this method has a lot of difficulties such as the low amount of obtained cells or their complicated *in vitro* cultivation and expansion [132]. Stem cells are, however, easier to harvest, expand, and they have the capability to differentiate into numerous phenotypes – these characteristics makes stem cells more promising for cell therapies, than the differentiated adult cells [132]. Stem cells are used generally by two methods: (1) direct administration of cells to the location of damaged tissue or (2) utilization of the cells on an artificial scaffold (tissue engineering) [132]. A third approach is also emerging, which is named (3) 'stem cell-based cell-free therapy'; the autocrine and paracrine products (growth factors and other bioactive molecules) of stem cells are collected from cell culture media [132, 135], named as the stem cell secretome or conditioned medium [136]. It was revealed that the secretome includes specific nanovesicles, exosomes, which consist of biologically active molecules in a lipid bilayer [137]. Biological factors and molecules of the secretome play a role in a wide variety of biological functions, such as the regulation of homeostasis, cell development, cellular signaling, angiogenesis, apoptosis and numerous other cellular function [136]. These make the secretome an ideal substrate for tissue repair, without the possible drawbacks of cell therapies, as tumorigenicity or adverse immune responses [136, 137]. The composition of the secretome is not constant; it varies between species, tissue type, or it can be artificially altered by *in vitro* biochemical or physical preconditioning of the producing cells [136].

The suspected beneficial role of factors secreted from stem cells were mentioned above. As in general, the effect of cell therapies in the cardiovascular system is also suspected to be a result of the combination of autocrine, paracrine and endocrine-like effects, while transdifferentiation, fusion or engraftment have minor effect due to the low

amount of cells that reside in the injured tissue [138]. Exosomes secreted by stem cells were also proven to have beneficial effect in the cardiovascular system by promoting cell proliferation, differentiation, survival, angiogenesis, while inhibition of apoptosis [137]. In a mouse model of myocardial infarction, extracellular vesicles collected from the conditioned media of hPSC-derived cardiovascular progenitor cells promoted survival of post-ischemic cardiomyocytes and improved angiogenesis [139]. The use of extracellular vesicles from progenitor cells cultured under hypoxic conditions were even more efficient, and it was revealed that these effects partially contribute to the expression of the long noncoding RNA (lncRNA) sequence of metastasis associated lung adenocarcinoma transcript 1 (MALAT1), via targeting microRNA-497 (miR-497) [139]. Interestingly, former studies from our laboratory found no improvement in the survival of post-ischemic rat cardiomyoblasts after treatment with hASC conditioned media – as a relevant difference, it was not possible to examine angiogenesis in our *in vitro* work, and by this, positive effects related to the progression of vascular supply in the peri-infarct area were not examined [140].

The potential role of cell-based therapies in cardiovascular diseases is an extensively studied field. Different types of stem cells were already examined, as embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), and numerous adult stem cells: skeletal myoblasts, bone marrow progenitor cells, hematopoietic stem cells, mesenchymal stem cells, endothelial progenitor cells, bone marrow mononuclear cells [141]. Cardiosphere-derived cells were also used in the experiments – the discovery of these cell types had changed the former belief that the heart is a terminally differentiated, post-mitotic organ with no possibility of self-renewal [141]. Except embryonic stem cells and iPSCs, all the mentioned cell types were examined in clinical trials [141]. Despite the great number of available stem cell types, the clinical studies were performed mainly on bone marrow-derived stem cells [141].

The way of delivery is either systemic (intravenous injection) or local (intracoronary infusion or imtramyocardial injection) [141]. Numerous pretreatment methods are under investigation, which should improve the effect of cell-based therapies [141]. There are important factors that are needed for the controlled differentiation of stem cells, these are (1) use of specific bioactive molecules and growth factors in the culture media; (2) mechanical stimulation during *in vitro* culturing (laminar shear stress,

vibrational stress, pressure stress); (3) use of the optimal scaffold in the case of tissue engineering (graphene and its derivatives are very promising materials as stem cell scaffolds) [130, 132]. In addition, in stem cell therapy there is still struggle to rule out some unfavorable condition, e.g. the possibility of tumorigenesis (particularly teratoma formation in ESCs and iPSCs), immunological rejection or low efficiency and their use is also limited by the lack of full understanding of their mechanism of action [130].

1.2.3. Potential role of hydrogen sulfide in cell-based therapies

Based on its pleiotropic effects, H_2S was implied in several models connected to cell-based therapies. In an *ex vivo* experiment with pressure myograph revealed that mesenchymal stem cells (MSCs) exert vasodilatative effect on mesenteric vessels through H_2S; inhibition of NO generation (by the use of vessels from eNOS KO animals) decreased the vasodilation [142]. In a rat model of cell therapy in isoprenaline-induced heart failure, NaHS was either delivered intraperitoneally to the animals at the same time as the rat bone marrow stem cells (BMSCs) as an *in vivo* preconditioning, or the BMSCs were pretreated with NaHS before administration [143]. Both methods had favorable results – after the *in vitro* delivery, stem cell homing, proliferation and cardiac functions were improved, while after the *in vivo* preconditioning stem cell homing was also improved, and histological alterations were ameliorated, while the expression of VEGF and eNOS were increased in this group compared to the animals received the *in vitro* preconditioned BMSCs [143].

In a study on intestinal ischemia, the H_2S production of therapeutic human BMSCs were inhibited by knockdown of the endogenous H_2S-producing enzymes, however, during hypoxia, the H_2S-producing capacity of these BMSCs was not suppressed [144]. According to these data, nonconventional mechanisms of H_2S production are suspected in the hypoxic environment, and H_2S is probably one of the paracrine factors secreted from stem cells [144].

NaHS treatment increased proliferation and prevented hypoxia-induced apoptosis of induced pluripotent stem cell-derived mesenchymal stromal cells [145]. In the background of the effects, H_2S decreased BK_{Ca} currents and increased the phosphorylation of Akt, while decreased the expression of Caspase 8 and Bax [145].

The survival and efficacy of rat therapeutic BMSCs were improved by NaHS treatment through increased proliferation. Under *in vitro* cirumstances, H_2S suppressed the apoptosis of BMSCs, decreased Bax/Bcl-2 ratio and HIF-1a expression, while brain-derived neurotrophic factor (BDNF) and VEGF release were increased; the ERK1/2 and Akt pathways were proven to take part in these effects [146]. In an in vivo rat ischemic stroke model the NaHS-preconditioned BMSCs improved neurological function and reduced infarct volume; here an anti-apoptotic effect, decrease of Bax/Bcl-2 ratio and increased BDNF and VEGF expression were also observed, similarly to the *in vitro* experiments [146].

1.2.4. Controversies and scandals around stem cell research

In relation with the therapeutic use of stem cells, it is necessary to mention the unfortunate events at Pierro Anversa's research group, from where unfounded publications were released about the differentiation of haematopoietic stem cells into cardiomyocytes, and the cardiac functions were supposedly improved by this de novo formed myocardium [147]. A few years later three independent laboratories claimed that the used stem cells are not able to differentiate into cardiomyocytes [148-150]. Despite these latter results, the clinical studies were continued. By the data from these clinical investigations it was proven that in general cardiac function was not improved by treatment with haematopoietic stem cells and – although in the publication of certain trials a moderate increase in the left ventricular ejection fraction (LVEF) was communicated – the studies were later proven to contain serious mistakes and inconsistencies [133]. A similar situation occurred in the laboratory of Haruko Obokata, who claimed that adult somatic cells can be reprogrammed to pluripotent cells under acidic conditions (STAP cells) [151, 152]. These findings were later unreproducible by other study groups, and significant errors were also found in the initial data and the publications were finally withdrawn [133].

1.3. Regulation of cerebrovascular tone

The cerebral circulation has unique properties when compared to the systemic circulation from functional aspects. The vascular tone is mainly controlled by local factors in the cerebral circulation; the neural control has less role than in the systemic circulation, and its effect is less obvious [153-155]. Local factors include myogenic, flow or shear mediated and also metabolic responses – the autoregulation of brain blood flow is based on a complex vascular answer, mediated by these factors [154, 156] (Fig. 7.). Under physiological circumstances, cerebral autoregulation is functioning between 50-60 mmHg and 140-150 mmHg mean arterial pressure [154, 157]. At lower pressure values the myogenic tone is absent, while above the autoregulation range forced dilation occurs (which is an active vasodilation process) in order to protect the arterial wall from the unfavorable effects of extremely high intraluminal pressure [157].

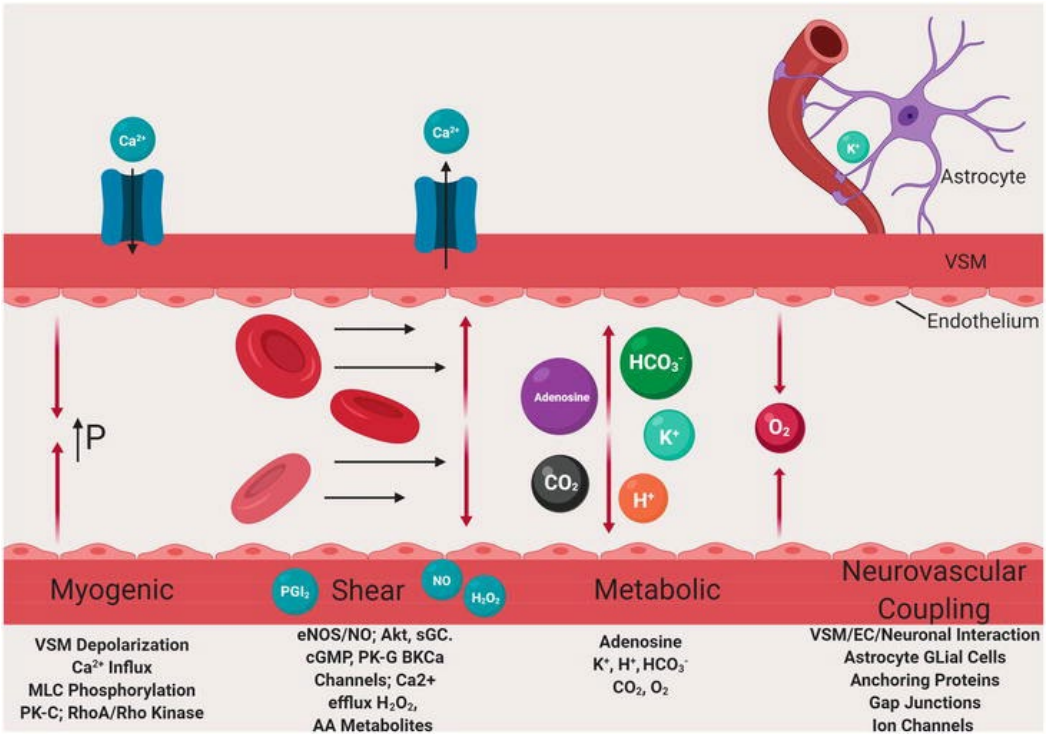

Figure 7. Components of cerebral autoregulation [157]. P: pressure, VSM: vascular smooth muscle, MLC: myosin light chain, PK-C: protein kinase C, RhoA: Ras homolog family member A, Rho Kinase: Ras homolog family member kinase, AA: arachidonic acid, EC: endothelial cell

Myogenic response in the cerebral vessels is based on the Ca^{2+}-influx mainly through $Ca_v1.2$ channels, after the increased intraluminal pressure induces depolarization

of smooth muscle cells [157]. The increased Ca^{2+}-concentration increases the phosphorylation of myosin light chain (MLC), and therefore vasoconstriction occurs [158].

Metabolic control of cerebral circulation is based on the effect of vasoactive metabolites on the vascular smooth muscle cells. Rise in the local CO_2 level exerts a vasodilatative response, while the decrease of the partial pressure of carbon dioxide ($PaCO_2$) results in vasoconstriction and decreased blood flow, through the alteration of perivascular pH level [154, 158]. Other important factors inducing vasodilatation are adenosine, potassium ion (K^+), and bicarbonate (HCO_3^-) [157].

The local regulation of cerebral blood flow is tightly connected to the actual neuronal activity; "neurovascular coupling" provides sufficient blood flow of active brain area by the interaction between the vascular smooth muscle, neuron and astrocytes [157, 159]. Elevated K^+ concentration due to increased frequency of neuronal depolarization exerts vasodilatation through hyperpolarization of the vascular smooth muscle cell membrane [157]. Numerous neurotransmitters and neuromodulators induce Ca^{2+} waves in astrocytes and neuronal dendrites, facilitating the release of vasoactive molecules as NO or arachidonic acid metabolites [154, 158, 160].

Perturbations in the regulation of vascular tone are frequently a consequence of systemic or cerebrovascular diseases that destroy the integrity of the vessels. These pathological changes can lead to further damage in the vessels or in the brain parenchyma due to insufficient blood supply. Approximately 3-4 days after the onset of subarachnoideal haemorrhage, cerebral vasospasm can develop, which is a life-threatening condition consisting of prolonged vascular smooth muscle contraction with damage and remodeling of the vascular wall, potentially causing ischemic lesions in the affected brain territory and inducing edema due to insufficient circulation [161]. Numerous substances were identified in the background of vasospasm (the so-called "spasmogens"), as OxyHb, hemoglobin breakdown products, arachidonic acid metabolites, reactive oxygen species (ROS), lipid peroxides, cytokines, endothelin-1 (ET-1), sphyngosylphosphorylcholine, and thromboxane A2 (TxA2), which latter is mainly synthetized by damaged endothelial cells and activated platelets [161].

Metabolic syndrome (hypertension, type II diabetes, obesity, dyslipidaemia) is an example of a systemic disease with pathological changes in the cerebral circulation [157].

General increase of the pro-inflammatory and oxidant state results in alterations in every aspect of local regulation of blood flow through endothelial cell dysfunction, vascular wall remodeling and the dysfunction of vascular smooth muscle [157]. As a result, myogenic constriction is increased, shear-induced and metabolic-induced dilation is decreased, while the neurovascular coupling is impaired [157]. Increased TxA2 production plays a role in the development of increased myogenic constriction and decreased dilatative potential of the vessels [157].

1.3.1. Potential role of hydrogen sulfide in the regulation of cerebrovascular tone

It is proven by animal studies that NO and CO has an impact on the activity of cerebral vessels [162]. With regard to the third gasotransmitter, H_2S, also numerous *in vitro*, *ex vivo* and *in vivo* studies had already reported its effect on cerebral circulation, however, the results were often conflicting [162]. Generally sulfide salts were used in these studies as exogenous donors of H_2S with only one exception, where GYY4137 was administered to vessel segments in a myograph study [115, 162].

According to studies on pig pial arteries and rat middle cerebral arteries, the endogenous H_2S production involves the CSE enzyme [163-165]. On the contrary, in mouse models the role of CBS-produced H_2S was suspected [166].

In pressure arteriography measurements on pig cerebral arterioles H_2S treatment resulted in vasodilation through K_{ATP} channels. The effect requires the SUR2 subunit of the K_{ATP} channel: in SUR2-null mice the vasodilation did not occur [44].

The underlying mechanism behind the vascular effects of H_2S was also investigated. The vasorelaxant effect of H_2S was observed in several studies (murine and pig *ex vivo* examinations). Mainly the role of voltage-gated Ca^{2+} channels, K_{ATP}- and other K^+-channels, and the interaction with the NO-pathway were suspected as underlying mechanism, similarly to the systemic circulation [162]. In another study it was proven that the vasodilation was independent from the existence of intact endothelial lining; it was partly mediated through L-type Ca-channels and involved K-channels also, which are other than the K_{ATP}, K_{CA}, K_V or K_{IR} channels [72]. ROS formation or scavenging did not alter the effect of H_2S. In a rat global cerebral ischaemia-reperfusion model, H_2S had vasodilatative effect through K_{ATP} channels [72]. The NO-H_2S interaction increased this

vasodilation by the activation of the NO/cGMP/sGC/PKG signaling pathway. In vessels with a diameter less than 20 μM, the effect of NO was moderate, while the greatest H_2S-induced dilation was observed in these vessels [72].

H_2S is supposed to act as an EDHF in the cerebral circulation with special emphasis on ischemia-reperfusion [167]. Besides the vasodilation, vasoconstrictive effect of H_2S - which was already documented in systemic vessels – was observed on cerebral vessels also [40, 162]. In rat basilar artery segments, exogenous H_2S donor treatment induced dose-dependent vasoconstriction at lower concentrations; NaHS treatment had the strongest effect, while GYY4137 had the weakest potential [115]. Isoprenaline and forskolin augmented the vasoconstriction, therefore it is suspected that the cAMP/adenylyl cyclase pathway has a role in this phenomenon – the involvement of the β-adrenergic receptor mediated pathways was also proven in human brain vascular smooth muscle cells (HBVSMCs) [115].

Besides the studies which focus on the physiological role of H_2S in the cerebral circulation, exogenous H_2S treatment was examined also in different cerebrovascular disease models [162, 168]. In a rat model of middle cerebral artery occlusion, H_2S had neuroprotective effects in the post-ischemic area, and also promoted angiogenesis together with endothelial cell synthesis and migration [169]. As another important finding, in a rat model of ischemia-reperfusion, the NO-mediated vasodilation was more severely affected than the H_2S-pathway in the pial arteries, underlining the importance of sulfide-mediated vascular effects [170].

Clinical studies were not performed on the cerebrovascular effects of H_2S, but examination of the serum cysteine levels (the universal substrate of endogenous H_2S-production) of acute stroke patients demonstrated a positive correlation between cysteine levels and poor clinical outcome [171]. In a rat model, cysteine treatment increased the infarct volume in post-stroke animals, while the inhibition of CBS had protective effect [171]. In a rat model of subarachnoideal hemorrhage, NaHS effectively decreased the vascular tone in cerebral vasospasm [172]. Taken together, the studies about the role of H_2S in tissue damage after a cerebrovascular insult are controversial.

2. Objectives

The aim of our studies was to investigate the potential role of hydrogen sulfide in the cardiovascular system regarding two of its potential aspects: as a pretreatment on therapeutic stem cells in an *in vitro* myocardial ischemia model, and also as a player in the regulation of cerebrovascular tone.

The detailed objectives were:

- To study the effect of hydrogen-sulfide pretreatment, both on the survival of therapeutic cells and the postischemic cells in an *in vitro* myocardial ischemia-reperfusion model
- To study the impact of endogenously produced or exogenously administered hydrogen sulfide on the *in vitro* proliferation of human adipose-derived stem cells
- *Ex vivo* pressure myograph examination of the effect of hydrogen sulfide on the vascular tone of rat anterior cerebral artery segments

3. Methods

3.1. Cells and cell cultures

The cells used in the *in vitro* experiments were kept in homogenous cultures, in adequate culture medium. The conditions of incubation were: 37 °C in a humidified atmosphere of 5% CO. All the required manipulations were done in a class II. safety cabinet. Cell culture media was changed at every 2-3 days, the passage of the cells were done at 70-80% of confluency.

H9c2 rat cardiomyoblast cells (ATCC, Wesel, Germany) were used in the *in vitro* ischemia-reperfusion model. Cells were kept in high glucose (4,5 g/l) culture medium (Dulbecco's Modified Eagle Medium, DMEM, PAA), supplemented with 10% fetal bovine serum (FBS, Gibco), 4 mM L-glutamine, 100 U/ml penicillin (Biochrom AG) and 100 µg/ml streptomycin (Biochrom AG). In the experiments, cells from passage 9-11 were used.

Primarily isolated human adipose-derived stem cells (hASCs) were used as therapeutic cells. Cells were harvested from healthy 22-50 years old female volunteers, during an esthetic liposuction. The isolation of cells were made during a collaboration in the Universitätsklinikum Hamburg-Eppendorf. After isolation, cells were directly characterized to stem cell surface markers by flow cytometry [140]. ASCs were kept in 1 g/l glucose DMEM (Dulbecco's Modified Eagle Medium, DMEM, PAA); other supplementary components of the medium were the same as above. In the experiments, cells from passage 9-13 were used.

3.2. Animals

All the following procedures were conformed to the Guide for the Care and Use of Laboratory Animals (Guide for the care and use of the laboratory animals, 8th edition, ELAR/NRC 2011), the legal and institutional guidelines for animal care and were approved by the Animal Care Committee of the Semmelweis University and Hungarian authorities (PE/EA/1430-7/2018).

Experiments were perfomed on freshly isolated anterior cerebral artery segments from the A2 stage, prepared from 3-4 months old/ 295-385 g male Wistar rats (n= 4-9). All animals had the same rat chow ad libitum (S8106-S011 SM, Ssniff Spezialdiaten, Soest, Germany).

3.3. Experimental protocols

3.3.1. *In vitro* ischemia-reperfusion model

During the experiments, we used our formerly configured study model, where controlled oxygen-glucose deprivation was performed on the examined cells, followed by restitution of the ordinary cell culture environment [173]. During the ischemic period, the standard media was changed to glucose-free, while the atmosphere was changed to 0,5% O_2 and 99,5% N_2 in an incubation chamber (PECON incubation system, Erbach-Bach, Germany) under the confocal microscope (Zeiss LSM 510 META, Carl Zeiss, Jena, Germany). After a distinct time, media of the cells was changed back to the standard 4,5 g/l glucose DMEM, and the incubation was continued at normal cell culture conditions (37 °C, 5% CO_2). The experimental protocol is summarized on Fig. 8.

The structural and intracellular changes of the cells were monitored continuously using confocal microscope, by morphological changes and fluorescent probes as well. During the pilot studies, the sufficient cell number and adequate time of ischemic period were determined. Before any manipulation, cells were labeled with a double fluorescent dye. Calcein acetoxymethyl ester (Calcein AM, part of the LIVE/DEAD Viability/Citotoxicity assay Kit, Molecular Probes, ex/em 494/517 nm), indicates living cells. It is a colorless substance in its initial form, which gains its green fluorescence after cleavage by intracellular esterases of living cells. The other dye was ethidium homodimer (EthD, part of the LIVE/DEAD Viability/Citotoxicity assay Kit, Molecular Probes, ex/em 495/695 nm) for labeling necrotic cells. EthD is only able to enter cells with disorganised membranes, and therefore its red fluorescence signs irreversibly damaged or dead cells. According to the results of the pilot stuides, the optimal cell density was 12x30.000 H9c2 cell on a 12-well plate. The endpoint of the ischemia was when 50% of the cells were died; to achieve this point, 155 min long ischemia was needed in our model.

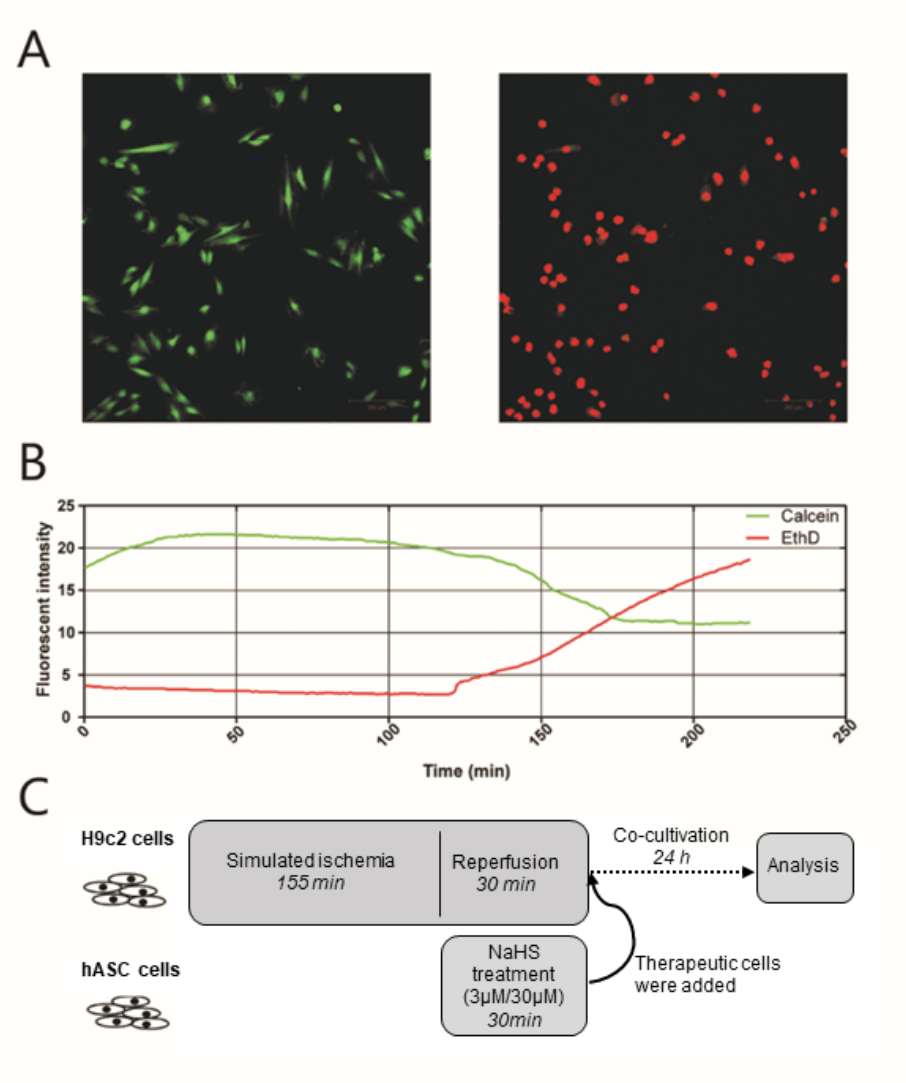

Figure 8. The *in vitro* controlled oxygen-glucose deprivation. **A:** confocal microscopic images of viable cells (green fluorescence by Calcein-AM) and necrotic cells (red fluorescence by EthD) from the identical field of view; **B:** changes in fluorescence intensity throughout the simulated ischemia, fluorescent intensity measured by *field of view %*; **C:** experimental protocol of the *in vitro* ischemia-reperfusion with stem cell therapy.

The administration of therapeutic cells was done after 30 minutes from the beginning of simulated reperfusion, when approx. 20 000 hASC cells were added to every well of H9c2 cells. Cells were kept in co-culture for 24 hours. In order to the latter distinction of the hASC cells, they were labeled with Vybrant® DiD (excitation/emission: 633/665 nm, Molecular Probes) fluorescent dye before their administration to the co-

culture. As a pretreatment, watery solution of NaHS (Sigma-Aldrich) was used for 30 min prior to co-cultivation. Because of the volatile character of H_2S, the final concentrations from the stock solution were always made immediately before the treatment.

Flow cytometry measurements (FACSCalibur™; Becton-Dickinson, Franklin Lakes, NJ, USA) were performed after 24 h co-cultivation of the H9c2 with hASC cells. Because of the previous Vybrant DiD labeling of hASC cells, the survival of the two cell type (H9c2 and hASC) was measured separately.

3.3.2. Cell proliferation experiments

During these experiments, either exogenous H_2S donor (NaHS), or an inhibitor of endogenous H_2S synthesis (PAG) were used in hASC cells (Fig.9). Both substances were dissolved in the standard culture medium of the cells. In the case of NaHS measurements, cells were seeded on 24-well plates in a density of 2000 cells/well, and were treated with 0.3 -3 -30-300 µM NaHS (except the control group, which received saline). During the PAG measurements, cells were seeded on a 12-well plate in a density of 5000 cells/well, and cells were treated with 1 or 5 mM PAG (except the control group, which received saline). Measurements were perfomed for 9 days in both setup. Culture media was changed every third day, and then 3x3 tile scan images were taken from the identical points of the wells by confocal microscope.

The proliferation rate was defined by the following equation:

(1-(initial cell count/actual cell count))x100

Evaluation of the images were performed with ImageJ software (NIH).

In the proliferation experiments, the metabolic activity of the cells was also studied. Total mitochondrial activity was measured of the 0.3- 3- or 30 µM NaHS-treated cells and from the control cells.

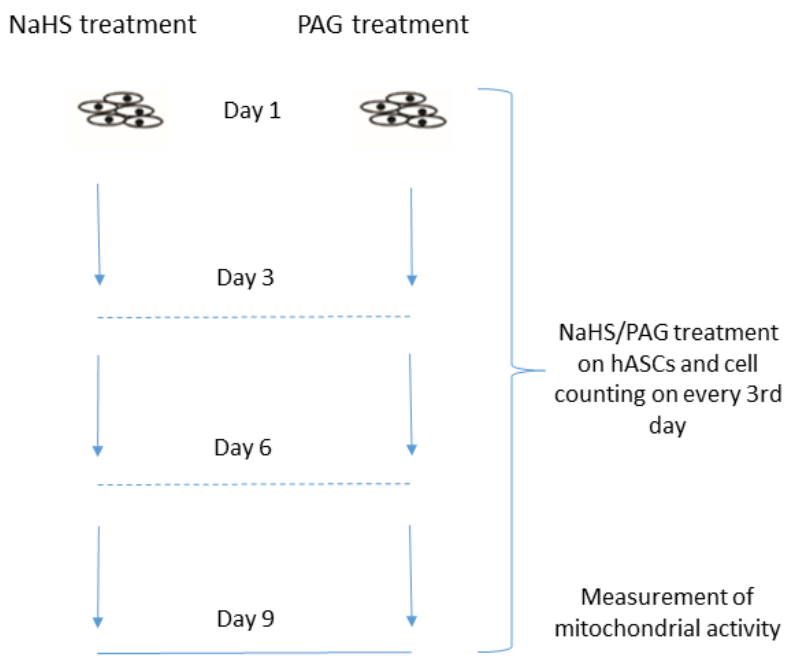

Figure 9: Protocol for the proliferation studies on ASCs.

3.3.3. Pressure myograph experiments

Wistar rats were anaesthetised with pentobarbital (Nembutal, Ceva Santé Animale, Libourne, France, 45 mg/kg body weight, administered i.p.). Then the left atrium was cut open, and the vessels were perfused from the left ventricle with heparinized cold Krebs-Ringer solution, in the end the animals were decapitated. Occipital craniotomy was performed, then the brain was removed together with the intact meninges, and was put in ice-cold Krebs solution. The right anterior cerebral artery (ACA) was cleared from the surrounding tissue and cut out. An approx. 2 mm long segment from the A2 section was used in the further experiments. After preparation, the artery segments were immediately put into an organ chamber filled with Krebs-Ringer solution. Side-branches were ligated, vessels were cannulated at both ends with microcannulas, and extended to in vivo length.

The organ chamber containing the cannulated vessel segment was placed on the stage of an inverted microscope (Leica, Wetzlar, Germany). Servo-controlled pumps (Living Systems, Burlington, VT, USA) with pressure transducer (Living Systems,

Burlington, VT, USA) were used to set the intraluminar pressure. Calibration of the experimental system was done with a mercurial manometer. The vessels were mounted in Krebs-Ringer solution bubbled with a gas mixture containing 5% CO2, 20% O2 and 75% N2, and were pressurized to 50 mmHg. Then the segments were left in the system due to equilibration for 30 min. During the examinations, temperature of the bath solutions was kept at 37 °C by a digital thermostate system (MLW UH8, Germany). Pictures were taken with a digital camera (Leica DFC 320) and were analyzed with Leica Qwin V3 software. The inner and outer diameters of the vessels were measured from these pictures. Calibration was made using a micrometer etalon (Wild, Heelbrugg, Switzerland).

U46619 was administered from its stock solution directly to the organ bath containing normal Krebs solution, the volume was calculated so as to reach the final concentration of 3 μM.

For the record of pressure-radius curves, vessel segments were examined in normal Krebs solution. After 30 min equilibration at 50 mmHg, pressure-radius curves were recorded. During the measurements, the intraluminar pressure was gradually increased as follows: 0-10-20-30-40-50-60-70-80-90-100 mmHg. Inner and outer diameters of the vessels were measured either offline using the ImageJ software from photos taken from the microscope (measurements with NaHS) or online with a picture analyzing software (Leica Qwin V3) from the camera connected to the stage of the microscope (Leica DFC 320).

At the end of experiments, vessel segments were rinsed and then, as the last step, they were incubated in Ca-free Krebs solution for 30 minutes to achieve maximal relaxation.

3.4. *In vitro* measurements in the myocardial ischemia model

3.4.1. Flow cytometry

Cells were trypsinized well by well, then resuspended in 500 µl phosphate buffered saline (PBS), which contained the previously mentioned calcein AM and EthD fluorescent dyes. Cytometry results were evaluated with the Weasel software (WEHI, Australia). According to the relevant literature and our previous observations, three group of cells were distinguished (Table 4, Fig. 10.):

Table 4: Definition of cell viability by fluorescent dyes. Calcein AM: Calcein acetoxymethyl ester. labeling living cells, EthD: ethidium homodimer, labeling dead cells.

Cell population	Calcein AM	EthD
living	positive	negative
dead	negative or slightly positive	intensely positive
apoptotic	positive	positive

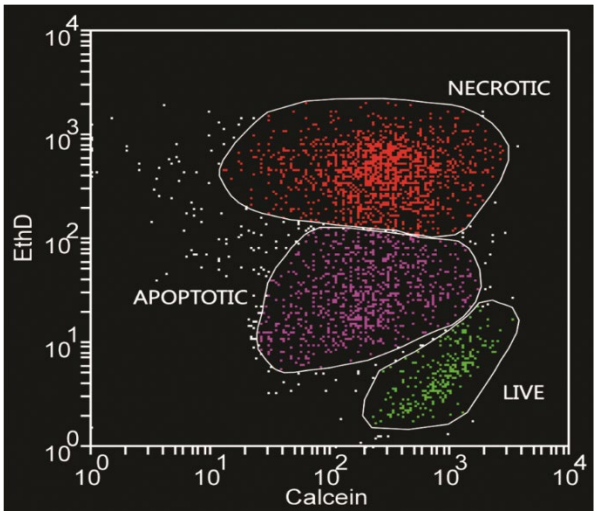

Figure 10. Scatter dot plot from flow cytometry measurements. According to the EthD staining, the positive (red) population consists of necrotic cells, the negative (green) population refers to the viable cells, while the intermediate staining ("double positive", purple) represents the apoptotic population.

3.4.2. Lactate dehydrogenase (LDH) assay

LDH release was measured in the hASC cells after NaHS treatment was perfomed in order to examine its possible toxic effect, and to determine the safe concentration of NaHS in hASC cells. This measurement was also used to investigate the possible effect of NaHS on the survival of cells.

During this cytotoxicity test the hASC cells were treated with 0.3 µM, 3 µM, 30 µM, 300 µM or 3 mM NaHS for 30 minutes, which was followed by spectrophotometric measurement from the cell culture supernatant with the LDH assay Kit (LDH Cytotoxicity Kit II; PromoCell, Heidelberg, Germany), according to the manufacturer's protocol. Toxic effect was measured as follows:

cytotoxicity%= [(sample-negative control) / (positive control-negative control)] x 100

For examination of cell survival, 3 µM and 30 µM NaHS treatment was used for 30 min, which was followed by a 2 mM hydrogen peroxide (H_2O_2) treatment for 120 min. This was followed by the LDH assay.

3.4.3. Measurement of mitochondrial activity

Mitochondrial activity was examined using the PrestoBlue cell viability reagent (Invitrogen, CA, USA). This is a resazurin-based assay, which is used to assess cell viability, cell proliferation or mitochondrial activity. Resazurin is blue with no fluorescent activity, and it is transformed to resurofin, a pink, fluorescent product by mitochondrial enzymes. Formation of resurofin can be detected by fluorescence or absorbance readers as well.

First, the standard culture medium was changed to phenol red-free, low glucose 1g/l medium, which was supplemented with 10% PrestoBlue reagent. After 6 hours of incubation, 200 µl of the supernatant was transported to a 96-well plate. It was followed by a spectrophotometric measurement of absorbancy at 570 nm and 600 nm wavelengths, and the value from the 600 nm was normalized with the value from 570 nm.

3.5. *Ex vivo* measurements in the pressure myograph system

3.5.1. Measurements on vessel segments with NaHS

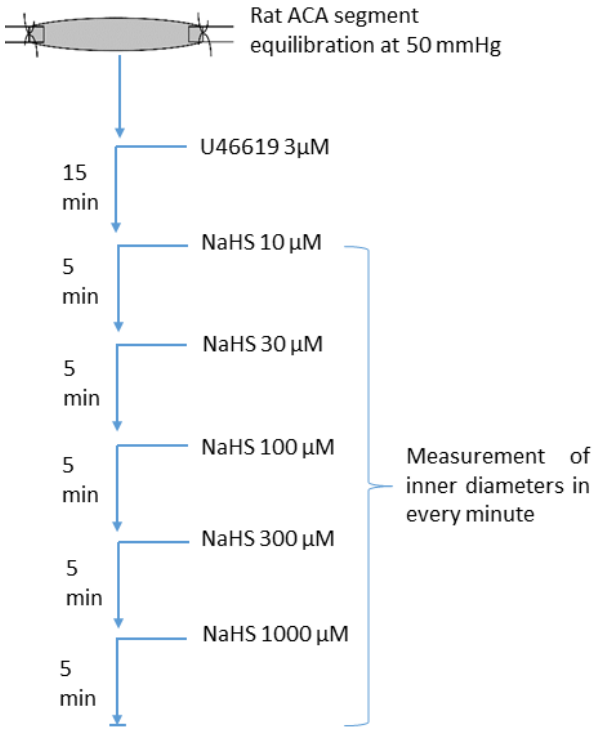

Figure 11: Protocol for NaHS treatment on the rat a. cerebri anterior segments.

The ACA segments were mounted on the microscope, and equilibrated with 50 mmHg as detailed before. After precontraction of the vessels with 3 µM U46619, NaHS was administered to the organ bath resulting in increasing concentrations (10-30-100-300-1000 µM), and the dose-response curves were recorded by measurement of vessel diameters after the addition of each dose of NaHS in every minute for 5 minutes (Fig. 11).

3.5.2. Measurements on vessel segments with PAG or DIDS

The endogenous H_2S-synthesis inhibitor, propargylglycine (PAG) was used to assess the potential role of endogenous H2S in the cerebral vascular tone. In these experiments, the intraluminal pressure was set to 50 mmHg. After 20 min equilibration, the pressure-radius curves were recorded between 0-100 mmHg intraluminar pressure values, increased by 10 mmHg steps.

DIDS is a nonselective anion exchanger inhibitor molecule. In the examinations with DIDS, first the vasorelaxation induced by 1000 µM NaHS was recorded. It was followed by pretreatment with 300 µM DIDS, and after 20 min equilibration, 1000 µM NaHS was administered to the vessel segments, and the results were compared to the vasorelaxation exerted by 1000 µM NaHS without DIDS treatment.

3.6. Materials

Dulbecco's modified eagle medium (DMEM) with standard (4,5 g/L) and low (1 g/L) glucose content and fetal bovine serum (FBS), penicillin and streptomycin were purchased from Sigma-Aldrich/Merck KGaA (Darmstadt, Germany).

U46619 was purchased from Tocris Bioscience (Bristol, UK). Sodium hydrosulfide (NaHS), DL-propargylglycine (PAG) and 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid disodium (DIDS) was obtained from Sigma-Aldrich/Merck KGaA (Darmstadt, Germany). All the materials above were stored under recommended conditions - according to the manufacturer - as aliquots, from which solutions were prepared freshly immediately before use.

The composition (in mmol/l) of the Krebs-Ringer solution used in the myograph experiments was the following: Na^+ 144; K^+ 4.7; SO_4^- 1,2; $H_2PO_4^-$ 1.2; Mg^{2+} 1.2; HCO_3^- 24; Ca^{2+} 2.5; glucose 5.5; and EDTA 0.02, and for the Ca-free solution: Na^+ 144; K^+ 4.7; SO_4^- 1,2; $H_2PO_4^-$ 1.2; Mg^{2+} 1.2; HCO_3^- 24; glucose 5.5; and EDTA 0.025, EGTA 2.0.

3.7. Statistical analysis

Results are shown as mean ± SEM. For the data from the experiments in the *in vitro* myocardial ischemia model, unpaired t-test and one-way or factorial ANOVA was used with Bonferroni-, Newman-Keuls- or Tukey's post hoc test. On the data from the *ex vivo* pressure myograph experiments, ordinary one-way and two-way ANOVAs were used with Tukey post-hoc tests.

The p value < 0.05 was considered as statistically significant. Statistical analysis was performed with GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA).

4. Results

4.1. Effect of the H_2S donor sodium hydrosulfide (NaHS) pretreatment on the survival of hASCs and on the postischemic H9c2 cells

4.1.1. Cytotoxicity test with NaHS

According to the lactate dehydrogenase (LDH) cytotoxicity test, only the highest examined concentration, 3 mM of NaHS was proven to have toxic effect on hASC cells (control: 0,0±1,6 %; 3 mM NaHS: 12,72 ± 1.23 %), resulting in cell necrosis. The NaHS concentrations that were selected for the further examinations (3 µM and 30 µM) did not cause toxicity (3 µM: 0,9230 ± 3.73 %; 30 µM: 1,860 ± 3.24 %; 300 µM; 0,5097 ± 1.82 %) (Fig. 12.).

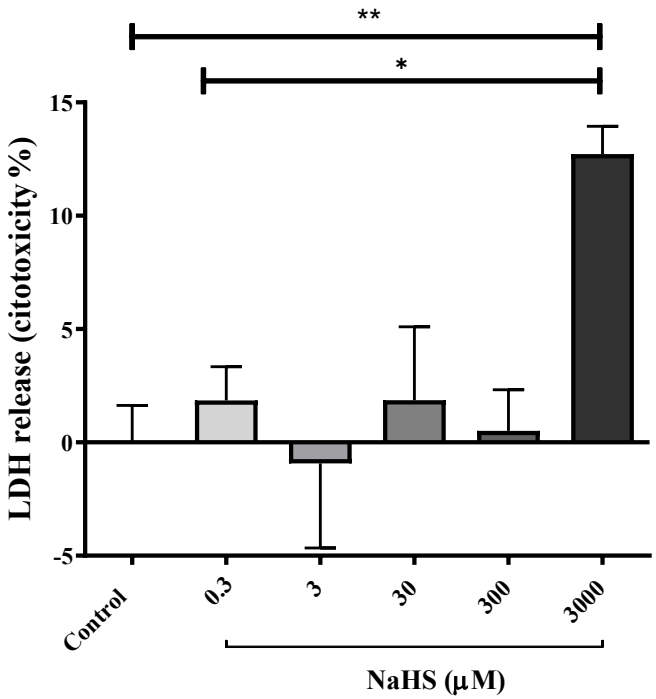

Figure 12. LDH release of cells after NaHS treatment (mean ± SEM, n=6; one-way ANOVA with Tukey' multiple comparison test; control vs. 3000 µM: ** $p < 0.01$; 0.3 µM vs. 3000 µM: * $p < 0.05$) [174].

4.1.2. Effect of NaHS pretreatment on the survival of both the therapeutic hASC cells and the postischemic H9c2 cardiomyoblasts.

After co-culture with the postischemic H9c2 cardiomyoblasts, the number of necrotic hASC cells decreased significantly in the NaHS-pretreated groups (Fig. 12.C). 3 μM NaHS treatment decreased the ratio of dead cells more effectively (3.892 ± 0.48 %) than 30 μM NaHS (4.415 ± 0.62 %), the difference was significant in both concentrations compared to the control group (6.648 ± 0.67 %,). No significant change was observed in the ratio of living (control: 87.21 ± 0.8 %; 3 μM NaHS 87.37 ± 3.96 %; 30 μM NaHS 90.88 ± 0.84 %, Fig. 13.A) and apoptotic cells (control: 6.140 ± 0.42 %; 3 μM NaHS: 8.735 ± 4.03 %; 30 μM NaHS: 4.707 ± 0.53 %, Fig. 13.B). Furthermore, co-cultivation with hASC cells pretreated with 3 μM NaHS significantly lowered the number of necrotic H9c2 cells (control: 10.67 ± 0.96%; 3 μM NaHS: 7.020 ± 1.11%) however this beneficial effect was absent when co-cultivating the postischemic cardiomyoblasts with 30 μM NaHS-pretreated hASCs (4.415 ± 0.62%, Fig. 13.F). Similarly, no significant change was measured in the percentage of living (control: 72.25 ± 3.8%; 3 μM NaHS: 80.29 ± 2.29%; 30 μM NaHS: 80.73 ± 2.51%, Fig. 13.D) and apoptotic (control: 17.09 ± 4.0%; 3 μM NaHS: 12.70 ± 2.17%; 30 μM NaHS: 10.95 ± 2.29% Fig. 13.E) cell populations of H9c2 cells after addition of therapeutic cells from either group.

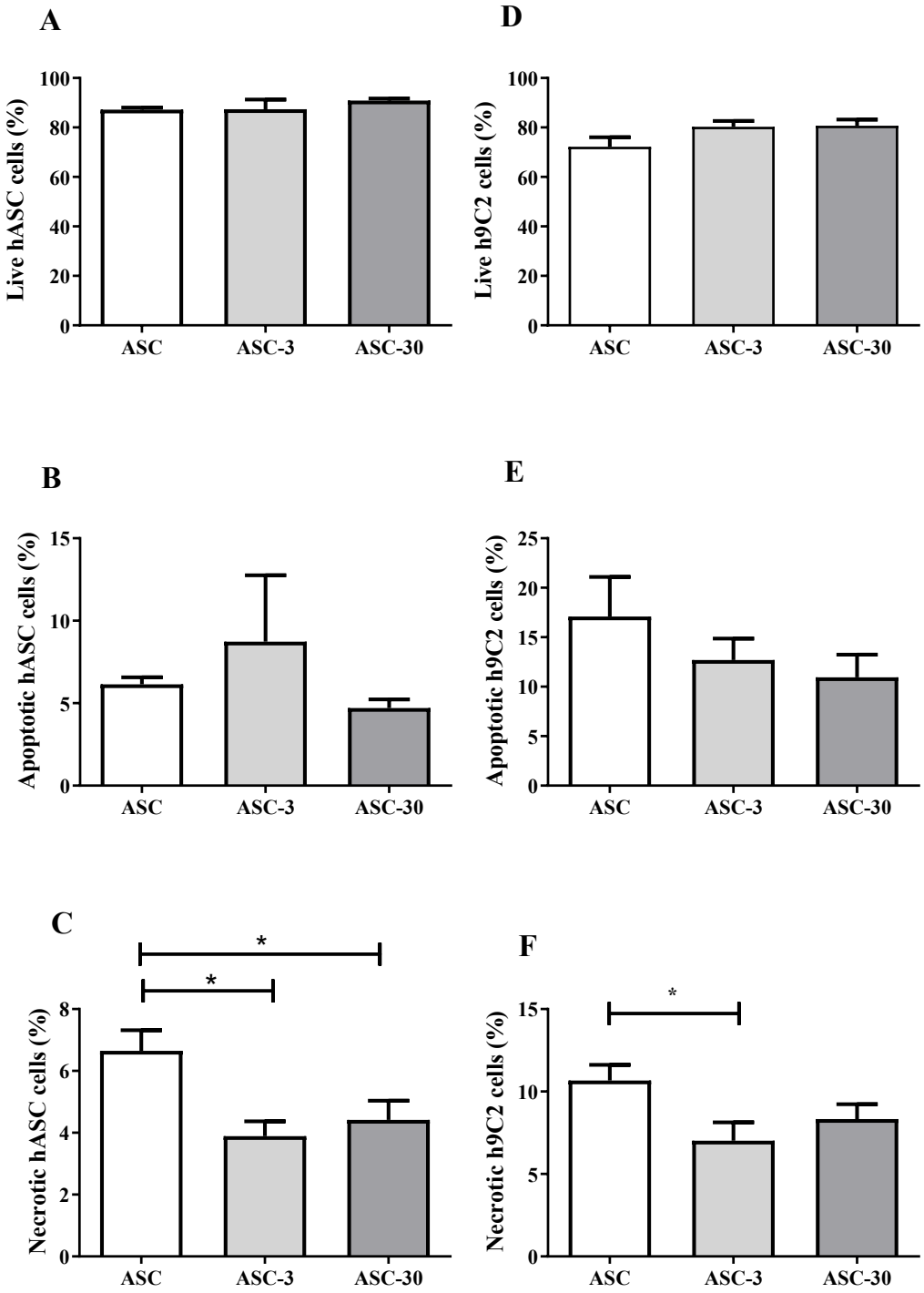

Figure 13. Ratio of living, apoptotic and necrotic hASC cells **(A-C)** and H9c2 cells treated with ASCs **(D-F)**. **A-C:** *ASC:* untreated hASC cells, *ASC-3:* ASCs pretreated with 3 µM NaHS, *ASC-30:* ASCs pretreated with 30 µM NaHS; **D-F:** *ASC:* H9c2 cells co-cultivated with untreated ASCs, *ASC-3:* H9c2 cells co-cultivated with 3 µM NaHS pretreated ASCs, *ASC-30:* H9c2 cells co-cultivated with 30 µM NaHS pretreated ASCs (mean ± SEM, n=6/group, repeated measures one-way ANOVA with Newman-Keuls multiple comparisons test, * $p<0.05$)[174]

4.1.3. Effect of NaHS pretreatment of hASC cells on antioxidant effectivity

3 µM NaHS treatment for 30 min increased the survival of hASCs after 2 mM hydrogen peroxide (H_2O_2) treatment for 120 min (control: 90.33 ± 3.93%; 3 µM NaHS: 66.98 ± 2.88%), however this beneficial effect was absent after 30 µM NaHS treatment (30 µM NaHS: 89.46 ± 5.11%, Fig. 14)

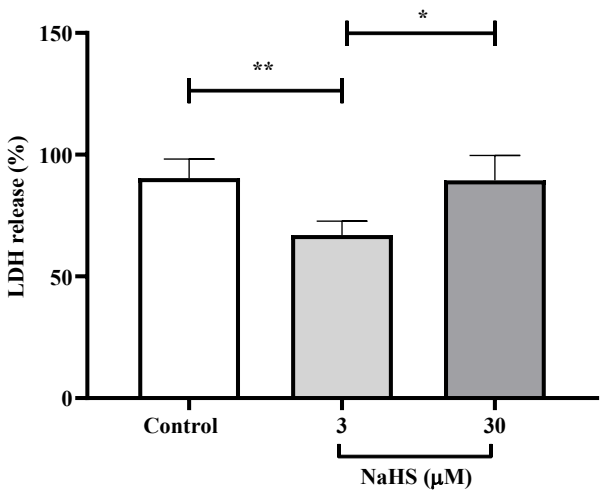

Figure 14. The ratio of LDH release from ASC cells after H_2O_2 treatment (mean ± SEM, n=4/group, one-way ANOVA with Bonferroni multiple comparison test, 3 µM NaHS vs. 30 µM NaHS *P<0.05; control vs. 3 µM NaHS **p<0.01) [174].

4.2. Effect of NaHS treatment on the *in vitro* proliferation of hASC cells

4.2.1. Effect of long-term NaHS treatment on cell proliferation

0,3-30 µM NaHS treatment of ASCs increased cell proliferation in a concentration-dependent manner (Fig.15); the differences became significant on the 9th day of treatment (control 234.5 ± 12.69%; 0.3 µM NaHS: 331,2 ± 33.72%; 3 µM NaHS: 405.6 ± 30.7%; 30 µM NaHS: 470.9 ± 16.71% on the 9th day) however this effect was absent in the group treated with the highest, 300 µM NaHS concentration (301.0 ± 22.47%).

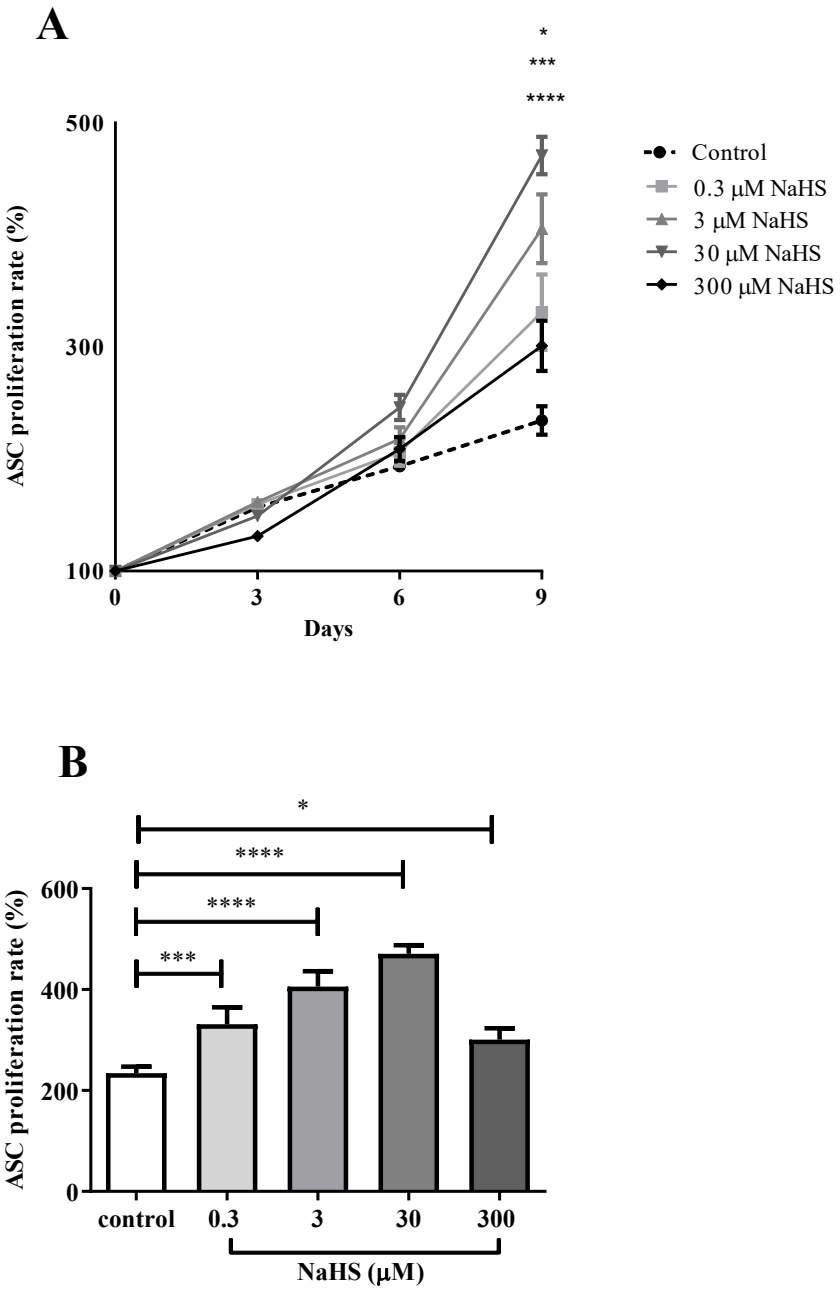

Figure 15. Effect of NaHS treatment on the proliferation of hASC cells. NaHS treatment was administered on the 3rd, 6th and 9th days. **A:** proliferation rate on the 3rd , 6th , and 9th day; **B:** proliferation rate on the 9th day (mean ± SEM, n=4/group, two-way ANOVA with Bonferroni multiple comparisons test; control vs. 300 μM NaHS: *P<0.05, control vs. 0.3 μM NaHS: ***P<0.001,; control vs. 3 μM and control vs. 30 μM NaHS: ****P<0.0001,) [174].

4.2.2. Effect of propargylglycine (PAG) treatment on the proliferation of hASC cells

Treatment with the endogenous H_2S synthesis inhibitor propargylglycine (PAG) decreased the proliferation of hASCs; this effect was observed from the 6th day of treatment, and became significant on the 9th day (control: 147.0 ± 4.43 %; 1 mM PAG: 125.6 ± 8.92 %; 5 mM PAG: 81.92 ± 10.59 % on the 9th day, Fig. 16).

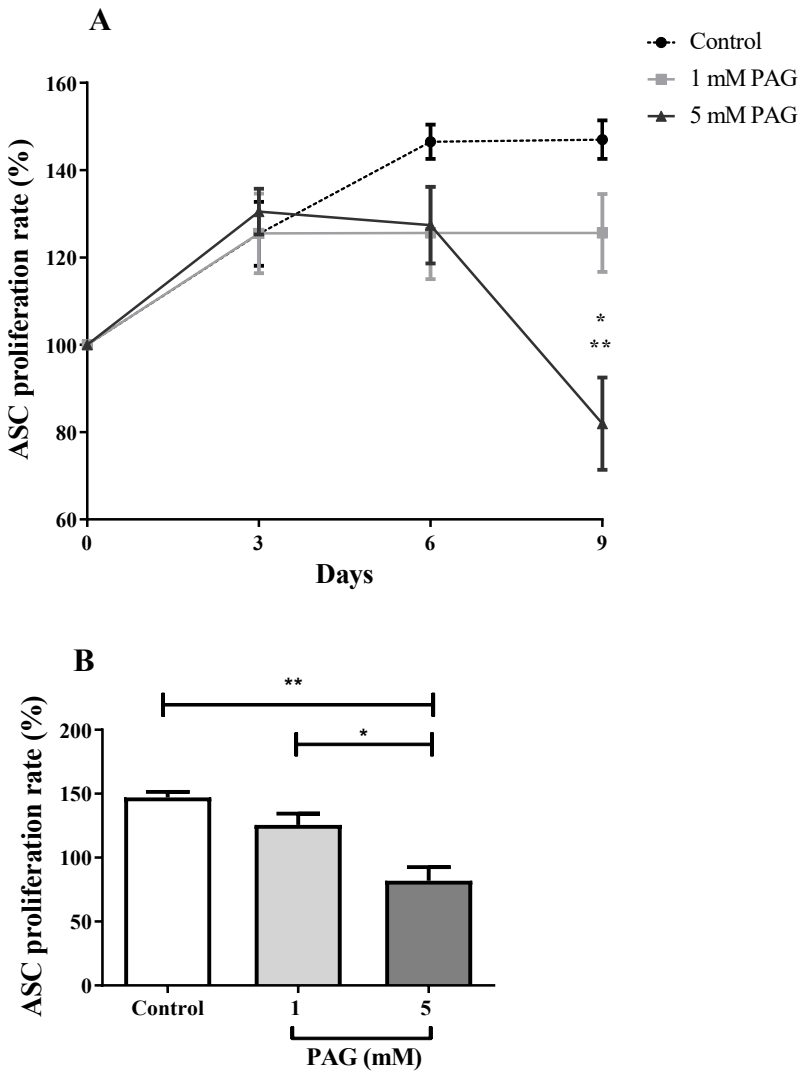

Figure 16: Effect of PAG treatment on the proliferation of hASC cells. A ratio of proliferation on the 3rd, 6th and 9th days. B: ratio of proliferation on the 9th day . (mean ± SEM; n=4/group; one-way ANOVA with Tukey's multiple comparison test, 1 mM vs 5 mM PAG *P<0.05, control vs. 5 mM PAG **P<0.01) [174].

4.2.3. Effect of the 9 day-long NaHS treatment on total mitochondrial activity

The mitochondrial activity of the cells did not increase linearly with the proliferation rate during the 9 day long treatment (control: 471.4 ± 131.3%; 0.3 µM NaHS: 510.0 ± 46.1%; 3 µM NaHS: 455.0 ± 121.1%; 30 µM NaHS: 607.9 ± 144.00% on the 9th day, Fig.17)

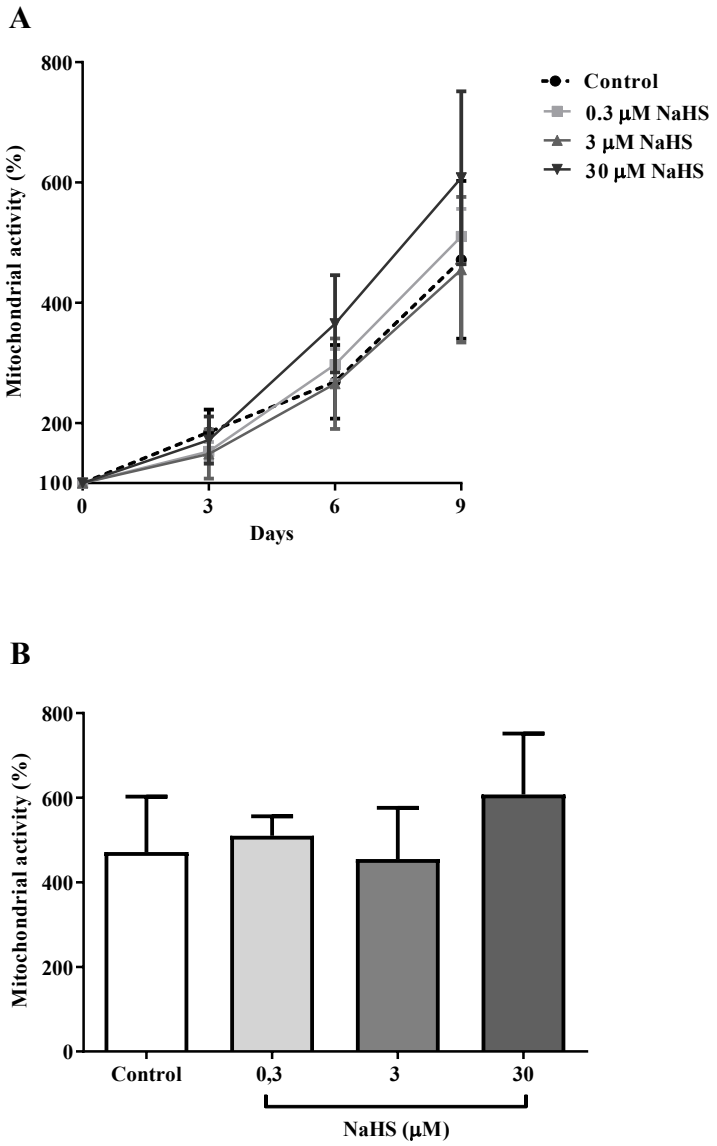

Figure 17: Effect of NaHS treatment on the mitochondrial activity of hASC cells measured by the PrestoBlue assay. **A:** changes in mitochondrial activity through the 9 days of NaHS treatment, **B:** Mitochondrial activity on the 9th day of NaHS treatment (mean ± SEM; n=6/group, ns) [174].

4.3. Effect of endogenous hydrogen sulfide on the vascular tone of rat a. cerebri anterior (ACA) segments

Treatment of ACA segments with 10 mM propargylglycine (PAG, endogenous inhibitor of H_2S synthesis) tended to increase the vessel diameter at intraluminal pressure values above 50 mmHg, compared to the untreated control as follows (Fig. 18):

0 mmHg: control: 0,446 ± 0,23%, PAG: 0,559 ± 0,17%;

10 mmHg: control: 0,587 ± 0,17%, PAG: 0,595 ± 0,17%;

20 mmHg: control: 0,748 ± 0,04%, PAG: 0,763 ± 0,05%;

30 mmHg: control: 0,810 ± 0,04%, PAG: 0,779 ± 0,02%;

40 mmHg: control: 0,789 ± 0,02%, PAG: 0,806 ± 0,04%;

50 mmHg: control: 0,834 ± 0,04%, PAG: 0,857 ± 0,04%;

60 mmHg: control: 0,810 ± 0,04%, PAG: 0,937 ± 0,11%;

70 mmHg: control: 0,794 ± 0,06%, PAG: 0,960 ± 0,09%;

80 mmHg: control: 0,815 ± 0,05%, PAG: 0,945 ± 0,09%;

90 mmHg: control: 0,772 ± 0,09%, PAG: 0,914 ± 0,09%;

100 mmHg: control: 0,856 ± 0,06%, PAG: 0,948 ± 0,07%;.

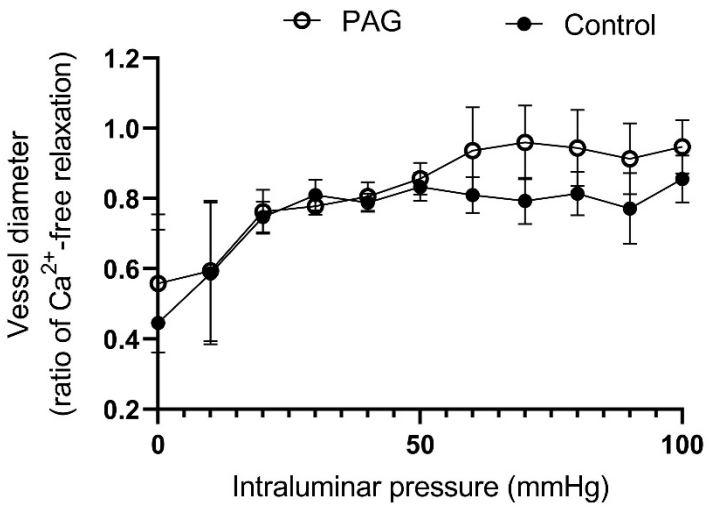

Figure 18. Effect of 10 mM PAG treatment on the vascular tone. Pressure-radius curves of ACA segments (n=4/group, ns.)

4.4. Effect of exogenous hydrogen sulfide on the vascular tone of rat ACA segments

4.4.1. Precontraction with U46619

Treatment with the thromboxane A2 receptor agonist U46619 in a concentration of 3 µM significantly decreased the outer and inner diameters of the vessel segments in minutes, and exerted a prolonged vasoconstrictive effect at 50 mmHg intraluminar pressure on the rat arteria cerebri anterior segments (*Outer diameter:* control: 309.3 ± 24.62 µM, U46619: 268.7 ± 19.42 µM; *inner diameter*: control: 212.3 ± 29.01 µM, U46619 163.7 ± 21.43 µM; measured 15 min after administration of U46619, Fig. 19).

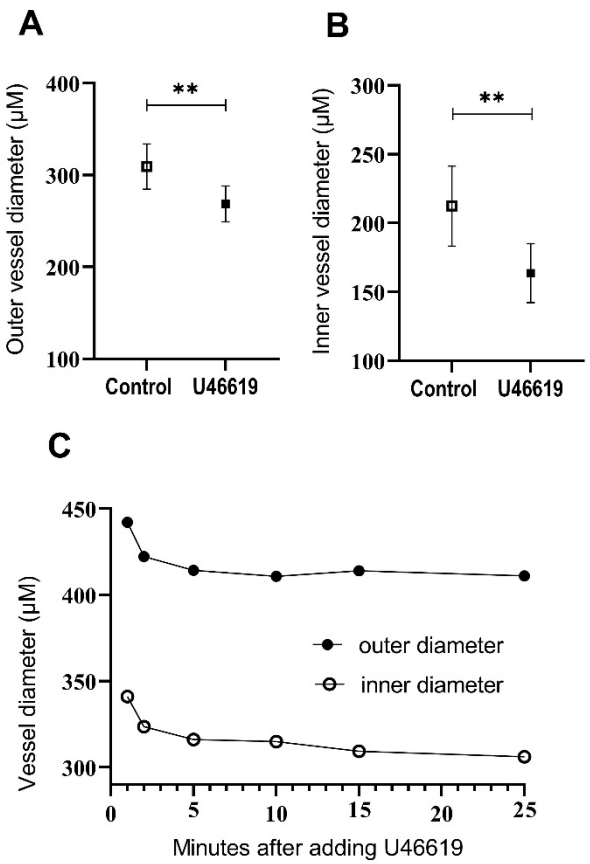

Figure 19: Effects of U46619 on the pressurized vascular segments regarding **(A)** outer **(B)** inner diameters and **(C)** over time (mean ± SEM, n=9/group; Ratio paired t-test, control vs. U46619: **p <0.01)

4.4.2. Exogenous H_2S treatment on pressurized vessel segments

NaHS was administered into the Krebs-Ringer solution of the organ bath in a concentration range of 10 µM – 1000 µM in a cumulative manner, and doses were increased every 5 minutes. The effect of the NaHS treatment was recorded in every minute by measurement of the inner vessel diameters, compared to the U46619-induced precontraction as follows:

1st minute: 10 µM NaHS: 1.002 ± 0.03%; 30 µM NaHS: 0.9498 ± 0.05%; 100 µM NaHS: 0.9128 ± 0.05%; 300 µM NaHS: 0.8331 ± 0.08%; 1000 µM NaHS: 1.295 ± 0.35%; Fig.20.A;

2nd minute: 10 µM NaHS: 0.9932 ± 0.04%; 30 µM NaHS: 0.9517 ± 0.05%; 100 µM NaHS: 0.9097 ± 0.05%; 300 µM NaHS: 0.8504 ± 0.09%; 1000 µM NaHS: 1.278 ± 0.36%; Fig20.B,

3rd minute: 10 µM NaHS: 0.9830 ± 0.03%; 30 µM NaHS: 0.9414 ± 0.05%; 100 µM NaHS: 0.9139 ± 0.04%; 300 µM NaHS: 0.8919 ± 0.08%; 1000 µM NaHS: 1.355 ± 0.39% Fig20.C;

4th minute: 10 µM NaHS: 0.9357 ± 0.05%; 30 µM NaHS: 0.9166 ± 0.04%; 100 µM NaHS: 0.8668 ± 0.04%; 300 µM NaHS: 0.9807 ± 0.11%; 1000 µM NaHS: 1.405 ± 0.42% Fig.20.D;

5th minute: 10 µM NaHS: 0.9535 ± 0.03%; 30 µM NaHS: 0.9521 ± 0.05%; 100 µM NaHS: 0.9418 ± 0.06%; 300 µM NaHS: 1.172 ± 0.16%; 1000 µM NaHS: 1.574 ± 0.36%, Fig.20.E.

The collected data showed a biphasic effect, slight vasocontraction in the lower concentrations followed by a pronounced relaxation above 300 µM. The change from constriction to relaxation was clearly observed at 300 µM (1st minute: 0.8331 ± 0.08%; 2nd minute: 0.8504 ± 0.09%; 3rd minute: 0.8919 ± 0.08%; 4th minute: 0.9807 ± 0.11%; 5th minute: 1.172 ± 0.16%; Fig. 20.F)

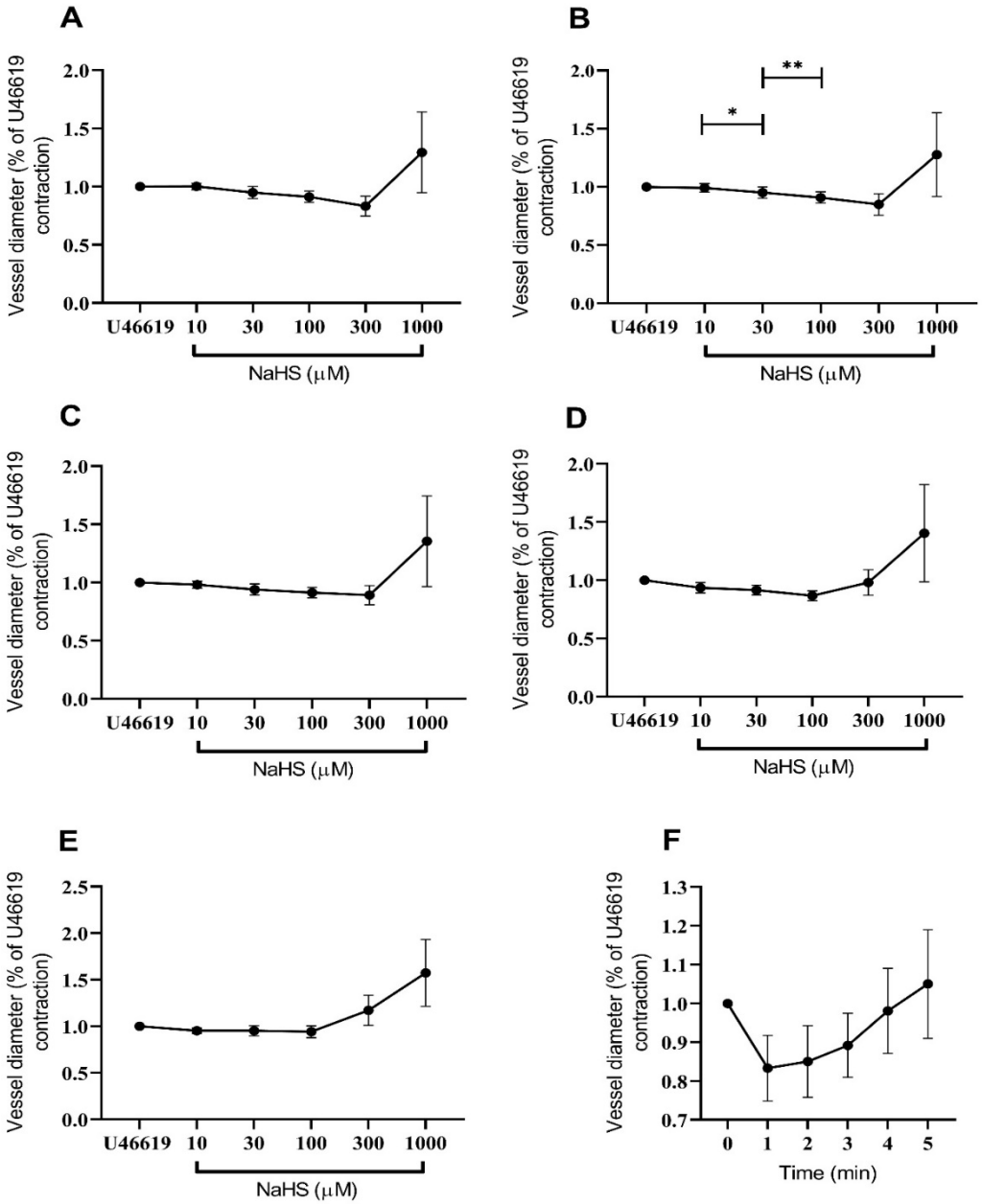

Figure 20. Effect of 10 μM-1000 μM NaHS treatment on vessel segments.

A-E: vascular diameters of the ACA segments, measured in the 1st – 5th minute after the addition of 10 μM – 1000 μM NaHS; **A:** vascular diameters measured after 1 min of NaHS treatment; **B:** vascular diameters measured after 2 min of NaHS treatment **C:** vascular diameters measured after 3 min of NaHS treatment **D:** vascular diameters measured after 4 min of NaHS treatment **E:** vascular diameters measured after 5 min of NaHS treatment (mean ± SEM, n=5/group, one-way ANOVA with Tukey's multiple comparison test, 10 μM vs 30 μM: * $p < 0.5$; 30 μM vs 100 μM: ** $p < 0.01$). **F:** Vascular diameters measured 1-5 minutes after the administration of 300 μM NaHS (mean ± SEM, n=5, ns).

4.4.3. Role of DIDS on the effect of H_2S treatment

Pretreatment of the vessel segments for 20 min at 50 mmHg intraluminar pressure with the nonselective anion channel blocker 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) in 300 µM concentration attenuated the vasorelaxation exerted by the administration of 1000 µM NaHS; and this effect was significant at the outer diameter (*Outer diameter:* control: 1.335 ± 0.15%; DIDS: 1.034 ± 0.02%; *inner diameter:* control: 1,753 ± 0.4%, DIDS: 1,008 ± 0.05%, Fig. 21).

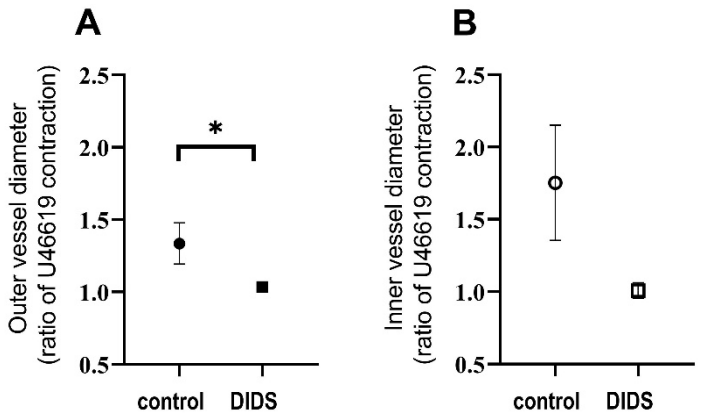

Figure 21. Effect of 300µM DIDS treatment on the 1000 µM NaHS-induced vasorelaxation, measured after 20 min equilibration at 50 mmHg intraluminar pressure. **A:** outer vessel diameter (ratio of U46619-induced contraction, mean ± SEM, n=5/group, unpaired t-test, control vs. DIDS, * $p < 0.5$); **B:** inner vessel diameter (ratio of U46619-induced contraction, mean ± SEM, n=5/group, control vs. DIDS, ns).

4.4.4. Effect of a non-ligated side branch on the vascular tone of a pressurized vessel segment

In particular vessel specimens, where non-ligated minor side branches were left, pressure difference and flow occurred between the proximal cannulated end and the location of the side branch. As a result of intraluminal flow, vasoconstriction was observed in this part of the vessel, however the distal parts remained in dilated position during 1000µM NaHS treatment (Fig. 22.).

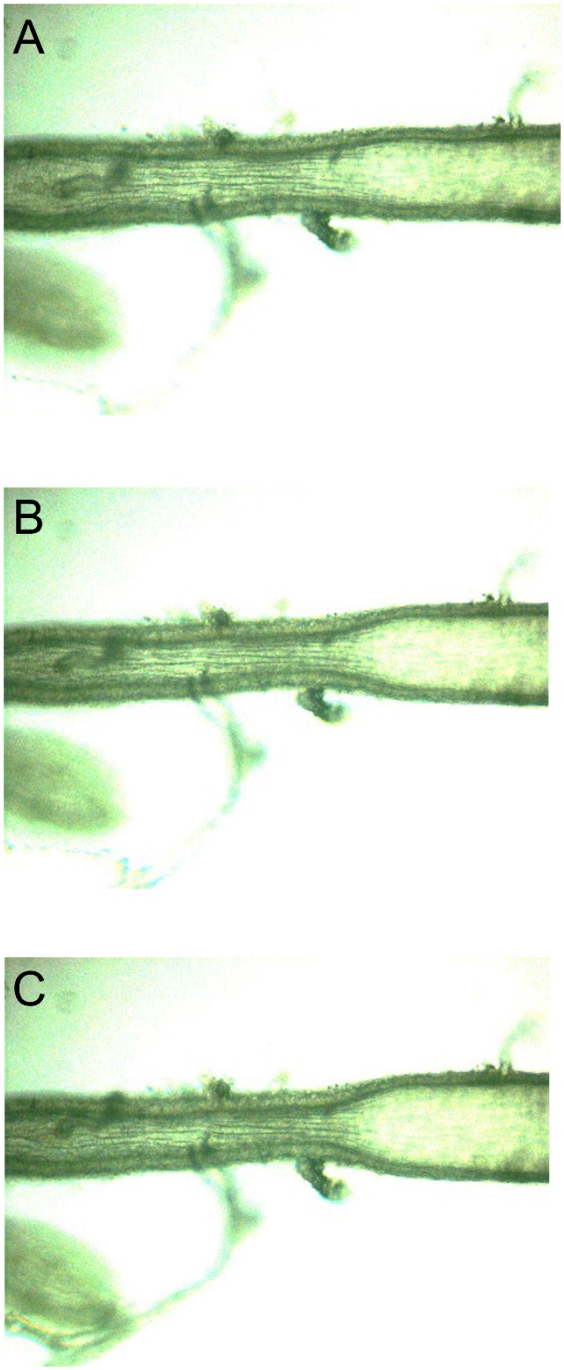

Figure 22: Effect of intraluminar flow due to a non-ligated side branch, observed during the pilot studies. **A-C:** pictures taken after 1000 μM NaHS treatment at the 1st, 3rd and 5th minutes, respectively.

5. Discussion

The experiments presented in this thesis examined the possible role of hydrogen sulfide in the cardiovascular system from two important aspects: as a pretreatment in an *in vitro* model of cell therapy after myocardial infarction, and its possible effect on rat cerebral artery segments. Experimental questions similar to these were already studied using several models in the related literature, however these earlier results were not fully congruent. The main cause of the inconsistent results is probably related to the pluripotent nature of H_2S in the used animal models, tissue- and cell types or experimental circumstances [12, 40].

5.1. Hydrogen sulfide as a pretreatment in an *in vitro* model of cell-based therapy

Numerous *in vitro, ex vivo* and *in vivo* ischemia-reperfusion models have been developed and used in cardiovascular research. *In vitro* methods include examination on primarily isolated cardiac cells, or coronary artery ligation on isolated, working heart with or without reperfusion, or the use of the Langendorff setup [175]. The *in vivo* examinations were performed mainly on rat models (Wistar, Sprague-Dawley, Fisher and Lewis), however other studies included experiments on mouse, rabbit, dog, primate models also [176]. In this latter group, myocardial infarction in most cases was induced by complete or temporary surgical ligation; other methods are cryoinjury, isoproterenol, microembolisation, thrombosis induction, cauterization [175, 176]. In our *in vitro* ischemia-reperfusion model, the h9C2 rat cardiomyoblast cells underwent a period of controlled oxygen-glucose deprivation, followed by a simulated reperfusion period, when the O_2 and the glucose was again administered to the cells, simultaneously with the NaHS-pretreated therapeutic hASC cells. Analysis was focused on the survival rates of both cell types.

ASC cells were selected as therapeutic cells due to their favorable characteristics. In former studies, the efficacy of hASC cells were similar to the human bone marrow derived stem cells (hBMSCs) in the same experimental setup [140]; cells are easy to

harvest from the human body through minimal-invasive methods, and this cell type has emerging role in studies of cell-based therapies as well.

The low survival of therapeutic cells after their delivery represents a major problem in cell-based therapies: the cytokine-rich environment, local inflammation and the infiltration of immune cells in the post-ischemic tissue are the main cause [177]. First, the hypoxic preconditioning was proven to have beneficial effects, however today a wide variety of pretreatment methods are under investigation in order to increase the proportion of viable therapeutic cells, these are physical, chemical, genetic and pharmacological manipulation [177-179]. These methods have more favorable effect for the therapeutic cells than the increased viability only: in former studies, the paracrine effect and the homing function of the cells were also enhanced, while the host immune responses were suppressed [178]. Preconditioning of stem cells in 3D structures, as aggregate formation or hydrogel encapsulation are effectively reducing the apoptosis of therapeutic cells [179].

According to our results, chemical pretreatment with the hydrogen sulfide donor NaHS enhanced the survival rate of the treated hASC cells, and this beneficial effect also applied to the postischemic H9c2 cardiomyoblasts. As an interesting finding, the protective role of H_2S on cell survival was more pronounced in the group treated with the lower concentration of NaHS (3 μM vs. 30 μM), similarly to the observations made after the H_2O_2 treatment. Due to the bell-shaped effects of H_2S, the increasing sulfide concentration augments the possibility of its disadvantageous and even toxic effects [34].

About 20 years ago, when the idea of cell-based therapies began to develop rapidly, the main effect of the therapeutic cells was supposed to be differentiation and tissue-replacement. As it was mentioned before, former studies claimed that the therapeutic stem cells are capable of significant engraftment and transdifferentiation into cells of the host myocardial tissue, however these data turned out to be misleading, and even more, seriously inaccurate. Although these publications were withdrawn later, these scandals had a clearly negative effect on the reputation of stem cell-based therapies and also highlighted the fact that the beneficial effect of therapeutic cells on the host tissue may involve other mechanisms of action. Today the autocrine, paracrine and endocrine factors secreted by the therapeutic cells are considered to play the major role behind the effects of stem cells instead of their differentiation and tissue replacement function [180].

In our former studies, the treatment of postischemic H9c2 cells with hASC concentrated media did not resulted in significant increase on survival of the postischemic cells – according to the literature, it is supposed that these paracrine factors have beneficial effects in immunomodulation and angiogenesis rather than in quantitative cell survival [140], which are conditions that cannot be examined in our *in vitro* model.

Extended H_2S treatment improved the *in vitro* proliferation of the hASCs after 9-day treatment, while their mitochondrial activity was not altered. In our model, the duration of co-cultivation of the therapeutic and postischemic cells was only 24 hours, and therefore the increased proliferation is definitely cannot be related to the observed pro-survival effect of NaHS on the pretreated stem cells, however it could be an important aspect in cell therapies under *in vivo* circumstances. On the contrary, administration of the CSE-inhibitor PAG decreased the proliferation rate, confirming the effect of CSE-produced endogenous hydrogen sulfide on hASC cell proliferation.

Interestingly, former studies revealed that low nanomolar doses of H_2S treatment increases the mitochondrial function by H_2S being an electron donor molecule [181]. However at higher doses this beneficial effect is absent, and H_2S has toxic effects on the respiratory chain by the blockade of complex IV [182]. In our system, the mitochondrial activity was not increased simultaneously with the cell proliferation, indicating that the mitochondrial functions were decreased in the cells. As a potential benefit, this can reduce the effects of ROS generation (which takes place in the mitochondria). It is also worth to mention that we administered micromolar concentrations of NaHS on the ASC cells in our experiments, which are a magnitude higher than the doses used in the above mentioned publication about the role of sulfide as a mitochondrial substrate. Another important aspect is the timing: even if H_2S was able to increase the mitochondrial activity of the ASC cells immediately after treatment in our model, it is possible that this effect was over by the time we performed the PrestoBlue measurement on the cells three days later.

These results suggest that the advantage of the pretreatment with the lower concentration of NaHS (3 μM) is connected to its more efficient antioxidant defense mechanisms through the decreased mitochondrial activity and an increased ERK1/2 phosphorylation and decreased AKT phosphorylation [174]. The effect of NaHS treatment on the proliferation of the cells was significant only on the 9th day, which makes

it less likely to have a role in the increased cell survival of the therapeutic or the post-ischemic cells after 24 hours of cocultivation.

There are important limitations of this study as well. First of all, the h9C2 rat cardiomyoblast cells which were used in the ischemia-reperfusion model are partially similar to mature cardiomyocytes, but also have common characteristics with skeletal myoblasts – it indicates the need of human cardiac cell lines in the future. As it was mentioned before, in our *in vitro* experiments we suggested that the efficacy of cell therapy is based on the survival of hASC cells, however, it is probable that therapeutic cells exert beneficial effect on the postischemic cells not only by their physical proximity, but also by paracrine and endocrine manners [137, 138]. Anoikis (cell death because of the loss of cellular connections with the extracellular matrix) is a further possible cause of low survival rates in cell therapies, – the above-mentioned 3D preconditioning techniques are probably able to eliminate this effect [179, 183]. These latter possibilities were not examined here, but should be considered in further studies. Moreover, this rather reductionist *in vitro* model is not suitable for examination of the angiogenetic or the systemic effect (as immune response) of cell therapies, therefore studies focusing on these factors in an appropriate (*in vivo*) model of ischemia-reperfusion would be an interesting next step.

5.2. Hydrogen sulfide in the cerebral circulation

In our experiments, the inhibition of endogenous H_2S production by the CSE-inhibitor molecule PAG resulted in increased vessel diameters in the intraluminal pressure range of 50 mmHg to 100 mmHg. It indicates a slight vasoconstrictive effect of endogenous H_2S on the examined vessel segments. Taking into account the possibility of H_2S-NO interaction, low amounts of endogenous H_2S could be able to scavenge NO, therefore counteract the endothelium-dependent relaxation even in the presence of intact endothelium. The role of H_2S in the decrement of the NO-mediated vasorelaxation is suspected to take part by two main methods under physiological conditions: inhibition of nitric oxide synthase and scavenging NO [33]. In our experimental setup it was not possible to examine the vessel segments at higher intraluminal pressure than 100 mmHg due to the technical limitations of our system. CSE is considered to be the main enzyme

form in the vasculature, however in certain studies the vascular H_2S production was related to CBS [162, 166, 171]. PAG is an inhibitor of CSE, therefore our result – although not significant – might indicate the role of CSE enzyme in the endogenous H_2S production of rat anterior cerebral arteries.

The TxA2-agonist compound U46619 caused a pronounced contraction on the examined ACA segments at 50 mmHg intraluminal pressure. The effect had a prompt onset and was sustained over a long period of time: the vasoconstriction was measured to be still stable after 25 minutes after the administration of TxA2. The used concentration of U46619 was similar to other studies, where it elicited an optimal precontraction of cerebral vessels [184-186]. Considering this robust, vasospasm-like contraction observed in our recent experiments, it is suspected that differences between the vessel segments or animals are responsible for these discrepancies in the vasoconstrictive potential of U46619. It is an interesting question if lower concentrations of U46619 had the similar effect on ACA, and how would a weaker TxA2 precontraction affect the vascular answer to NaHS treatment.

When NaHS was administered to the vessel segments, a biphasic effect was detected, which consisted of vasoconstriction at lower doses of H_2S, followed by vasorelaxation after the H_2S concentration increased above the level of 300 μM. Vasoconstrictive effect of H_2S is a well-known phenomenon from the related literature: it was formerly observed in other cerebral vessel studies as well, while the biphasic effect was proven by measurements on vascular segments from the systemic circulation [40, 162]. A possible underlying mechanism behind this phenomenon is the regulatory effect of H_2S on NO production and bioavailability, resulting in decreased NO-mediated vasorelaxation [115, 187]. Scavenging of NO, production of a biologically inactive H_2S-NO common product, inhibition of enzymatic NO production through eNOS, or blockade of its signal transduction are all potential mechanisms [114, 188]. This theory of H_2S-NO interaction would be congruent with our own suggestions about the mechanism of the observed vasodilatative tendency after treatment with the endogenous H_2S inhibitor PAG. At higher NaHS doses (approx. above 100-300 μM according to the literature), when the vasodilation occurs in the biphasic effect, it is suggested that other targets are got in the focus from the pleiotropic effects of H_2S, as the ion channel effects (K^+- and Ca^{2+}-

channels, TRP-channels), and formation of H_2S-NO common products with vasodilatative potential, e.g. nitroxyl (HNO) [40, 188].

The nonselective anion exchanger inhibitor 4,4'-diisothiocyanostilbene-. 2,2'-disulfonate (DIDS) has widespread biological effects. Its most known effects are the blockade of Cl^-channels and inhibition of Cl^-/HCO_3^- exchangers [72, 189]. It was also proven that this compound inhibits Na^+-current and the mitochondrial inner membrane anion channel, increases Akt phosphorylation, and has effect on the function of ryanodine receptors and TRPV1 channels also [190-194]. According to our results, a significant decrement was measured in the NaHS-induced relaxation on the outer vessel diameter after pretreatment with DIDS, and a similar (close to significant) trend was observed in the inner diameter. This inhibitory effect of DIDS on the vasorelaxant effect of NaHS was proven earlier by a few other studies. However, there are conflicting results about the underlying mechanisms; especially about the involvement of the Cl^-/HCO_3^- exchanger [72, 100, 195]. In our experiments, the mechanism of action behind the observed effect of DIDS was not yet investigated. According to the related literature mentioned before, the inhibition of Cl^-/HCO_3^- exchanger, blockade of Na^+ currents or the effect of DIDS on the ryanodine receptors should be considered and studied as feasible mechanism of action in the first place.

The observation about the flow generated vasoconstriction due to a non-ligated side branch of ACA segments highlights the importance of flow-mediated regulation of cerebral vessels. In these experiments, H_2S induced vasoconstriction on that part of the mounted vessel segment, where the intraluminal flow occurred. Based in the current knowledge about the H_2S-NO interaction discussed above, H_2S can exert vasoconstrictive effect instead of the flow-mediated dilatation mediated by NO as a result of their complex interaction. It is a similar observation to our results that shear stress-induced vasodilation was hampered in mouse coronary arteries by exogenous H_2S [113]. This effect of H_2S was absent in the presence of L-NAME or in vascular segments from eNOS-KO animals, and therefore it is supposed to be a result of the interplay of the two gasotransmitter molecule [113]. The exact effect of H_2S in our experiments and its mechanism of action should be studied precisely on a pressure myography model with constant intraluminal flow, which is a potential future step in our experiments.

Our present work includes some important limitations as well, which are mainly related either to the experimental setup, or to the used compounds.

In the pressure myograph system, several technical problems complicated our experiments such as leaking, drying out of vessels, bubble formation and cerebral arteries are extremely sensitive to physical stress. These factors resulted in serious disturbances in the function of examined vessel segments, making the measured data unreliable. Therefore several measurements finally got excluded from the statistical calculations and these technical difficulties had an impact on the sensitivity of our setup as well.

Taken together, our results revealed that the used exogenous hydrogen sulfide treatment had measurable effect both in the *in vitro* ischemia-reperfusion model and also on the pressure myography experiments on rat ACA segments.

Our results show that H_2S had complex effects in both experimental setup. The role of hydrogen sulfide in the cardiovascular system was proven earlier in numerous aspects; these previous data are supplemented now with our own observations.

6. Conclusions

Hydrogen sulfide is reported to have effect on numerous tissue and cell types throughout the mammalian and human body. Here we demonstrated its potential role as a pretreatment on the therapeutic human adipose-derived stem cells, increasing the survival of the therapeutic cells and postischemic cardiomyocytes in an *in vitro* model of cell-based therapies. The exogenous NaHS treatment had also positive impact on the proliferation of the therapeutic cells.

In another sort of experiments, the effect of H_2S was studied on isolated rat anterior cerebral artery segments in an *ex vivo* pressure myograph setup. These results showed a biphasic vascular response after administration of exogenous H_2S, and a scavenging effect against NO was also suggested in view of the observed changes in the vascular tone after administration of PAG.

In conclusion, the diverse effects of H_2S in the cardiovascular system were further confirmed by our own observations.

7. Summary

Hydrogen sulfide, which was formerly known only as an environmental hazard because of its toxicity, is today considered as a gasotransmitter molecule amongst nitrogen oxide and carbon monoxide, which are produced endogenously in the living organisms. H_2S regulates numerous biological functions under physiological conditions, as its increased or decreased level is responsible for the development of different pathological processes. Regulation of the vascular tone is of great importance; in this complex function H_2S acts through different targets, from the direct regulation of certain ion channels to modulation of signaling pathways. Furthermore, its supportive role in cell proliferation and survival, its anti-apoptotic effect are also quite important aspects, which have potential significance in ameliorating the - yet rather low - efficacy of cell-based therapies.

In this work the role of hydrogen sulfide in the cardiovascular system was studied. On one hand it was investigated as a preconditioning drug on therapeutic cells of cell-based therapies, and on the other hand, its role in the regulation of cerebrovascular tone was explored by pressure myography. According to the results, H_2S used in a non-toxic concentration is able to improve the survival of therapeutic and postischemic cells. In cerebral vessels, the biphasic effects of H_2S and their DIDS sensitivity were further supported along with the observation of the possibility that these effects could be highly flow-dependent.

Comparing our recent data with those in the literature, H_2S has proved again that it can profoundly alter living systems therefore it might have great therapeutic potential in the cardiovascular system. However, due to its pleiotropic character and its highly dose-dependent effects – which are often biphasic in nature – further extensive examinations of the underlying complex mechanisms and interactions are needed to convert this molecule into a widely utilizable compound in medicine and drug development.

8. Összefoglalás

A korábban kizárólag erősen toxikus környezeti hatásairól ismert kénhidrogént ma már a nitrogén-oxidhoz és a szén-monoxidhoz hasonlóan az élő szervezetekben endogén módon termelődő gáztranszmitter molekulaként tartják számon. A H_2S számos biológiai funkciót szabályoz élettani körülmények között; a szükségesnél alacsonyabb - vagy ritkább esetben magasabb - szintje kóros folyamatok oki tényezője lehet, mely már számos korábbi vizsgálat esetén bizonyítást nyert. Hatása az értónus szabályozására kiemelt jelentőségű, ebben a komplex működésben több támadásponton hat, ioncsatornák aktivitásának direkt szabályozásától kezdve a különböző, sejten belüli jelátviteli utak befolyásolásáig. Emellett pedig a sejtek túlélését és proliferációját segítő, anti-apoptotikus hatása is igen fontos aspektus, melynek a sejtalapú terápiák egyelőre alacsony hatékonyságának növelésében lehet szerepe.

A jelen dolgozatban bemutatott kísérletes munka a kénhidrogén lehetséges szerepét vizsgálja a kardiovaszkuláris rendszerben. Egyrészt a terápiás sejtek előkezeléseként vizsgáltuk sejtterápia-modellben, másrészt pedig nyomás arteriográf segítségével az agyi erek értónus-szabályozásában betöltött szerepét kívántuk feltérképezni. Eredményeink alapján a megfelelő dózisú, nem toxikus mennyiségű exogén kénhidrogén-kezelés képes javítani a terápiás sejtek és a posztiszkémiás sejtek túlélését. Az agyi ereken a kénhidrogén bifázisos hatása és a DIDS ezt befolyásoló hatása igazolódott, megfigyelésünk alapján ezen hatások áramlásfüggése valószínű.

Saját eredményeinket az irodalmi adatokkal összevetve a kénhidrogén mélyrehatóan képes befolyásolni az élő szervezetek működését, melynek alapján ígéretes terápiás lehetőségnek mutatkozik a kardiovaszkuláris rendszer betegségeinek kezelése terén. Ugyanakkor az igen összetett jellemzői és erősen dózisfüggő hatásai miatt – melyek gyakran bifázisos jellegűek – a komplex hatásmechanizmusainak és interakcióinak további kiterjedt vizsgálatai szükségesek ahhoz, hogy ez a molekula széleskörűen felhasználhatóvá válhasson az orvostudomány és a gyógyszerfejlesztés területén.

9. References

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10. Publication list

10.1 Publications related to the present thesis

Dongo, E., Hornyák, I., Benkő, Zs., Kiss, L.: *The cardioprotective potential of hydrogen sulfide in myocardial ischemia/reperfusion injury (review).* Acta Physiol Hung, 2011. **98**(4): p. 369-81. IF:0,821

Dongo, E., Benkő, Zs., Csizmazia, Á., Marosi, G., Grottke, A., Jücker, M., Schumacher, U., Kiss, L.: *H2S preconditioning of human adipose tissue-derived stem cells increases their efficacy in an in vitro model of cell therapy for simulated ischemia.* Life Sci, 2014. **113**(1-2): p. 14-21. IF: 2,702

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Dongo, E. and Kiss, L. *The Potential Role of Hydrogen Sulfide in the Regulation of Cerebrovascular Tone.* Biomolecules, 2020. **10**(12). IF: 4,082

Dongo, E., Harasztos, L., Nadasy, Gy., Kiss, L: *The effect of hydrogen sulfide on the contractility of cerebral arterioles. A pilot study.* Physiology International, 2022. DOI:10.1556/2060.2022.00190. IF: 2,090* *(in 2020)*

10.2 Publications not related to the present thesis

Kiss, L., **Dongó, E.**, Janicsek, Z., Szepes, M., Benkő, Z., Cselenyák, A., & Lacza, Z. (2010). *Őssejtterápia alkalmazásának eredményei perifériás artériás érbetegségben.* Érbetegségek / Hungarian Journal of Vascular Diseases, 17(3), 33–38.

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