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HELIUM-MEDIATED REGULATION OF ENDOTHELIAL PERMEABILITY:  
INVOLVEMENT OF  
CAVEOLIN-1, VE-CADHERIN AND CONNEXIN-43

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## Abbreviations

a.u.	Arbitrary unit
AJ	Adherens junctions
AMC	Academic Medical Center
AMI	Acute myocardial infarction
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
°C	Degree celsius
cAMP	Cyclic adenosine monophosphate
Cav	Caveolin
Cav-1	Caveolin-1
CSD	Caveolin scaffolding domain
Cx	Connexin
Cxs	Connexins
Cyt C	Cytochrome C
DNA	Deoxyribonucleotide acid
DTT	Dithiothreitol
ECGM	Endothelial cell grows medium
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
Fig.	Figure
FITC-BSA	Fluorescein isothiocyanate labelled bovine serum albumin
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GJ	Gap junctions
HUVEC	Human umbilical vein endothelial cells
I/R	Ischemia/reperfusion
kDa	Kilodalton
l	Liter
M	Molar
MAC	Minimal alveolar concentration
MEK-1	Mitogen-activated protein kinase kinase 1

## Abbreviations

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mg	Milligram
min	Minute
ml	Milliliter
MOPS	3-(N-morpholino)propanesulfonic acid
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger RNA
MW	Molecular weight
nm	Nanometer
PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
pH	Potential of hydrogen
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PMSF	Phenylmethylsulfonyl fluoride
PTGS	Posttranscriptional gene silencing
RIPA	Radioimmunoprecipitation assay
RISK	Reperfusion injury salvage kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
SPTB	Beta-spectrin
TJ	Tight junctions
V	Volt
VE-cadherin	Vascular endothelial - cadherin
vs.	Versus
µg	Microgram
µl	Microliter

# 1. Introduction

## 1.1 Ischemia and ischemia/reperfusion injury

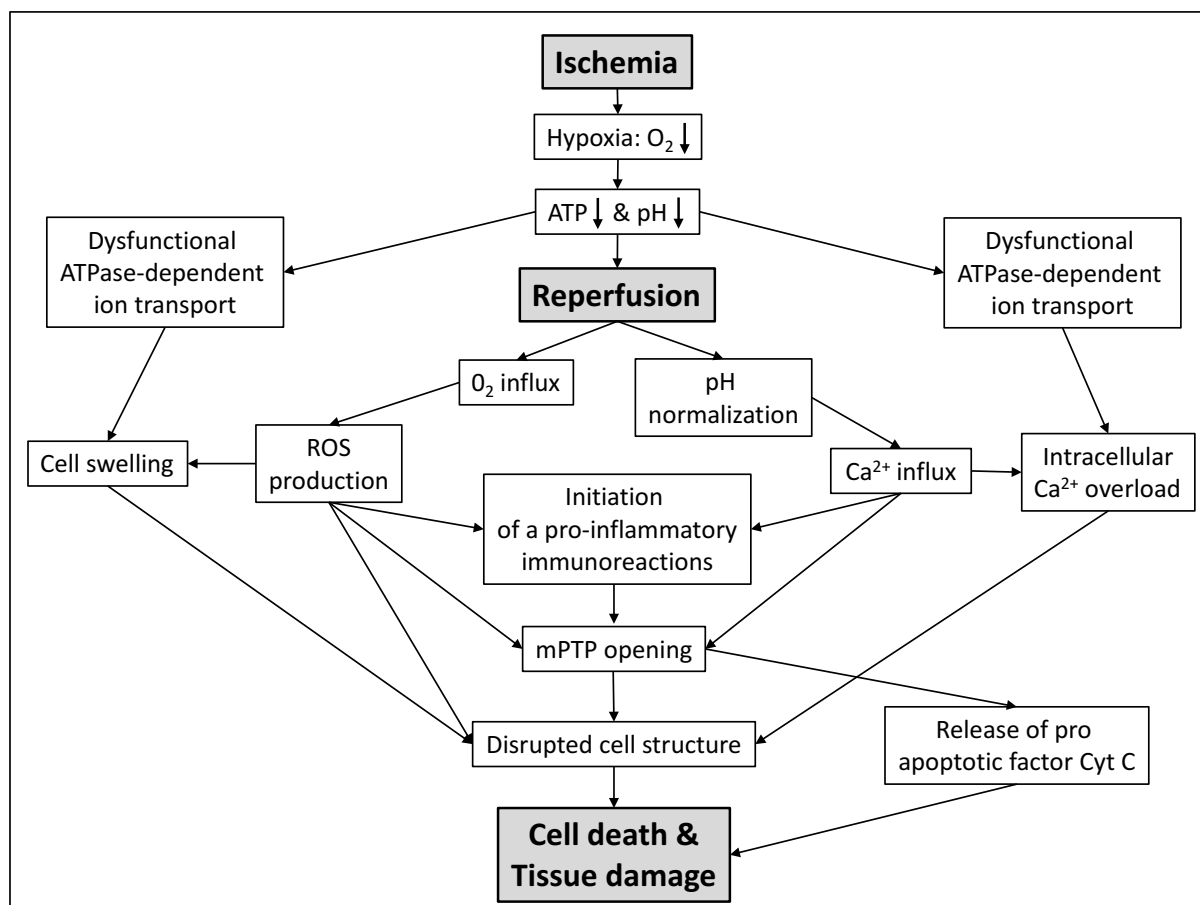
Organ dysfunctions characterized by ischemia/reperfusion (I/R) are among the leading causes of death (1). The term ischemia describes an impaired or interrupted blood supply to tissues as a result of reduced or obstructed arterial inflow (2,3). This leads to an insufficient supply of the tissue with essential substances such as oxygen and as a result subsequent depletion of cellular adenosine triphosphate (ATP) (4). The main therapeutic strategy to reduce ischemic injury is the rapid restoration of blood flow (2), in the heart for example by percutaneous coronary intervention (PCI) or thrombolysis in patients with acute myocardial infarction (AMI) (5). Surprisingly, reoxygenation by restoration of the arterial blood flow can cause an extensive inflammatory response and exacerbation of the tissue injury (6). This phenomenon is called “reperfusion injury” (6,7). Experimental studies showed, that almost 50% of the final infarct size after myocardial infarction is due to the reperfusion injury (7).

I/R-injury is of high clinical relevance because it contributes to pathologies in various conditions (6). Examples are myocardial infarction, stroke, multiple organ I/R-injury due to trauma, or organ transplantation as well as other surgical interventions that cause a temporary arrest of arterial blood flow (2,6).

The pathophysiological mechanisms underlying I/R-injury are complex. Anaerobic metabolism and lactate accumulation during extended ischemia result in decreased ATP levels and a lower intra-cellular pH (1). This leads to impaired ATPase-dependent ion transport mechanisms and intra-cellular and mitochondrial calcium ( $\text{Ca}^{2+}$ ) overload as well as cell swelling (1,8). Upon reperfusion, reactive oxygen species (ROS) are produced and a pro-inflammatory immunoreaction is initiated (8). I/R triggers the opening of the mitochondrial permeability transition pore (mPTP) (8). Finally release of the pro apoptotic factor cytochrome C (Cyt C) is induced by the mPTP opening and results in cell-death and tissue damage (9). The pro-inflammatory immunoreaction along with a leukocyte infiltration and activation of the complement system leads to a further aggravation of the tissue damage (10,11) (Fig. 1).

I/R is associated with hypoxia, which is characterized by a reduced partial pressure of oxygen in the arterial blood, or reduced oxygen content in a specific tissue or organ (3). In the endothelium hypoxia results in a decreased endothelial adenylate cyclase activity and decreased intra-cellular cyclic adenosine monophosphate (cAMP) levels (6,12,13). This leads to a weakened barrier function of endothelial cells and results in increased vascular permeability and vascular leakage (6,12,13).





**Figure 1: Ischemia/reperfusion-injury and cell death**

Simplified diagram of the pathophysiological mechanisms induced by the ischemia/reperfusion-injury (I/R-injury). Based on Kalogeris T et al. (8).

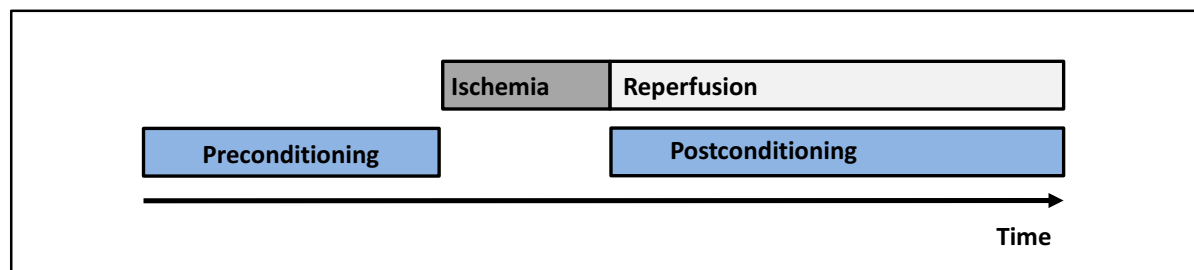
## 1.2 Organ protective strategies

Due to the high mortality connected to I/R-injuries (1), it is of great clinical interest to establish therapeutic strategies to protect organs from I/R-injury. A very promising method to protect cells and tissues, but also organs like the heart from I/R-injury is ischemic conditioning (14,15). Conditioning refers to a mechanism that enables an organ or tissue to develop adaptation processes to cope better with an ischemia (16).

Depending on the temporal context to the ischemia, conditioning can be divided into pre- and postconditioning (16). Protective interventions that occur before the ischemia are referred to as preconditioning, protective interventions that occur at the time of reperfusion are referred to as postconditioning (16) (Fig. 2). Furthermore, conditioning can be divided according to (i) the location where the protective stimulus is applied: Short and possibly repeated ischemia to the organ or tissue itself (local pre- and postconditioning), or distal to the organ or tissue (remote

pre- and postconditioning) and (ii) the type of intervention which is applied: Ischemia, or pharmacological stimuli such as anesthetics and noble gases (16,17).

Different pharmacological stimuli such as volatile anesthetics (isoflurane, sevoflurane) and noble gases (xenon, helium) can protect from the I/R-injury (18–21). The underlying mechanisms are very similar to the ones described for ischemic conditioning (17).



**Figure 2: Organ protective strategies: Pre- and postconditioning**

### 1.2.1 Conditioning with helium

Helium is a colorless, odorless and tasteless monoatomic gas with a lower density than air (22). Along with neon, argon, krypton, xenon and the radioactive radon it belongs to the noble gases, which have in common that their outer shell of valence electrons is filled (23,24).

The noble gas helium has been shown to mimic the early and late effects of ischemic preconditioning on the human endothelium which makes helium a promising reagent for pharmacological conditioning (21).

In the context of pharmacological conditioning helium is of great interest as helium, in contrast to the volatile anesthetics isoflurane and sevoflurane and the noble gas xenon, has no anesthetic properties and can therefore also be used in awake patients (22). Helium is called a “non-immobilizer”, meaning that it is intrinsically without anesthetic effect (24,25). Furthermore no hemodynamic side effects of helium have been described (23), which makes helium appealing for the use in patients with cardiovascular risk factors (22). Even though helium is called an inert gas, experimental studies in animals and humans have shown that it is not biochemically inert and can exert biologic effects (22).

Recent studies postulated that helium conditioning is able to protect the heart against I/R-injury and reduces infarct size (26–36). Preconditioning by three times 5 min of helium inhalation before a subsequent ischemia of the heart leads to reduced infarct size in rabbits (26). Furthermore helium preconditioning induces mitochondrial uncoupling and reduces infarct size in young rats (33). In aged rats, however, this effect is lost (33). Different concentrations of helium can protect the heart from I/R-injury (35). 70%, 50% and 30% of helium are potent to

induce preconditioning of the rat heart, however, 10% of helium are not potent enough (35). Helium can also protect the hypertensive myocardium from I/R-injury (36). However, in contrast to normotensive hearts only a combination of pre-, post- and late preconditioning by helium has a protective effect in hypertensive hearts (36).

Beside the protective effect of helium on the heart, animal and in-vitro experiments showed that helium also protects the brain from ischemic stroke (37): Various animal and in-vitro studies revealed that helium conditioning can reduce cerebral infarct size (37–40) and increases the number of viable neurons after a cerebral ischemia (38). Furthermore animal studies suggested that helium improves neurological outcomes after cerebral ischemia (38,40–42).

Unfortunately, the large amount of experimental data on helium-induced conditioning has not been translated to the clinical situation (23). However, a study by Smit et al. demonstrated in healthy volunteers, that helium can protect the human endothelium from I/R-injury by mimicking the early and late effect of ischemic preconditioning (21).

In the human endothelium in-vitro, helium leads to reduced endothelial permeability (43). As I/R-injury can cause endothelial dysfunction and reduce endothelial barrier function (6,44), the effect of helium on the human endothelial permeability might be one of the underlying mechanisms of helium-induced conditioning.

### **1.3 Target cells for helium conditioning: The endothelium**

The endothelium is the first target organ (45,46) to get in contact with circulating blood and the respective circulating factors and hypoxic conditions. Therefore, the human endothelium might play a key role in helium-mediated organ protection.

Blood vessels are mainly composed of endothelial cells, pericytes, smooth muscle cells and the basal membrane (47). The endothelium lines the inner surface of blood vessels and acts as a selective permeable barrier to fluids, solutes and bacteria, separating blood from underlying tissue (48–50). Alterations of the endothelial barrier function can cause irregular flow of fluids and proteins resulting in tissue edema and dysfunction (51) as well as inflammation by bacterial crossing of the barrier (52). Adhesions between the cells as well as the actin cytoskeleton are critically involved in regulating endothelial permeability (49,50).

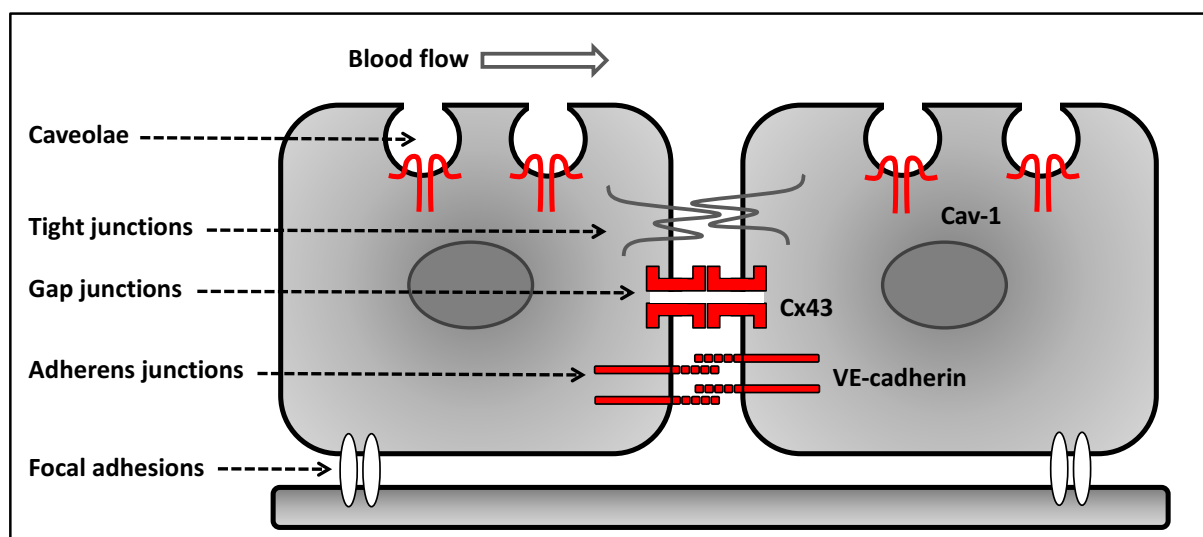
In a classical way endothelial permeability results from two different routes across the vessel wall (51). The first way across the endothelium is trans-cellular, by transcytosis and the second way is para-cellular, through the inter-endothelial space (51). However, the para-cellular and

the trans-cellular pathways seem to be dependent on each other to maintain tissue fluid homeostasis (51).

Trans-cellular transport or transcytosis is a receptor-mediated transport through the endothelial cells. It enables trafficking of macromolecules like albumin, insulin, lipids and hormones and is enabled by vesicular transport mechanisms (51,53).

Para-cellular permeability is maintained mainly by inter-endothelial junctions, which connect neighboring endothelial cells into a continuous monolayer (51). Inter-endothelial junctions include tight- (TJ), gap- (GJ) and adherens junctions (AJ) (51) (Fig. 3). TJ are composed of transmembrane proteins including occludin, claudin and small junctional immunoglobulins (54). GJ are formed by two connexons each consisting of six connexin (Cx) molecules (55,56) and AJ are formed mainly by vascular endothelial - cadherin (VE-cadherin) (54). AJ and TJ promote adhesions of adjacent endothelial cells and maintain the restrictiveness of the endothelial barrier, GJ form channels between adjacent cells allowing the exchange of water, ions and other small molecules and are able to transmit signals between the cells of the endothelial monolayer (51). Under physiological conditions para-cellular transport only allows to transport solutes smaller than 3 nm in radius (51,57). Well-functioning AJ are important for the regulation of para-cellular permeability and disruption of their VE-cadherin homophilic bindings leads to interstitial edema (51).

Focal adhesions connect the endothelial cells to the basement membrane (58). Caveolin-1 (Cav-1) is located in caveolae and is involved in vesicular uptake, cell signaling and maintaining cholesterol homeostasis (59) (Fig. 3).



**Figure 3: Endothelial barrier**

Cav-1 = caveolin-1, Cx43 = connexin-43, VE-cadherin = vascular endothelial - cadherin. Molecules pictured in red are investigated within this study.

### 1.3.1 Caveolae and Caveolins

Caveolae are lipid enriched invaginations of the plasma membrane (60) and caveolins (Cavs) are the structural membrane proteins essential for the formation of caveolae (24,61) (Fig. 3). In a traditional way caveolae are mainly responsible for vesicular uptake associated with transcytosis, pinocytosis and potocytosis (59). Caveolae contain Cav-1 and traffic macromolecules from the luminal side of the endothelium facing the blood flow, to the basal surface facing the extra-vascular space (62). However, recent findings revealed, that Cavs are also involved in other functions, like the regulation of cell signaling and maintaining cholesterol homeostasis (59).

Cavs were originally named according to their detection in caveolae, but today it is widely excepted, that they are also present elsewhere in the cell (63). Cavs, which have a molecular weight of 21 - 24 kDa (64), are currently known to consist of three isoforms: Cav-1, Cav-2 and Cav-3 (65). The isoforms Cav-1 and Cav-2 are found in various cell types, including endothelial cells, whereas Cav-3 is mainly found in striated muscle cells (cardiac and skeletal) and certain smooth muscle cells (65).

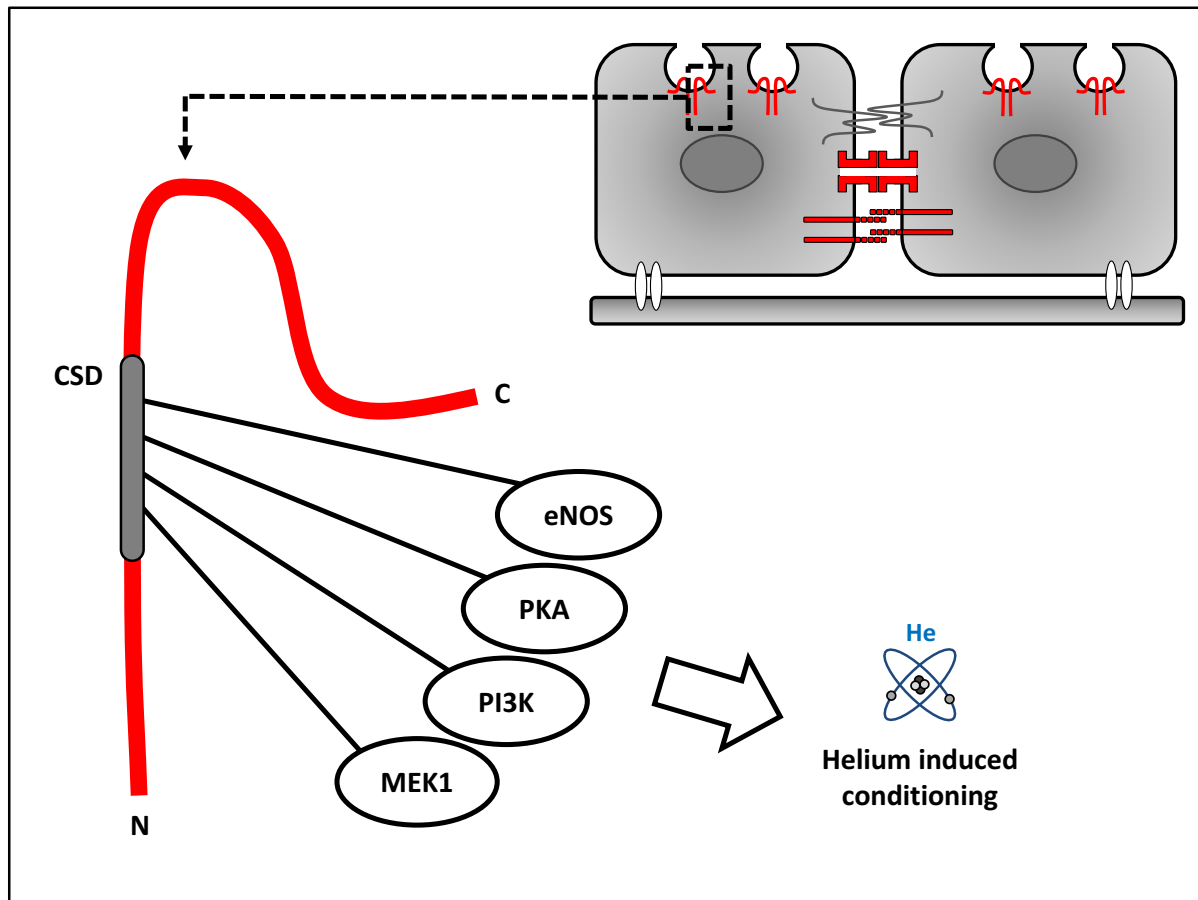
Over the last years, evidence was provided, that (i) caveolae and the Cav isoforms 1 and 3 are critically involved in cardiac protection from myocardial I/R-injury (66). Furthermore, evidence was provided, that (ii) stimuli producing preconditioning, including short periods of I/R and the exposure to volatile anesthetics as well as the noble gas helium alter the numbers of membrane caveolae and the expression rates of Cavs (43,67).

Cavs anchor signaling molecules and regulate multiple cellular processes such as inter- and intra-cellular signal transduction (24,59). Furthermore, Cavs play a key role in anesthetic-induced organ protection (23,68). Through their scaffolding domain (caveolin scaffolding domain (CSD)), Cavs can operate as chaperons and attract different signaling molecules to caveolae thereby directly influencing the spatial and temporal regulation of signal transduction (66). Signaling molecules bind to the CSD in an inactive state and can then be activated and released by conformational changes (63,69).

Some of the proteins known to interact with the CSD, are also known to be involved in helium-induced conditioning (24,63). These proteins include the endothelial nitric oxide synthase (eNOS), protein kinase A (PKA) and the enzymes of the reperfusion injury salvage kinase (RISK) pathway: phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase kinase 1 (MEK-1) (24,63,70) (Fig. 4).

Helium has been shown to induce various biological changes, and membrane proteins like Cavs might be important in the helium-induced organ protection (24). Animal and cell culture experiments revealed that helium leads to a release of Cavs (especially Cav-1/3) and suggest

that Cavs are specifically secreted from endothelial cells into the circulation from where they can reach various organs and exert protective effects (43,67).



**Figure 4: Caveolin scaffolding domain (CSD)**

CSD = caveolin scaffolding domain, eNOS = endothelial nitric oxide synthase, He = helium, MEK1 = mitogen-activated protein kinase kinase 1, PI3K = phosphoinositide 3-kinase, PKA = protein kinase A. Based on Patel HH et al. (63).

## 1.3.2 Junctional molecules

### 1.3.2.1 Vascular endothelial - Cadherin (VE-cadherin)

VE-cadherin is the main molecule to form AJ by  $\text{Ca}^{2+}$ -dependent homophilic bindings between VE-cadherin molecules of adjacent endothelial cells (54) (Fig. 3). VE-cadherin is found only in endothelial cells and is part of the super-family of classical cadherins (47). Within the AJs VE-cadherin is essential for the endothelial barrier function and regulates the expression and spatial distribution of different junctional molecules, among which are N-cadherin and claudin-5 (47). Primarily  $\beta$ - and p120-catenins bind to the cytosolic tail of VE-cadherin and connect it

to the actin cytoskeleton via actin binding proteins such as vinculin,  $\alpha$ -catenin and eplin (47). Phosphatases and kinases regulate the activity of VE-cadherin adhesions in endothelial cells according to extra-cellular stimuli (51). Different receptors at the cell surface of the endothelium modulate the stability of VE-cadherin adhesions and organize the actin cytoskeleton in response to various stimuli (51). Decreased VE-cadherin levels are correlated with barrier disruption and increased permeability (47,71).

VE-cadherin is involved in conditioning mechanisms (72–74). The volatile anesthetic sevoflurane as well as angiotensin-1 can preserve endothelial barrier function by maintaining VE-cadherin levels (72,73). Furthermore, the herbal medicine extract tongxinluo, which is known to induce preconditioning, leads to an upregulation of VE-cadherin in the endothelium (74).

### **1.3.2.2 Connexin-43 (Cx43)**

Cxs are the protein family that forms GJ between cells and enables inter-cellular communication between endothelial cells, as well as cardiomyocytes (75). This cell-cell communication regulates vascular tone, endothelial function, metabolic interchange and preserves cardiac rhythm (75,76). GJ are formed by two facing hemichannels or connexons, each of the communicating cells contributing one of them (55,56). Every connexon is formed by six Cx molecules (56) (Fig. 3). GJ allow the inter-cellular exchange of molecules with a molecular weight of less than 1.5 kDa, such as signaling molecules as  $\text{Ca}^{2+}$  and inositol trisphosphate ( $\text{IP}_3$ ) (55). The nomenclature of Cxs is based on their molecular weight (55). Cx43 therefore refers to the Cx with a molecular weight of 43 kDa (55).

Cx43 resides both in the plasma membrane, where its main function is the inter-cellular communication through GJ (75) and in the mitochondria, where it regulates mitochondrial physiology and myocyte apoptosis (75,77).

Ischemic preconditioning leads to a preserved sarcolemmal Cx43 activity during sustained ischemia, and protects the cell by attenuating cellular edema (78). Furthermore ischemic preconditioning translocates Cx43 to the mitochondria, where it increases ATP formation and contributes to the release of ROS, which are two important mechanisms of conditioning (78). Interestingly, in addition to the proteins already known to be involved in helium-induced conditioning, also Cx43 interacts with the scaffolding domain of Cav-1 (CSD) and additionally interacts with the C-terminal domain of Cav-1 (79).

### 1.3.3 Caveolins and junctional molecules in conditioning

Cavs as well as the junctional molecules VE-cadherin and Cx43 are involved in various conditioning mechanisms (24,72,74,78).

In 2001 Young et al. demonstrated in isolated rat hearts that infusion of the CSD peptide of Cav-1 into ischemia/reperfused hearts increases the recovery of the cardiac function (80). A study in rats in-vivo showed that I/R of the myocardium down-regulates the Cav-1 levels in ischemic as well as non-ischemic zones of the myocardium (81). Isoflurane is known to induce pharmacological conditioning of the heart and Patel et al. demonstrated in 2007 that a Cav-1 knock-down abolishes the isoflurane-induced infarct size reduction, indicating that conditioning by isoflurane is dependent on Cav-1 (82). In addition to Cav-1, Cav-3 is also involved in pharmacological conditioning by isoflurane. Preconditioning by isoflurane reduces infarct size and cardiac troponin levels in mice hearts after I/R (68). A Cav-3 knock-out in mice abolishes this isoflurane-induced cardiac protection, indicating that conditioning by isoflurane is also dependent on Cav-3 (68). Furthermore, Cav-1 plays a role in pharmacological conditioning by helium. After helium inhalation decreased levels of Cav-1/3 were found in mice hearts and increased levels were found in the serum of mice (67). A following study in rats partly supported these findings by demonstrating that helium postconditioning increases Cav-3 in the serum of rats (83). The same study, furthermore, revealed that helium postconditioning increases the amounts of Cav-1/3 in the membrane fraction of ischemic cardiac tissue in rats (83). Jiang et al. demonstrated the relationship between Cav-1 and endothelial permeability in a preeclampsia study employing hypoxic trophoblast conditioned medium (84). This medium increases endothelial permeability and Cav-1 levels in endothelial cells (84). However, a Cav-1 knock-out abrogates the effect on the endothelial permeability (84). This indicates that Cav-1 is a main part of the pathway that regulates endothelial monolayer permeability (84). Overexpression of Cav-1 in mice leads to endogenous cardiac protection similar to wild-type mice undergoing ischemic conditioning, confirming the key role of Cav-1 in ischemic conditioning (85). In 2015 Hamaguchi et al. confirmed the role of Cavs in isoflurane-induced preconditioning in mice by demonstrating that conditioning by isoflurane increases Cav-3 in the heart after infarct induction and is abolished in Cav-3 knock-out mice (86).

VE-cadherin is involved in pharmacological conditioning by angiotensin-1 (73). Lee et al. showed that angiotensin-1 prevents vascular leakage through a regulation of VE-cadherin phosphorylation (73). Sevoflurane-induced preconditioning preserves endothelial barrier function and maintains VE-cadherin levels by preventing VE-cadherin internalization (72). This demonstrates the key role of VE-cadherin in this conditioning strategy. The herbal medicine extract tongxinluo leads to an upregulation of VE-cadherin, which might be one of the key targets of pharmacological conditioning by tongxinluo (74).



The role of Cx43 in ischemic conditioning was postulated in various publications. Infarct size reduction induced by ischemic conditioning in mice is abrogated in Cx43 deficient mice, pointing towards a key role of Cx43 in ischemic conditioning (87). Schulz et al. showed that ischemic conditioning induces Cx43 phosphorylation during ischemia in pic hearts in-vivo (88). This Cx43 phosphorylation induced by ischemic conditioning was confirmed in isolated buffer-perfused rabbit hearts and in addition a reduction in infarct size was found (89). In isolated rat hearts ischemic conditioning enhances the mitochondrial localization of Cx43 (90). Rodriguez-Sonovas et al. demonstrated that ischemic conditioning transports Cx43 to the inner mitochondrial membrane and furthermore, revealed that normal mitochondrial Cx43 content is crucial for cardioprotection by ischemic conditioning (91). The involvement of Cx43 in ischemic conditioning of the heart was, furthermore, supported by findings in aged mice hearts (92). Left ventricular content of Cx43 is reduced in hearts of aged animals and in addition, the effect of ischemic preconditioning on infarct size reduction is abolished in these hearts (92). This led to the conclusion that the reduced Cx43 levels might be responsible for the age related loss of ischemic preconditioning, once more demonstrating the involvement of Cx43 in ischemic conditioning (92).

Table 1 gives an overview of Cav-1/3, VE-cadherin and Cx43 in different conditioning strategies.

<b>Molecule</b>	<b>Conditioning strategy</b>	<b>Mechanism of regulation/ action</b>	<b>Reference</b>
Cav-1	Pharmacological: CSD peptide of Cav-1	Infusion of CSD peptide of Cav-1 into ischemic/reperfused hearts increases recovery of cardiac function	Young LH, 2001 (80)
Cav-1	Ischemic	I/R-injury down-regulates Cav-1	Ballard-Croft C, 2006 (81)
Cav-1	Pharmacological: isoflurane	Cav-1 knock-out abolishes isoflurane-induced infarct size reduction	Patel HH, 2007 (82)
Cav-1/3	Pharmacological: helium	Conditioning with helium leads to decreased level of Cav-1/3 in the mice hearts and increased levels in the serum of mice	Weber NC, 2013 (67)
Cav-1	Ischemic	Hypoxic trophoblast conditioned medium increases endothelial permeability and Cav-1 levels in endothelial cells, this effect is abrogated by a Cav-1 knock-down	Jiang R, 2014 (84)
Cav-1/3	Pharmacological: helium	Helium postconditioning regulates Cav-1/3 levels in cardiac tissue of rats and increases Cav-3 in the serum of rats	Flick M, 2016 (83)
Cav-3	Pharmacological: isoflurane	Cav-3 knock-out abolishes isoflurane-induced reduction of infarct size and cardiac troponin levels	Horikawa YT, 2008 (68)
Cav-3	Ischemic	Endogenous cardiac protection in Cav-3 overexpressed mice is similar to wild-type mice undergoing ischemic conditioning	Tsutsumi YM, 2008 (85)
Cav-3	Pharmacological: isoflurane	Isoflurane-induced preconditioning increases Cav-3 in the heart and is abolished in Cav-3 knock-out mice	Hamaguchi E, 2015 (86)
VE-cadherin	Pharmacological: angiotensin-1	Angiotensin-1 prevents vascular leakage through a regulation of VE-cadherin phosphorylation	Lee SW, 2011 (73)

VE-cadherin	Pharmacological: sevoflurane	Sevoflurane-induced preconditioning preserves endothelial barrier functions by maintaining VE-cadherin levels	Huang Y, 2015 (72)
VE-cadherin	Pharmacological: tongxinluo	Herbal medicine extract tongxinluo leads to an upregulation of VE-cadherin and protects against myocardial I/R-injury	Qi K, 2015 (74)
Cx43	Ischemic	Cx43 knock-out abrogates effect of ischemic conditioning in mice	Schwanke U, 2002 (87)
Cx43	Ischemic	Ischemic conditioning preserves Cx43 phosphorylation during ischemia in pig hearts in-vivo	Schulz R, 2003 (88)
Cx43	Ischemic	Ischemic conditioning induces Cx43 phosphorylation and reduces infarct size in isolated buffer perfused rabbit hearts	Miura T, 2004 (89)
Cx43	Ischemic	Ischemic conditioning enhances mitochondrial localization of Cx43 in isolated rat hearts	Boengler K, 2005 (90)
Cx43	Ischemic	Ischemic conditioning transports Cx43 to the inner mitochondrial membrane and normal mitochondrial Cx43 content is important for cardioprotection	Rodriguez-Sinovas A, 2006 (91)
Cx43	Ischemic	Cardioprotection by ischemic conditioning is lost in aged mice hearts, possibly due to reduced left ventricular Cx43 content in the aged heart	Boengler K, 2007 (92)

**Table 1: Caveolins and junctional molecules in conditioning**

## 1.4 Aim of the study

The noble gas helium has been shown to mimic the early and late effects of ischemic preconditioning on the human endothelium which makes helium a promising reagent for organ protective strategies. Recent studies suggested that helium decreases protein levels of caveolin-1 (Cav-1) in the human endothelium and attenuates the permeability of human umbilical vein endothelial cell (HUVEC) monolayers. However, the underlying cellular and molecular mechanisms and the precise role of Cav-1 in helium-mediated regulation of endothelial barrier integrity are still unclear.

The aim of this study was to gain deeper insight into the mechanisms responsible for the barrier protective functions of helium. Therefore, employing in-vitro cultured primary HUVEC and small interfering RNA (siRNA) transfection experiments, we investigated (i) whether Cav-1 is involved in helium-mediated stabilization of the endothelial barrier and (ii) whether the expression of the key junctional molecules vascular endothelial - cadherin (VE-cadherin) and connexin-43 (Cx43) is regulated by helium and might be involved in the effects of helium on the endothelial barrier function.

The results of this study will lead to a better understanding of the cellular and molecular mechanisms behind the barrier protective effects of helium and in combination with further translational trials could help to implement helium into organ protective strategies in humans.

## 2. Material and Methods

### 2.1 Material

A detailed list of manufacturers is included in the appendix section (7.1 List of manufacturers).

#### 2.1.1 Cell culture

##### Devices

Capnomac Ultima (Datex), Centrifuge 5810 R, Rotor A-4-81, Radius 18.0 (Eppendorf), Clean Air, Biological safety cabinet, class II (Telstar Laboratory Equipment), Heracell 150 I CO<sub>2</sub> incubator (Thermo Scientific), Leica DM-RA(X) Microscope (Leica Microsystems), Microplate fluorescence reader FLx800 (BioTek).

##### Consumables

12-well-plate transwell inserts, pore diameter size: 0.4 µm (Corning Costar), 6-well- and 12-well-plates (Greiner Bio One), Cell culture flasks 25 cm<sup>2</sup> and 75 cm<sup>2</sup> (Corning), Cell scrapers (Corning Life sciences), OptiPlate 96 Black (Packard BioScience).

##### Chemicals

Amphotericin B (Sigma-Aldrich), calcium chloride (MERCK Millipore), collagenase A (Roche), ECGM (Promocell), ECGM supplement mix (Promocell), fetal bovine serum (PAA), FITC-BSA (Sigma-Aldrich), gas mixture: 5% CO<sub>2</sub>, 25% O<sub>2</sub>, 70% He (Linde Gas Benelux), gas mixture: 5% CO<sub>2</sub>, 25% O<sub>2</sub>, 70% N<sub>2</sub> (Linde Gas Benelux), gelatine (BD Diagnostic system), KH<sub>2</sub>PO<sub>4</sub> (Merck), L-glutamine (Sigma-Aldrich), lipofectamine RNAiMAX (Invitrogen), medium 199 (PAN), milliQ-water made by Milli-Q Synthesis: Ultrapure Water, System, type 1 (MERCK Millipore), Na<sub>2</sub>HPO<sub>4</sub> (Sigma-Aldrich), NaCl (Merck), penicillin-streptomycin (Sigma-Aldrich), silencer negative control siRNA #1 (Ambion), siRNA Cav-1 (Ambion), Triton X-100 (Sigma-Aldrich), trypsin-EDTA (Invitrogen).

**Buffer solutions and cell culture medium**

<b>B1</b>	10x PBS	437.5 g NaCl, 71.1 g Na <sub>2</sub> HPO <sub>4</sub> x 2H <sub>2</sub> O, 10.5 g KH <sub>2</sub> PO <sub>4</sub> , add 5 l milliQ-water, pH 7.4
<b>B2</b>	PBS+	100 ml 10x PBS ( <b>B1</b> ), 900 ml milliQ-water, 100 mg MgCl <sub>2</sub> , 100 mg CaCl <sub>2</sub>
<b>B3</b>	Umbilical cord buffer	500 ml PBS+ ( <b>B2</b> ), 5 ml penicillin-streptomycin, 5 ml amphotericin B
<b>M1</b>	Stop medium	428 ml medium 199 (M199), 50 ml fetal bovine serum (FBS), 5 ml penicillin-streptomycin, 5 ml amphotericin B, 5 ml L-glutamine
<b>M2</b>	Cell culture medium with antibiotics and antimycotics	428 ml endothelial cell grows medium (ECGM), 50 ml FBS, 5 ml penicillin-streptomycin, 5 ml amphotericin B, 12.2 ml of ECGM supplement mix
<b>M3</b>	Starving medium	96 ml ECGM, 2 ml FBS, 1 ml penicillin-streptomycin, 1 ml amphotericin B
<b>M4</b>	Cell culture medium without antibiotics and antimycotics	87.6 ml ECGM, 10 ml FBS, 2.4 ml of ECGM supplement mix
<b>M5</b>	Stop medium without antibiotics and antimycotics	89 ml M199, 10 ml FBS, 1 ml L-glutamine

**Table 2: Buffer solutions and cell culture medium used for cell culture**

**SiRNA, transfection reagents and controls**

<b>Name</b>	<b>Description</b>	<b>Company</b>
Lipofectamine RNAiMAX	Transfection reagent	Invitrogen
Silencer Negative Control siRNA #1 Cat#: AM4611	Negative control siRNA	Ambion
Cav-1 (sense) 5'CCCUGAACACCUCAACGAU(dT)(dT)3' Cav-1 (antisense) 5'AUCGUUGAGGUGUUUAGGG(dT)(dT)3'	SiRNA Cav-1	Sigma-Aldrich

**Table 3: SiRNA, transfection reagents and controls used for HUVEC transfection****2.1.2 Protein biochemistry****Devices**

Centrifuge 5430R (Eppendorf), Criterion Blotter Gel Holder Cassette (Bio-Rad), Criterion cell (Bio-Rad), Criterion Blotter (Bio-Rad), Heaterblock III (MFG by Henry Troemner), Odyssey CLx imaging system (LI-COR Biosciences), microplate fluorescence reader FLx800 (BioTek).

**Consumables**

96-well-plates (Fisher Scientific), Criterion XT precast gel (Bio-Rad), filter paper (Bio-Rad), Immobilon FL (MERCK Millipore).

**Chemicals**

Aprotinin (Sigma-Aldrich), bromophenol blue (Merck), bovine serum albumin (BSA) (PAA), C<sub>2</sub>H<sub>6</sub>OS (Sigma-Aldrich), CuSO<sub>4</sub> x H<sub>2</sub>O (Merck), deoxycholate (Sigma-Aldrich), DTT (Sigma-Aldrich), EDTA (Sigma-Aldrich), Folin & Ciocalteu's phenol reagent (Sigma-Aldrich), glycerol (Sigma-Aldrich), glycine (Appli-Chem), KNa-tartrate (Merck), leupeptin (Sigma-Aldrich), MOPS (Sigma-Aldrich), Na<sub>2</sub>CO<sub>3</sub> (Merck), Na<sub>3</sub>VO<sub>4</sub> (Sigma-Aldrich), NaOH (Merck), nonidet P40 (Sigma-Aldrich), Odyssey blocking buffer (LI-COR Biosciences), pepstatin A (Sigma-Aldrich), PMSF (Sigma-Aldrich), precision plus protein all blue standards (Bio-Rad), SDS (Sigma-Aldrich), Sigma 7-9 (Sigma-Aldrich), SPTB (Sigma-Aldrich), Tris-HCl (Sigma-Aldrich), Trizma base (Sigma-Aldrich), Tween-20 (Sigma-Aldrich).

**Antibodies used for western blot analyses**

<b>Primary antibodies</b>				
<b>Target protein</b>	<b>MW (kDa)</b>	<b>Species</b>	<b>Dilution</b>	<b>Company</b>
VE-cadherin	130-140	Rabbit	1:1000	Cell Signaling Technology
Cx43	39-44	Rabbit	1:1000	Cell Signaling Technology
Cav-1	22	Rabbit	1:10000	Abcam
GAPDH (loading control)	40	Rabbit	1:5000	Abcam
Alpha-tubulin (loading control)	50	Mouse	1:10000	Sigma-Aldrich
<b>Secondary antibodies</b>				
<b>Name</b>		<b>Species</b>	<b>Dilution</b>	<b>Company</b>
Goat anti-rabbit (GAR) - red		Goat	1:5000	LI-COR Biosciences
Goat anti-rabbit (GAR) - green		Goat	1:5000	LI-COR Biosciences
Goat anti-mouse (GAM) - green		Goat	1:5000	LI-COR Biosciences

**Table 4: Antibodies for western blot analyses****Inase-inhibitor-mix**

Inase-inhibitor-mix	1 mg aprotinin, 1 mg leupeptin, 1 mg pepstatin A, 10 ml Tris-HCl solution, adjust to pH 7.4
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**Table 5: Composition of Inase-inhibitor-mix****Buffer solutions**

<b>B4</b>	RIPA-buffer	219 mg NaCl, 197 mg Tris-HCl, 250 mg nonidet P40, 62.5 mg deoxycholate, 25 mg SDS, add 25 ml milliQ-water
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<b>B5</b>	Lysis-RIPA-buffer	2500 µl RIPA-buffer ( <b>B4</b> ), 25 µl PMSF 100 mM, 50 µl Na <sub>3</sub> VO <sub>4</sub> 100 mM, 200 µl inase-inhibitor-mix, 25 µl DTT 100 mM, 25 µl SPTB 100 mM
<b>B6</b>	Sample buffer	1 g SDS, 25 mg bromophenol blue, 150 mg Trizma base, dissolve in 4 ml milliQ-water, add 5 ml glycerol and heat to 50°C, adjust pH to 6.8, add 1 ml C <sub>2</sub> H <sub>6</sub> OS
<b>B7</b>	10x MOPS-running buffer	60.6 g Sigma 7-9, 104.6 g MOPS, 10 g SDS, 3 g EDTA, add 1000 ml milliQ-water
<b>B8</b>	10x transfer buffer	30 g Sigma 7-9, 144 g glycine, add 1000 ml milliQ-water
<b>B9</b>	1x transfer buffer	200 ml 10x transfer buffer ( <b>B8</b> ), 400 ml methanol, add 1400 ml milliQ-water
<b>B10</b>	Blocking buffer	Purchased ready for use (Odyssey blocking buffer by LI-COR Biosciences)
<b>B11</b>	Antibody buffer solution	15 ml blocking buffer ( <b>B10</b> ), 150 µl Tween-20 (10%, diluted in milliQ-water)
<b>B12</b>	PBS-T	1000 ml 1x PBS+ ( <b>B2</b> ), 2 ml Tween-20

**Table 6: Buffer solutions used for protein biochemistry**

## Reagents for Lowry protein determination

<b>Solution 1</b> (Lowry reagent A:B:C in relation 100:1:1)	
Lowry reagent A	10 g Na <sub>2</sub> CO <sub>3</sub> , 500 ml 0.1 M NaOH
Lowry reagent B	2 g KNaC <sub>4</sub> H <sub>4</sub> O <sub>6</sub> x 4H <sub>2</sub> O, 100 ml milliQ-water
Lowry reagent C	1 g CuSO <sub>4</sub> x 5H <sub>2</sub> O 100 ml milliQ-water

**Table 7: Composition of Lowry solution 1**

<b>Solution 2</b> (relation 1:1)	
Folin & Ciocalteu's phenol reagent (purchased ready for use)	
milliQ-water	

**Table 8: Composition of Lowry solution 2**

## 2.2 Methods

### 2.2.1 Cell culture

#### 2.2.1.1 Background

As a model for the human endothelium, human umbilical vein endothelial cells (HUVEC) were used. HUVEC were freshly isolated from umbilical cords and used for experiments in passages three to five. The use of HUVEC was approved by the ethical committee at the Academic Medical Center (AMC) in Amsterdam (Waiver: W12\_167# 12.17.0196, Ethical Committee AMC, Amsterdam). Working with HUVEC took place in a biological safety cabinet (Clean Air, biological safety cabinet, class II).

#### 2.2.1.2 HUVEC isolation

HUVEC were isolated from fresh human umbilical cords. Umbilical cords were collected from the maternity department of the AMC in Amsterdam. Prior to HUVEC isolation umbilical cords were kept in **B3** for up to two days at room temperature (RT).

Human umbilical cords physiologically have two umbilical arteries and one umbilical vein. The umbilical vein can visually be detected by its larger lumen and its thinner wall compared to the umbilical arteries. The umbilical vein was cannulated with a vascular irrigation cannula of a well-fitting size and fixated using two cable ties on each side. To clean the umbilical vein from remaining blood the lumen of the vein was rinsed with **B2**. Afterwards, tree-way-valves were connected to the vascular irrigation cannulas. For digestion of the umbilical cords 0.1 g/l collagenase A was injected into the umbilical vein and the umbilical cord was incubated at 37°C for 25 min in a CO<sub>2</sub> incubator (Heracell 150 I CO<sub>2</sub> incubator). After incubation, the enzymatic reaction of collagenase A was stopped by draining the solution inside the umbilical vein into **M1**. To separate cells from stop medium, the solution was centrifuged at 1100 rpm, 4°C, for 10 min (Centrifuge 5810 R, Rotor A-4-81, Radius 18.0). The supernatant was discarded and the pellet was resuspended in **M2**.

HUVEC from two to three different donors were pooled to reduce the effect of interindividual differences on the outcome of experiments and to obtain sufficient amounts of cells. 5 ml of cells diluted in cell culture medium was pipetted into a cell culture flask with a surface area of 25 cm<sup>2</sup>. The cell culture flasks were coated with gelatine for 20 min prior to this step.

### 2.2.1.3 HUVEC cultivation

Before cells reached confluency the cell culture medium (**M2**) was changed every two to three days, the old cell culture medium was discarded, cells were washed three times with **B2** and 5 ml of fresh cell culture medium (**M2**) was added. By the time the primary cell cultures had reached confluency in 25 cm<sup>2</sup> flasks they were passaged to a surface area of 75 cm<sup>2</sup> (splitting 1:3). By the time the cells had reached confluency in 75 cm<sup>2</sup> flasks they were passaged again into three cell culture flasks with a surface area of 75 cm<sup>2</sup> each (splitting 1:3). This procedure was repeated until passage two to four for transfection experiments and until passage three to five for experiments with non-transfected HUVEC.

For passaging HUVEC, the medium was discarded and cells were washed three times with **B2**. A trypsin-EDTA solution was heated to 37°C in a water bath and added to the cells (1 ml for 25 cm<sup>2</sup>, 3 ml for 75 cm<sup>2</sup>, 0.6 ml for one well in 6-well-plates). HUVEC were incubated with trypsin-EDTA solution for 1.5 min at 37°C in a CO<sub>2</sub> incubator (Heracell 150 I CO<sub>2</sub> incubator). Cells were detached by gently tapping the cell culture flasks onto the work bench. The reaction was stopped by pipetting the cells suspended in a trypsin-EDTA solution into **M1**. To separate cells from cell culture medium the solution was centrifuged at 1100 rpm, 4°C, for 10 min

(Centrifuge 5810 R, Rotor A-4-81, Radius 18.0). The supernatant was discarded and the pellet was resuspended in fresh **M2**.

## **2.2.2 SiRNA transfection**

### **2.2.2.1 Background**

In order to obtain a Cav-1 knock-down model of the human endothelium, small interfering RNA (siRNA) was introduced into HUVEC. SiRNA consists of double stranded RNA molecules (93) and allows to block the synthesis of a specific protein (94). SiRNA interferes with messenger RNA (mRNA), leads to a degradation of the mRNA and results in posttranscriptional gene silencing (PTGS) (94). The application of siRNA to knock-down protein levels of Cav-1 in HUVEC is well described in the literature (84,95–101).

### **2.2.2.2 Transfection with Cav-1 siRNA**

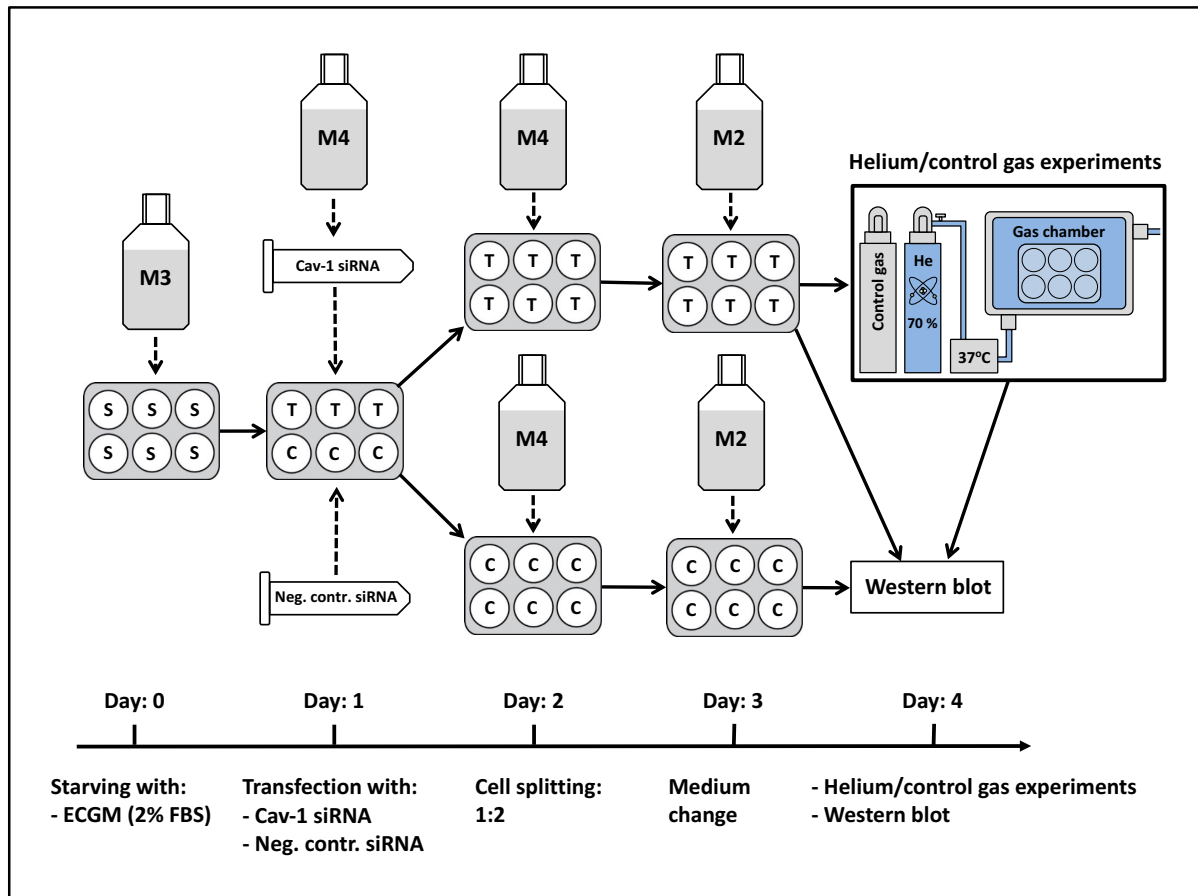
The experimental protocol used for Cav-1 siRNA transfection in HUVEC is outlined in Fig. 5. This protocol was developed based on a protocol from Dr. Zhenlong Chen, University of Illinois, Chicago, USA and was modified based on the results of pilot experiments performed in our laboratory. In the following protocol, all amounts refer to one well of a 6-well-plate (surface area 9.6 cm<sup>2</sup>).

#### **Day -1: Passage HUVEC into 6-well-plates**

48 hours prior to transfection with siRNA, HUVEC in passages one to three were seeded into gelatine coated 6-well-plates containing 3 ml of **M2** per well.

#### **Day 0: Starving**

20 hours prior to transfection with siRNA, cells in passages two to four were washed three times with **B2** and **M3** was added to each well (Fig. 5).

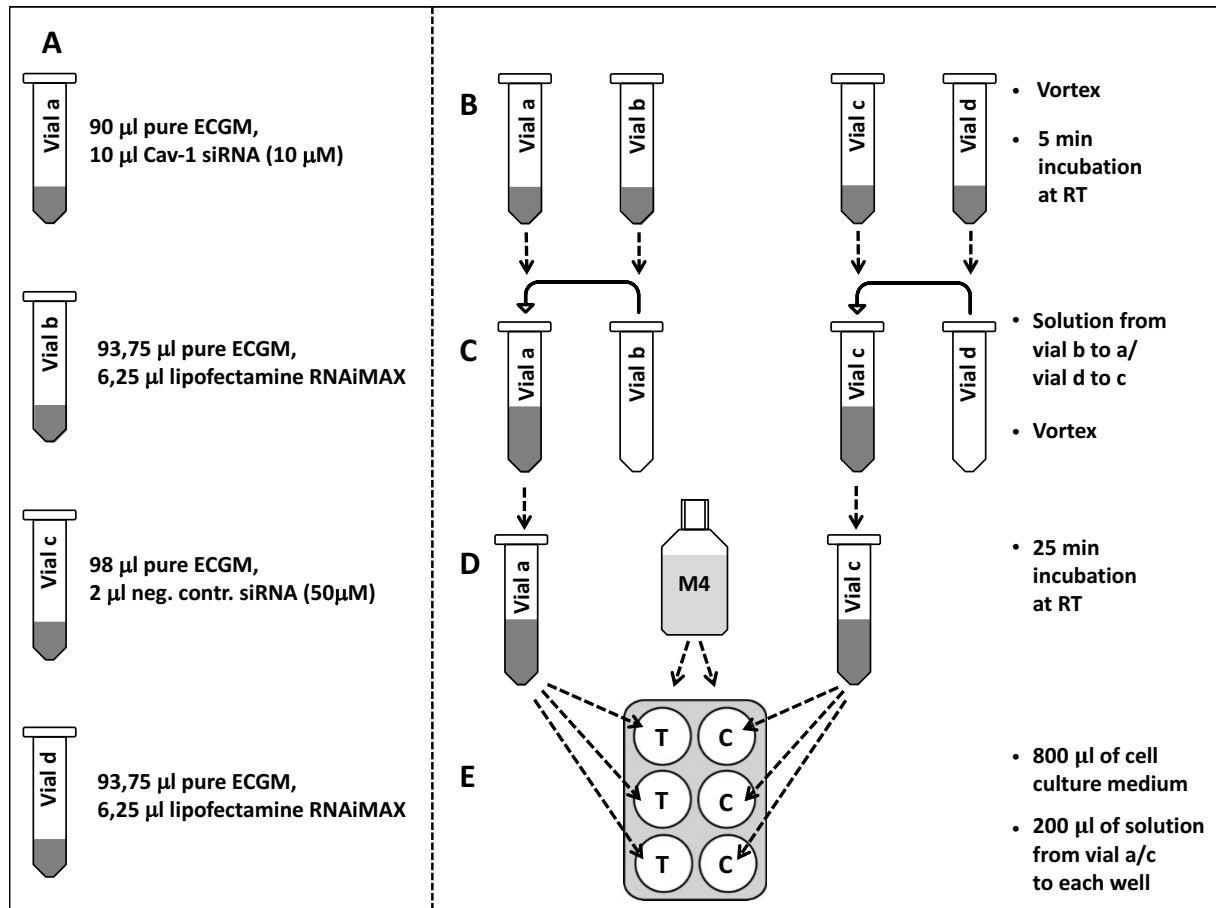


**Figure 5: Transfection with Cav-1 siRNA**

C = well containing negative control siRNA transfected HUVEC, HUVEC = human umbilical vein endothelial cells, S = well containing HUVEC starved with M3, T = well containing Cav-1 siRNA transfected HUVEC. For medium composition see table 2.

### Day 1: Transfection

HUVEC were transfected at a confluency of 50-80% with siRNA for Cav-1 or scrambled siRNA as negative control with the use of lipofectamine RNAiMAX. Immediately before adding the siRNA for Cav-1 or negative control siRNA **M3** was discarded, the cells were washed three times with **B2** and 800  $\mu$ l **M4** was added. Four vials (a, b, c and d) were prepared according to Fig. 6A. All vials were gently vortexed and kept at RT for 5 min (Fig. 6B). Then the solution in vial b was added to the solution in vial a and the solution in vial d was added to the solution in vial c and the remaining vials a and c were vortexed gently for 3 seconds (Fig. 6C). The resulting solutions were kept at RT for 25 min before they were added onto the cultured cells (Fig. 6D,E). This resulted in a final concentration of 100 nM Cav-1 siRNA or negative control siRNA on the cultured cells. Throughout the next 6 hours the 6-well-plates were gently shaken by hand every 2 hours to allow the siRNA to spread evenly. 6 hours after adding the siRNA for Cav-1 or negative control siRNA to the cells, 2 ml of **M4** were added to each well.



**Figure 6: Preparation of siRNA**

C = well containing negative control scrambled siRNA transfected HUVEC, Cav-1 = caveolin-1, ECGM = endothelial cell grows medium, RT = room temperature, siRNA = small interfering RNA, T = well containing Cav-1 siRNA transfected HUVEC. For medium composition see table 2.

**Day 2: Passage of transfected HUVEC**

24 hours after adding siRNA for Cav-1 or negative control siRNA to each well, the cells were split 1:2. Cells were either split into gelatine-coated 6-well-plates for western blot analysis or onto transwell membranes to perform permeability assays. During Day 1 and Day 2 of the transfection protocol no antibiotics or antimycotics were added to the cell culture medium (**M4**, **M5**).

**Day 3: Medium change**

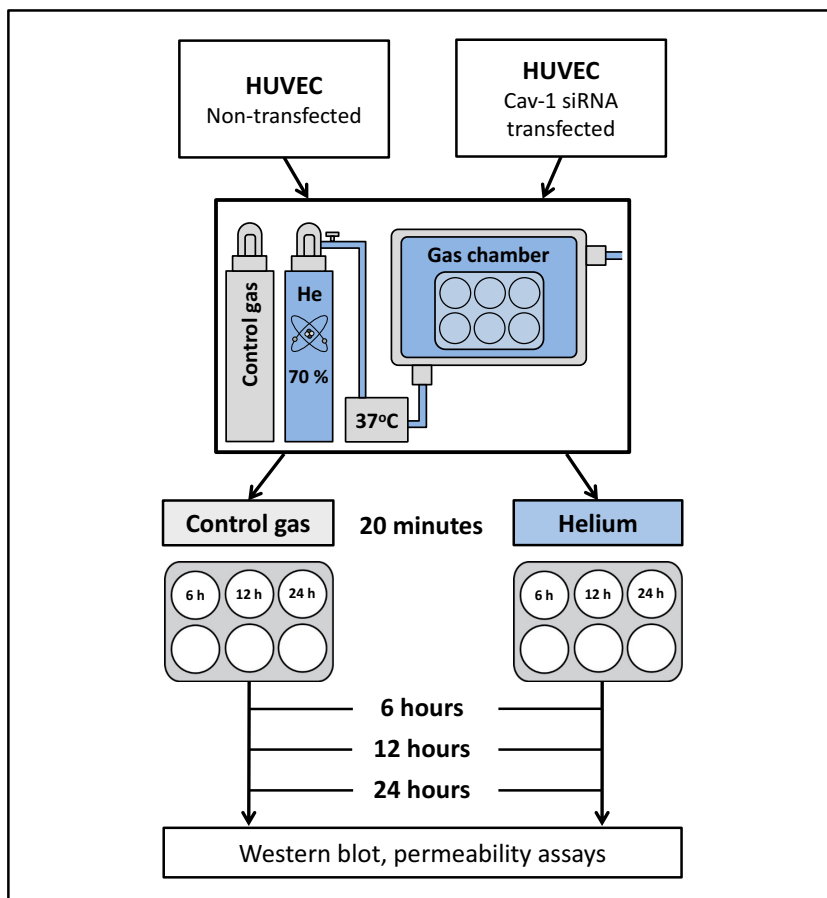
48 hours after adding siRNA for Cav-1 or negative control siRNA to each well, the cells were washed three time with **B2** and fresh **M2** was added to each well (Fig. 5).

**Day 4: Western blot analysis or helium/control gas experiment**

After 72 hours the cells were lysed to determine protein knock-down by western blot analysis or helium/control gas experiments were started (Fig. 5).

### 2.2.3 Helium and control gas treatment

Cav-1 siRNA transfected and non-transfected HUVEC were grown on gelatine coated 6-well-plates for western blot analysis of the junctional molecules VE-cadherin and Cx43, or on transwell membranes for permeability assays. Before treatment with helium or control gas, the cells were washed three times with **B2** and fresh **M2** was added. Confluent monolayers of Cav-1 siRNA transfected and non-transfected HUVEC were treated with either 20 min of helium (5% CO<sub>2</sub>, 25% O<sub>2</sub>, 70% He) or control gas (5% CO<sub>2</sub>, 25% O<sub>2</sub>, 70% N<sub>2</sub>) in a specially designed gas chamber (Fig. 7). The gas flow was set to 5 l/min and outlet gas concentrations were monitored by a gas analyzer (Capnomac Ultima). The gas temperature inside the gas chamber was kept at 37°C and was constantly monitored. After 20 min of treatment, the cells in 6-well-plates or permeability membranes were placed into a CO<sub>2</sub> incubator (Heracell 150 I CO<sub>2</sub> incubator) at 37°C for 6, 12, or 24 hours. At these time points the cells and supernatants were collected for western blot analysis or permeability measurements. If necessary, samples were frozen at -80°C.



**Figure 7: Helium experiments**

Cav-1 = caveolin-1, HUVEC = human umbilical vein endothelial cells, siRNA = small interfering RNA.

## 2.2.4 Western blot analysis

### 2.2.4.1 Background

Western blotting is a technique to separate and identify proteins in a sample. During the first step, which is called gel electrophoresis, proteins are separated according to their molecular weight. In the following step proteins are transferred to a membrane and unspecific binding sites of the membrane are blocked with a blocking buffer. A primary antibody binds specifically to the protein of interest, a secondary fluorescence-coupled antibody binds to the Fc part of this primary antibody and can be detected by its fluorescent signal. Data produced with this technique are semi-quantitative as they compare relative protein levels and do not measure an absolute quantity of the amount of a protein (102).

### 2.2.4.2 Western blot implementation

#### Sample preparation

At each time point (72 hours after transfection to determine transfection efficiency and 6, 12, or 24 hours after exposure to helium/control gas to measure expression of junctional molecules VE-cadherin and Cx43), the cell lysates and cell culture media were collected. HUVEC growing on 6-well-plates were washed three times with ice-cold **B2**. 400  $\mu$ l of ice-cold **B5** was pipetted into each well and the cells were detached with cell-scrapers. The cell lysate was centrifuged at 14000 rpm and 4°C for 10 min (Centrifuge 5430 R) and was collected and stored at -80°C until further analyses.

#### Lowry protein determination

The Lowry protein assay works through the formation of color complexes that react proportionally to the amount of protein in a sample (103).

Prior to the Lowry protein determination all samples were thawed and diluted (1:20) with milliQ-water. The required amount of **solution 1** was calculated and prepared. 500  $\mu$ l of **solution 1** for every sample and every standard of the calibration curve were needed. A standard curve was prepared by diluting BSA in milliQ-water, this standard was used as a reference for all samples. 500  $\mu$ l of **solution 1** was added to 100  $\mu$ l of each diluted sample and each of the standards, followed by an incubation time of 10 min at RT. During this first step, copper ions in alkaline solution react with proteins to form complexes (103). In the following step **solution 2** was prepared and 50  $\mu$ l of **solution 2** was added to all samples and standards. After a



second incubation time of 30 min at RT the samples and standards were transferred in duplicates to a 96-well-plate. The protein concentration was detected colorimetrically at an absorbance wavelength of 750 nm using the microplate fluorescence reader FLx800.

### Sample dilution

For western blot analyses, all samples were diluted with **B4** to obtain equal protein concentrations for related experiments.

### Electrophoresis

Samples were diluted 1:5 with **B6**. Subsequently, samples were heated at 95°C for 5 min (Heaterblock III) to denature proteins (102).

Commercially available gels (Criterion XT precast gel) were used for electrophoresis. **B7** was diluted 1:10 with milliQ-water and 500 ml per gel were filled into the criterion cell tank. After removing the combs 15 µl of the diluted samples were loaded into each pocket of the gel. Additionally, 2 µl of a protein marker (precision plus protein all blue standards) was loaded onto the gel to function as a reference for the molecular weight.

The electrophoresis leads to a separation of proteins according to their molecular weight and takes around 1 hour and 15 min with a voltage of 150 V. During electrophoresis, the negatively charged proteins migrate through the gel. Large molecules migrate slower and small molecules faster, which leads to the formation of the protein bands by a separation of the molecules along the gel. Bromophenol blue in **B6** allows to visualize how far the separation has marched (102).

### Protein transfer and blocking

Prior to the transfer, the membranes (Immobilon FL) were cut to the size of the gels and soaked in methanol for 5 min. Fiber pads and filter paper were soaked in cold **B9**. After electrophoresis was complete, the transfer cassette (Criterion Blotter Gel Holder Cassette) was loaded from cathode to anode as follows: fiber pad, two filter papers, gel, membrane, two filter papers, fiber pad (Fig. 8). One or two transfer cassettes together with an ice block and a magnetic stirrer were placed in a transfer tank (Criterion Blotter) and the tank was filled with cold **B9**. During the transfer the magnetic stirrer was constantly rotating, the protein transfer from the gel to the membrane took 30 min at a voltage of 100 V. Placing the membrane between the gel and the anode leads to a migration of the negatively charged proteins from the gel to the membrane (102). After the transfer the gel was discarded and the membrane was blocked in **B10** for one hour on a shaker.

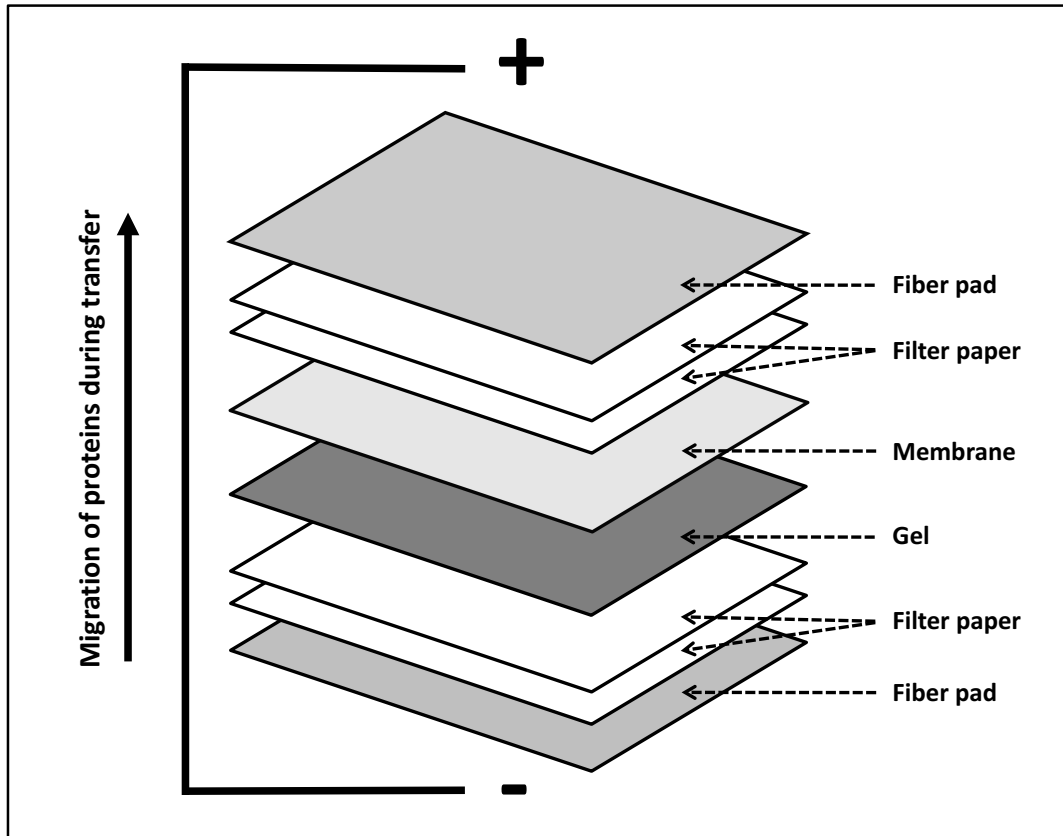


Figure 8: Assembly of gel and membrane for western blot transfer

### Protein detection

After blocking the membranes, they were incubated with the primary antibody in 15 ml of **B11** at 4°C overnight. This step allows the primary antibody to bind specifically to the protein of interest. The following primary antibodies were used: alpha-tubulin (1:10000), Cav-1 (1:10000), Cx43 (1:1000), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5000), VE-cadherin (1:1000) (table 4). The following day the membranes were washed three times for 5 minutes with cold **B12**. Washing removes unspecifically bound antibodies and excessive antibodies from the membrane and minimizes the background (102). After the third wash the membranes were incubated with a secondary antibody for 1 hour at RT in 15 ml of **B11**. This step leads to a specific binding of the secondary antibody to the Fc part of the primary antibody. On membranes with rabbit primary antibodies, anti-rabbit secondary antibodies were used and on membranes with mouse primary antibodies, anti-mouse secondary antibodies were used. For the primary antibodies binding to the proteins Cav-1, Cx43 and VE-cadherin a secondary antibody with a red fluorescent signal was employed; for the loading controls alpha-tubulin and GAPDH a secondary antibody with a green fluorescent signal was used. After washing three times with cold **B12** for 5 minutes, membranes were scanned with the Odyssey CLx imaging system.

### Signal quantification

Quantification of the signals was performed with Odyssey Imaging Studio software (LI-COR Biosciences, Bad Homburg, Germany). The results show the relation of VE-cadherin and Cav-1 to GAPDH. Because the molecular weights of Cx43 (39-44 kDa) and GAPDH (40 kDa) are close together, alpha-tubulin (50 kDa) was used as a loading control for Cx43.

### 2.2.5 Permeability assays

The permeability of confluent HUVEC monolayers was evaluated by employing fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA) flow through in combination with a microporous membrane (Fig. 9). Upon confluency, cells growing on microporous membranes with a pore diameter size of 0.4  $\mu\text{m}$  and a area of 1.12  $\text{cm}^2$  were exposed to either helium or control gas and grown in a  $\text{CO}_2$  incubator (Heracell 150 I  $\text{CO}_2$  incubator) at 37°C for 6, 12, or 24 hours. FITC-BSA was diluted in water (25 mg FITC-BSA + 2.5 ml milliQ-water) and excessive exposure to light was avoided. After each time point 100  $\mu\text{l}$  of FITC-BSA (10 mg/ml) was added to the upper compartment. After incubating for 2 hours at 37°C in a  $\text{CO}_2$  incubator, medium from the lower compartment was collected and stored at -80°C. Before permeability was measured a standard curve was prepared by diluting FITC-BSA in **M2**. Standards and samples were loaded in duplicates into a 96-well-plate (OptiPlate 96 Black) and read by a microplate fluorescence reader (FLx800) using KC4 software (BioTek). The concentration of FITC-BSA in the samples was calculated based on the FITC-BSA standard calibration curve.

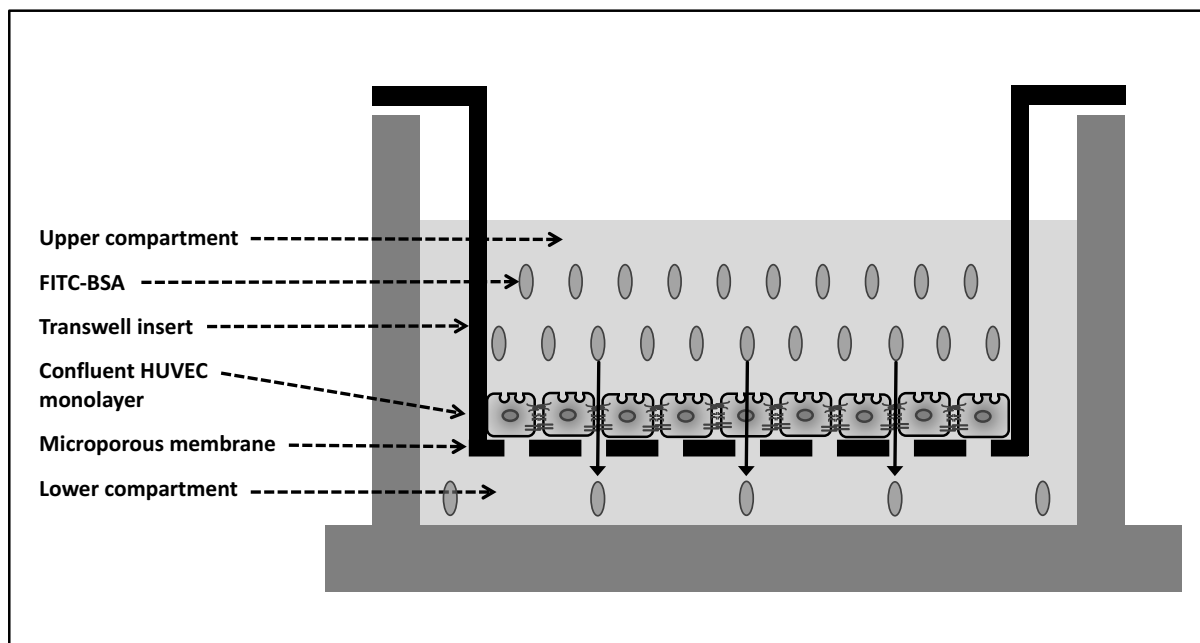


Figure 9: Set-up of the permeability assays

### **2.2.6 Statistics**

For experiments evaluating the effect of helium/control gas on permeability and the expression of junctional molecules in Cav-1 siRNA transfected and non-transfected HUVEC, 7 or more independent experiments were performed. The exact number of independent experiments performed for each set of experiments is listed in the respective figure legend.

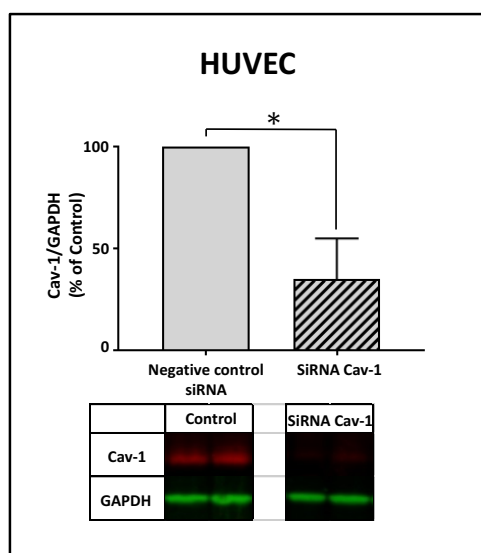
Statistical analysis was performed using the statistics software program GraphPad Prism 7 for Windows (GraphPad Software, La Jolla, CA, USA). Cav-1 knock-down experiments (3.1) were analyzed using t-tests. All other data (3.2 and 3.3) were analyzed using a two way analysis of variance (ANOVA) for repeated measures with a Sidak's multiple comparison test. Values of  $p < 0.05$  were considered statistically significant. Data are described as mean  $\pm$  SD (standard deviation).

### 3. Results

#### 3.1 Cell transfection and Cav-1 knock-down

In order to investigate the role of Cav-1 in the helium-mediated changes of the endothelial permeability and the expression rates of VE-cadherin and Cx43, a knock-down model of the human endothelium was established. The transfection efficiency was evaluated by western blot analysis using cell lysates.

HUVEC transfected with siRNA for Cav-1 contained significantly lower amounts of Cav-1 compared to controls ( $34.82 \pm 20.07\%$  vs.  $100.00\%$ ,  $p < 0.05$ , Fig. 10), which indicates successful Cav-1 knock-down.



**Figure 10: Cav-1 siRNA transfection efficiency in HUVEC**

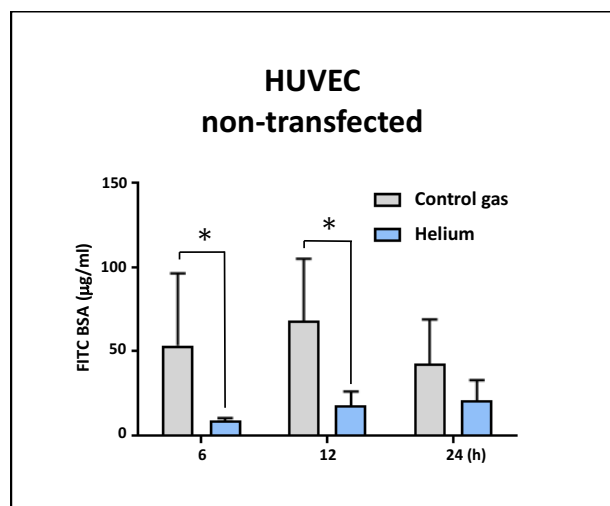
Western blot results of the ratio of Cav-1 compared to GAPDH loading controls in HUVEC transfected with Cav-1 siRNA and negative controls. Cav-1 protein expression is shown as percentage of the negative controls which were set at 100%. Representative western blot results are displayed below the respective graph.  $n=6$  (6 independent experiments, 6-10 replicated in separate wells per experiment). Data are represented as  $\text{mean} \pm \text{SD}$ , \* =  $p < 0.05$ , Cav-1 = caveolin-1, GAPDH = Glyceraldehyde 3-phosphate dehydrogenase, HUVEC = human umbilical vein endothelial cells.

## 3.2 Helium-induced attenuation of permeability of HUVEC monolayers is dependent on Cav-1

### 3.2.1 Effects of helium on permeability of non-transfected HUVEC

The permeability of confluent HUVEC monolayers was measured at different time points after treatment with 20 min of helium or control gas.

Helium treatment decreased cellular permeability in non-transfected HUVEC after 6 hours compared to controls (helium:  $8.69 \pm 1.68$  vs. control:  $53.62 \pm 42.61$ ,  $p < 0.05$ ; Fig. 11), and this effect remained present after 12 hours (helium:  $17.86 \pm 8.31$  vs. control:  $67.96 \pm 36.95$ ,  $p < 0.05$ ; Fig. 11), but not after 24 hours ( $p > 0.05$ ; Fig. 11).



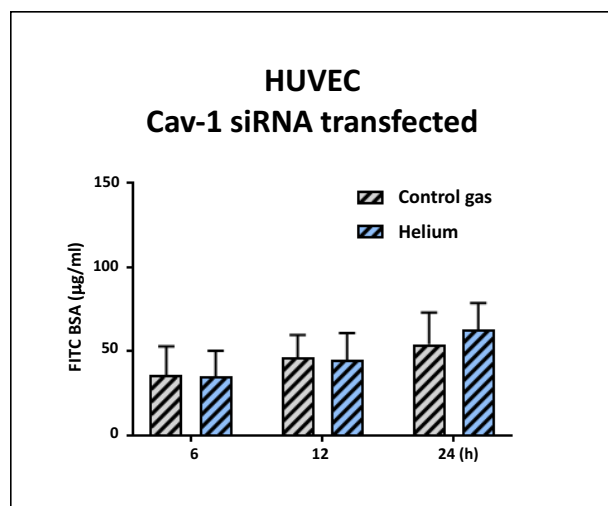
**Figure 11: Effect of helium on permeability of non-transfected HUVEC**

Results of helium on permeability of a confluent monolayer of non-transfected HUVEC, estimated by the transfer of FITC-BSA at different time points compared to control gas.  $n=9$  (7 independent experiments were performed by M. Landau, Laboratory of Experimental Intensive Care and Anesthesiology (L.E.I.C.A.), Amsterdam, while 2 independent experiments were performed as part of this thesis). Data are represented as mean  $\pm$  SD, \* =  $p < 0.05$ , HUVEC = human umbilical vein endothelial cells.

### 3.2.2 Effects of helium on permeability of Cav-1 siRNA transfected HUVEC

In order to investigate if the barrier protective effect of helium is dependent on Cav-1, HUVEC were transfected with Cav-1 siRNA prior to helium application. The effect of helium on the permeability of Cav-1 knock-down HUVEC monolayers was measured at different time points after treatment with 20 min of helium or control gas.

Cav-1 knock-down in HUVEC completely abolished the helium-mediated decrease in permeability described above and no significant differences between the helium and control group were detected (helium vs. control gas, all time points  $p > 0.05$ ; Fig. 12).



**Figure 12: Effect of helium on permeability of Cav-1 siRNA transfected HUVEC**

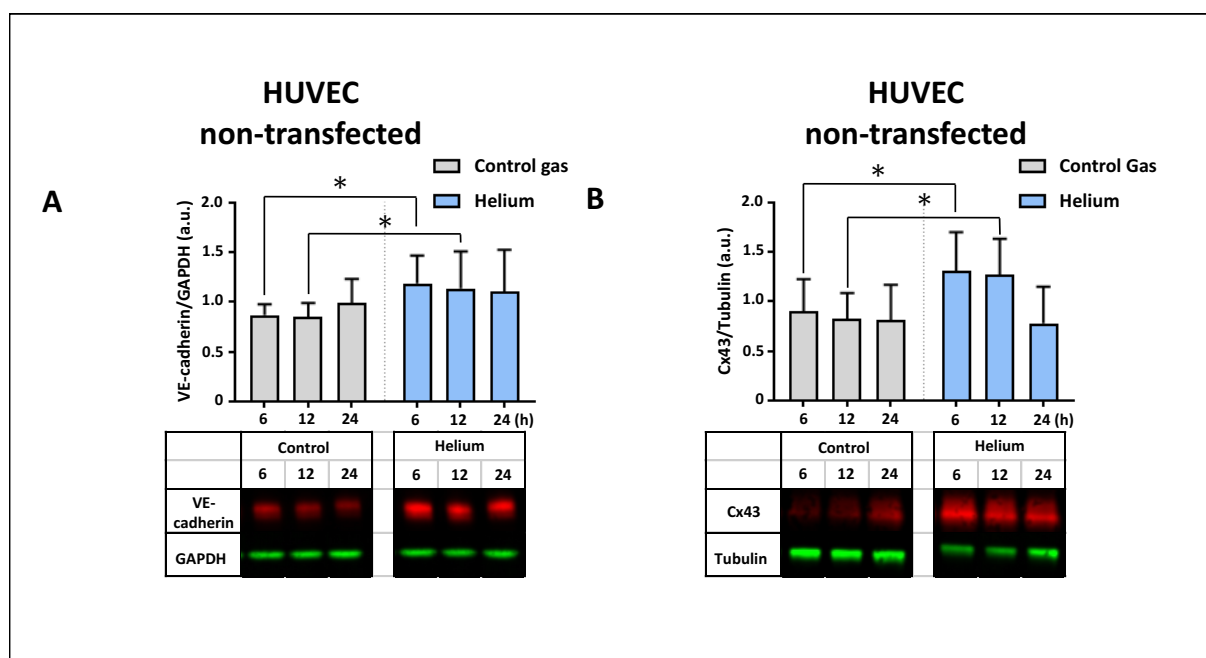
Results of helium on permeability of a confluent monolayer of Cav-1 siRNA transfected HUVEC, estimated by the transfer of FITC-BSA at different time points compared to control gas.  $n=8$  (8 independent experiments). Data are represented as mean $\pm$ SD, \* =  $p<0.05$ , Cav-1 = caveolin-1, HUVEC = human umbilical vein endothelial cells.

### 3.3 Helium increases VE-cadherin and Cx43 levels in HUVEC possibly via Cav-1

#### 3.3.1 Effect of helium on VE-cadherin and Cx43 levels in non-transfected HUVEC

To reveal a possible molecular mechanism behind the helium-mediated decrease in permeability, the effect of helium on the expression rates of the junctional molecules VE-cadherin and Cx43 was investigated.

Helium treatment of HUVEC showed significantly increased cellular levels of VE-cadherin and Cx43 at 6 hours and 12 hours after treatment compared to treatment with control gas (VE-cadherin in helium treated cells at 6 hours:  $1.19\pm 0.28$  and 12 hours:  $1.14\pm 0.37$ . VE-cadherin in control cells at 6 hours:  $0.87\pm 0.11$  and 12 hours:  $0.85\pm 0.14$ . Cx43 in helium treated cells at 6 hours:  $1.31\pm 0.39$  and 12 hours:  $1.27\pm 0.37$ . Cx43 in control cells at 6 hours:  $0.90\pm 0.32$  and 12 hours:  $0.82\pm 0.26$ .  $p<0.05$  for all; Fig. 13 A and B). This effect subsided 24 hours after treatment ( $p>0.05$ ; Fig. 13 A and B).



**Figure 13: Effect of helium on VE-cadherin and Cx43 levels in non-transfected HUVEC**

Panel A: Western blot results of the ratio of VE-cadherin compared to GAPDH loading controls in non-transfected HUVEC at different time points following helium or control gas treatment. n=14 (14 independent experiments). Panel B: Western blot results of the ratio of Cx43 compared to alpha-tubulin loading controls in non-transfected HUVEC at different timepoints following helium or control gas treatment. n=11 (11 independent experiments).

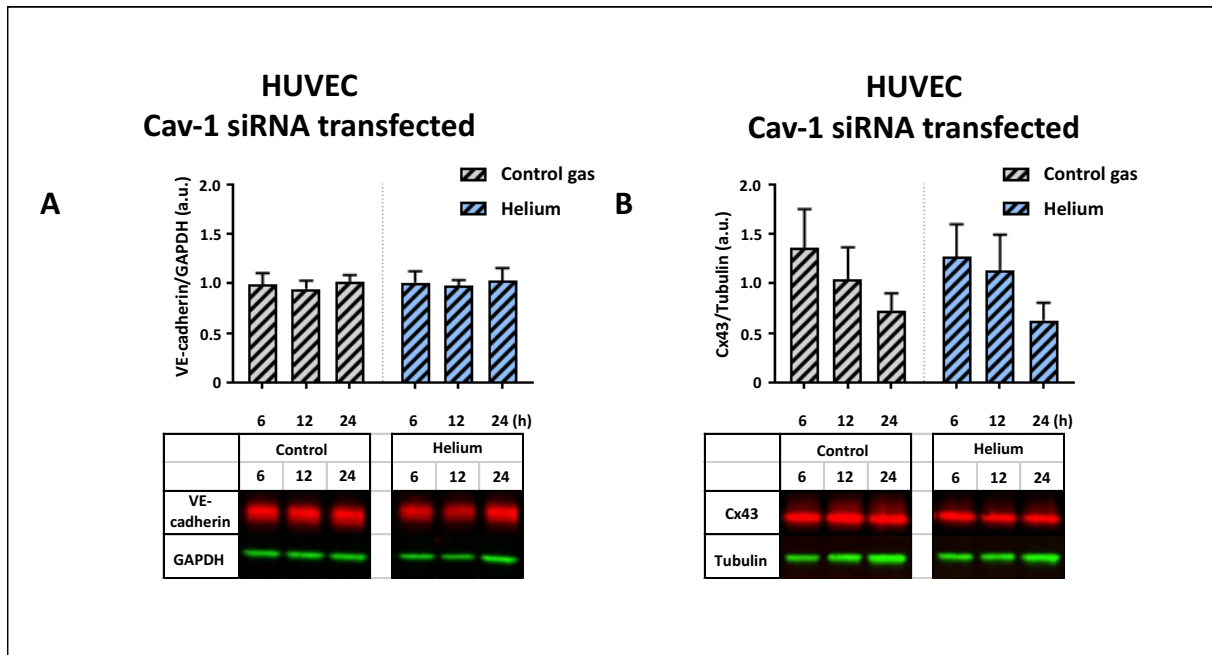
Representative western blot results are displayed below the respective graphs. Data are represented as mean $\pm$ SD. \* = p<0.05, Cx43 = connexin-43, GAPDH = Glyceraldehyde 3-phosphate dehydrogenase, HUVEC = human umbilical vein endothelial cells, VE-cadherin = vascular endothelial - cadherin.

### 3.3.2 Effect of helium on VE-cadherin and Cx43 levels in Cav-1 siRNA transfected HUVEC

In order to investigate if the observed effect of helium on the junctional molecules VE-cadherin and Cx43 is related to Cav-1, HUVEC were transfected with Cav-1 siRNA, to knock down the Cav-1 expression prior to helium application.

A knock-down of Cav-1 in HUVEC completely abolished the helium-mediated increase in the expression of the junctional molecules VE-cadherin and Cx43. Western blot analysis of VE-cadherin and Cx43 showed no significant differences between the helium and control gas group at any timepoint (helium vs. control gas, all time points p>0.05; Fig. 14 A and B). Interestingly, Cx43 showed a time dependent decrease in protein expression independently of helium or control gas application (Fig. 14 B).





**Figure 14: Effect of helium on VE-cadherin and Cx43 levels in Cav-1 siRNA transfected HUVEC**

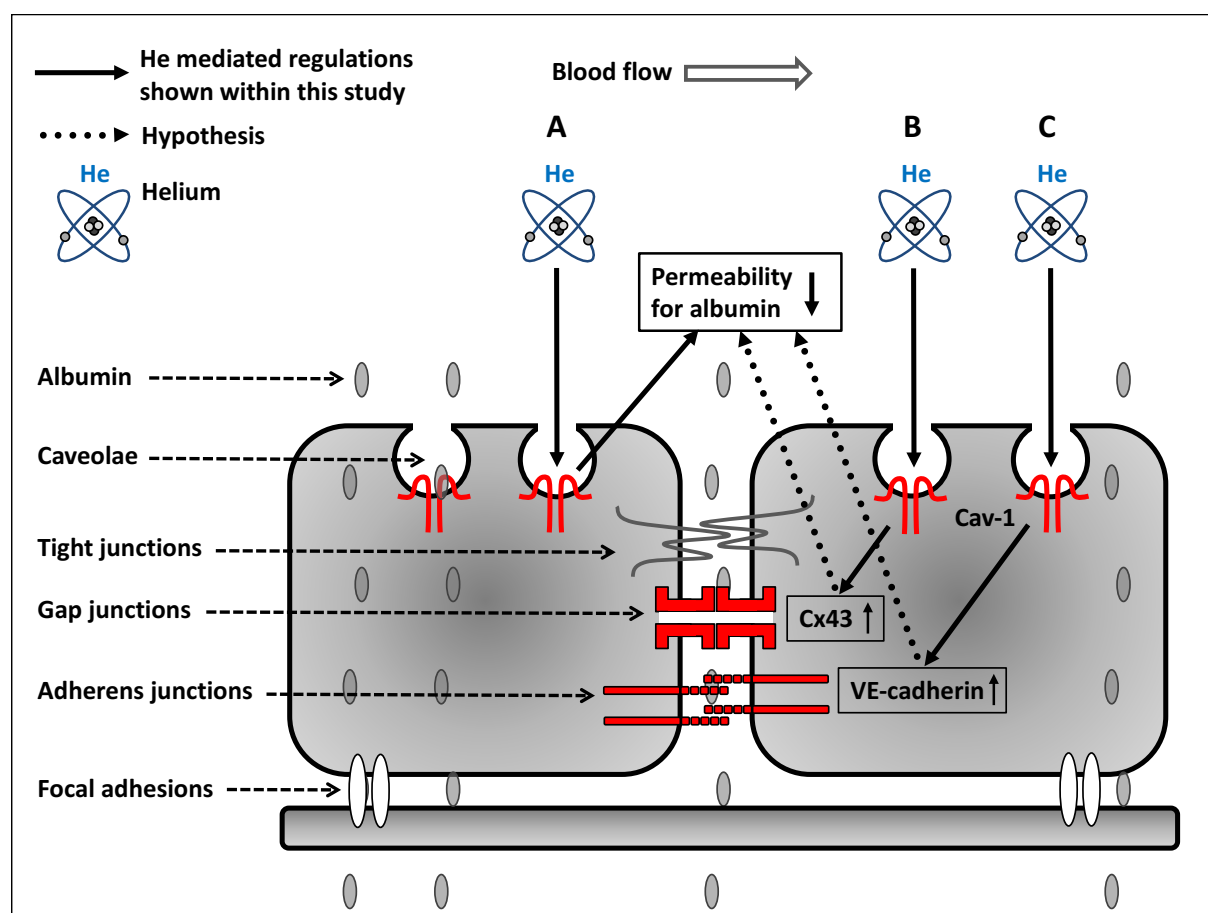
Panel A: Western blot results of the ratio of VE-cadherin compared to GAPDH loading controls in Cav-1 siRNA transfected HUVEC at different time points following helium or control gas treatment. n=7 (7 independent experiments). Panel B: Western blot results of the ratio of Cx43 compared to alpha-tubulin loading controls in Cav-1 siRNA transfected HUVEC at different timepoints following helium or control gas treatment. n=7 (7 independent experiments).

Representative western blot results are displayed below the respective graphs. Data are represented as mean±SD. \* = p<0.05, Cx43 = connexin-43, GAPDH = Glyceraldehyde 3-phosphate dehydrogenase, HUVEC = human umbilical vein endothelial cells, VE-cadherin = vascular endothelial - cadherin.

### 3.4 Summary of results

The results suggest that (i) helium decreases permeability of a confluent monolayer of HUVEC, (ii) helium increases VE-cadherin and Cx43 protein expression, and (iii) Cav-1 knock-down abrogates the effects of helium on permeability and VE-cadherin/Cx43 expression.

Since VE-cadherin and Cx43 are known to regulate endothelial function (47,54,75), we hypothesize that the helium-induced decrease in endothelial permeability, is mediated by the observed changes in the expression rates of VE-cadherin and Cx43.



**Figure 15: Helium-mediated alteration of endothelial permeability: Involvement of Cav-1, VE-cadherin and Cx43**

A: Helium attenuates permeability of HUVEC monolayers which is dependent on Cav-1. B: Helium increases Cx43 levels in HUVEC which is dependent on Cav-1. C: Helium increases VE-cadherin levels in HUVEC which is dependent on Cav-1. Cav-1 = caveolin-1, Cx43 = connexin-43, He = helium, VE-cadherin = vascular endothelial - cadherin. Molecules pictured in red are investigated within this study.

## 4. Discussion

### 4.1 Helium and organ protective strategies

The non-anesthetic noble gas helium has gained high interest in organ protective strategies, because in contrast to volatile anesthetics, it can be used also in awake patients (24). Experimental studies in animals have shown that helium can mimic ischemic preconditioning and protect different organs including the heart (26–36), brain (37–42), liver (104) and intestine (105) from I/R-injury.

Recent studies showed that helium is able to protect the heart against I/R-injury and to reduce infarct size (26–36). In 2007 Pagel et al. were the first who showed in animal experiments that helium can induce preconditioning of the heart (26). In their study the authors exposed rabbits to three times 5 min inhalation of 70% helium, each 5 min session separated by 5 min of air inhalation, followed by a coronary occlusion of the anterior descending coronary artery (LAD) for 30 min and 3 hours of reperfusion (26). Helium inhalation prior to the coronary artery occlusion significantly reduced the infarct size (26). Since this publication numerous studies by the same authors as well as others have been published, confirming the feasibility of helium-induced conditioning in animal models (27–42,104,105).

The protective effect of helium-induced conditioning on the brain is also well investigated in animal models (37–42). Pan et al. were the first to demonstrate the protective effect of helium-induced conditioning on the brain in animals (40). Focal cerebral ischemia was induced through 2 hours of middle cerebral artery occlusion and 1 hour of reperfusion (40). During artery occlusion and reperfusion rats inhaled 70% helium (40). In this animal model of focal cerebral ischemia, helium treatment reduced infarct volume and improved 24 hour neurological outcome compared to the control group inhaling 30% O<sub>2</sub>/70% N<sub>2</sub> gas mixture and compared to the normobaric hyperoxia group inhaling 100% O<sub>2</sub> (40).

In 2014 Zhang et al. revealed that helium preconditioning, furthermore, protects the liver from I/R-injury (104). Helium preconditioning was induced by inhalation of three times 5 min of 70% helium before a midline laparotomy and 90 min of occlusion of the structures left to the porta hepatis (hepatic artery, portal vein, and bile duct) in mice (104). The authors found that helium preconditioning significantly increased the actin phosphorylation in hepatocytes and resulted in attenuated hepatocellular injury and increased survival in the treated animals (104).

A more recent trial, published in 2015, demonstrated that helium preconditioning in animals also protects the intestine from I/R-injury (105). Rats were treated with three times 5 min of 70% helium inhalation before 60 min of intestinal I/R induced by superior mesenteric artery

occlusion and 60 min of reperfusion (105). Helium preconditioning reduced the I/R-induced increase in intestinal permeability, cell apoptosis and the inflammatory response (105).

The large amount of experimental data on helium-induced conditioning has so far not been translated to the clinical situation (23). However, a study by Smit et al. demonstrated that helium protects the human endothelium from I/R-injury by mimicking the early and late events of ischemic preconditioning (21). Healthy human volunteers were treated with 79% helium directly before or 24 hours before forearm ischemia and reperfusion induced by an inflated blood pressure cuff on the forearm for 20 min (21). Endothelial dysfunction was evaluated by measuring the blood flow in the forearm in response to infusion of acetylcholine before and after 20 min of I/R (21). In this study early and late preconditioning by helium inhalation successfully attenuated the endothelial dysfunction induced by I/R and thereby, mimicked the effect of ischemic preconditioning (21).

A following study by Smit et al. was intended to translate the protective effect of helium into the clinical setting (106). In this first clinical trial the effect of helium was evaluated in patients subjected to coronary artery bypass graft surgery (106). Patients inhaled 3 x 5 min of 79% helium before the start of cardiopulmonary bypass or at the timepoint of coronary artery reperfusion (106). Surprisingly, no effect of helium preconditioning, postconditioning, or a combination of both was seen in signal transduction kinases or postoperative troponin release (106). Therefore, further clinical studies are necessary to evaluate the feasibility of pre- and postconditioning by helium in this and other clinical situations.

## **4.2 HUVEC as an in-vitro model for the human endothelium**

To investigate the cellular and molecular mechanisms responsible for the barrier protective effects of helium, HUVEC monolayers were used as a model of the human endothelium. This cell culture model is widely used in preclinical research (107–111).

HUVEC are non-immortalized human cells and isolation without contamination of other cell types is comparatively simple to conduct (112). Human umbilical cords are resected after delivery, making human umbilical veins easy to obtain (112). HUVEC monolayers have been used in cell research for several decades. In 1963 Maruyama et al. were the first who published the successful isolation of HUVEC (107). The authors cannulated the umbilical vein after post-natal resection of the umbilical cords and detached HUVEC utilizing trypsin (107). By the time HUVEC are properly differentiated they obtain the so-called cobblestone phenotype (112). In this stage they mimic an endothelium in-vivo and can be utilized to study endothelial-related processes (112).

In the course of the present study, permeability for albumin was assessed for HUVEC monolayers after treatment with helium or a control gas. HUVEC were seeded on a microporous membrane with a pore diameter size of 0.4  $\mu\text{m}$ ; permeability was assessed by FITC-BSA flow-through in 2 hours. The described protocol used to assess permeability was already established in our laboratory and was initially developed based on Kiemer et al., with some modifications (113). In the study by Kiemer et al., as well as in the present study, FITC-BSA flow-through was applied to assess permeability (113). The use of different forms of labeled albumin to assess endothelial permeability of HUVEC monolayers was described in several publications (84,111,113–120). However, fluorescence-labeled dextrans are also frequently used as molecular tracers to assess permeability of HUVEC monolayers (111,121–127). The passage of dextrans occurs only by passive diffusion through the inter-cellular clefts, albumin however, passages by a combination of active vesicular transport and para-cellular transport (111). Since vascular leakage of plasma albumin is one of the consequences of I/R-injury (128), albumin flow-through was selected to assess HUVEC monolayer permeability within this study.

In order to investigate whether Cav-1 is involved in the helium-mediated stabilization of the endothelial barrier function, a Cav-1 knock-down model, using siRNA transfection in HUVEC, was established. The relatively low proliferative rate and limited lifespan of HUVEC make it more challenging to transfect them compared to immortalized cell lines (129). However, these characteristics imply that HUVEC may reflect the in-vivo situation of the endothelium better than immortalized cell lines (129).

After transfection, siRNA forms RNA-induced silencing complexes inside the cell, which block the expression of a target gene expression (130). Transfection can be achieved by biological, physical or chemical methods (130). Biological methods are virus-mediated and also known as transduction, whereas physical methods employ various physical instruments to transport nucleic acids across the cell membrane into the cytoplasm or nucleus (130). Chemical methods employ positively charged transfection reagents (cationic polymers, calcium phosphate, cationic lipids, or cationic amino acids) for the transport across the cell membrane (130). The positively charged transfection reagents form complexes with the negatively charged siRNA, building positively charged siRNA/transfection reagent complexes that are attracted to the negatively charged cell membrane (130). Endocytosis and phagocytosis are presumably involved in the passage of the siRNA/transfection reagent complexes through the cell membrane (130). However, the exact mechanism of the passage is unknown (130).

Transfections of Cav-1 siRNA into HUVEC by means of chemical methods has been described in various publications (84,95–101) and was, therefore, also employed in the present study. Sun et al. have employed Lipofectamine RNAiMAX to transfect HUVEC with Cav-1 siRNA (95); the same transfection reagent was employed in the present study. The authors investigated

the underlying mechanism of endothelial dysfunction caused by atheroprone (oscillatory) flow compared to atheroprotective (pulsatile) flow (95). Proteins in HUVEC exposed to atheroprone flow were analyzed and integrin alpha5 was the most significantly elevated protein in lipid rafts (95). Remarkably, a Cav-1 knock-down by siRNA transfection abrogated the integrin alpha5 translocation and activation induced by atheroprone flow (95). A preceding study employing Cav-1 siRNA transfection of HUVEC, investigated the role of Cav-1 in insulin and insulin-like growth factor 1 stimulated eNOS activity (100). A Cav-1 knock-down in HUVEC abrogated the insulin and insulin-like growth factor 1-induced eNOS phosphorylation (100).

In the present study protein levels of Cav-1 were reduced to 34.82% in Cav-1 siRNA transfected HUVEC compared to negative controls, indicating successful Cav-1 knock-down. Other publications reported Cav-1 levels remaining in a range between 10% and 33% after Cav-1 siRNA transfection: 10% Salani et al. (101), 19.8% Jiang et al. (84), 20% Beardsley et al. (99), 20% Repetto et al. (100), less than 33% van der Meer et al. (98).

The implementation of a permeability model in Cav-1 siRNA transfected endothelial cells was described by Liu et al. as well as Jiang et al. (84,131). Jiang et al. assessed permeability for albumin of monolayers formed by transfected HUVEC (84), while in the study by Liu et al. mouse brain microvascular endothelial cells were transfected and permeability was assessed by dextran flow-through (131). Further details about these studies will be given below.

### **4.3 Effects of helium on endothelial barrier function**

Previous experiments of our laboratory showed that helium treatment regulates the Cav-1/3 expressions in mice hearts (67). Within this study mice inhaled 70% helium which led to decreased levels of Cav-1/3 in the heart after 24 hours, while elevated levels were found in the serum of the animals (67). These findings led to the hypothesis that Cavs are secreted into the blood stream after helium inhalation. Increased amounts of Cav-3 in the serum after helium inhalation were confirmed in rats in a recent study by Flick et al. (83). However, the analysis of the membrane fraction of the area at risk in the study by Flick et al. showed increased levels of Cav-1/3 after helium inhalation which somehow contrasts with the findings in mice, where helium led to decreased levels of Cav-1/3 in hearts (67).

As the endothelium is the first target organ to get in contact with circulating blood, a following study of our laboratory translated the recent findings to the human endothelium. Within this study the effect of helium on the human endothelium was investigated in-vitro. Results for Cav-1 levels were in line with the findings in mice and showed that helium leads to decreased intracellular protein levels of Cav-1 and elevated extra-cellular levels (43). These findings

supported the hypothesis that Cav-1 might be released by the stimulus of helium. A translocation of Cav-1 in endothelial cells was also described for mouse brain microvascular endothelial cells (131). However, in these cells deprivation of oxygen and glucose led to a translocation of Cav-1 from the cytoskeleton to the cytosol (131). Even though this study did not show a translocation to the extra-cellular space, as shown for helium treatment, the findings still support the concept that different stimuli might translocate endothelial Cav-1.

Earlier findings of our laboratory (43) and a repetition of these experiments within this study showed decreased endothelial permeability for albumin after helium treatment, which is an indicator for improved endothelial barrier function (43). Since ischemia and reperfusion leads to a weakened barrier function of the endothelium and can result in increased vascular permeability and vascular leakage (6,12,13,128), the protective effect of helium treatment on the endothelium might be a key mechanism of organ protection by helium.

There is inconsistent experimental data regarding the effect of helium on endothelial function. An in-vivo study by Luchinetti et al. contrasts with our findings by not showing endothelial protection after helium inhalation in humans (132). Eight healthy male volunteers were exposed to 15 min of forearm I/R in a cross-over study design (132). Combined pre- and postconditioning by helium was applied by inhalation of 50% helium from 15 min before ischemia until 5 min after onset of reperfusion (132). Hyperemic reaction, assessed by venous occlusion plethysmography, was used as a marker of nitric oxide bioavailability and endothelial function (132). However, in this study the concentration of helium (50%) was relatively low compared to other studies and it cannot be ruled out that inhalation of helium in a concentration of 70% or higher might have provided endothelial protection (132). In the study by Smit et al. that demonstrated a protective effect of helium preconditioning on the human endothelium in-vivo, helium was inhaled in a considerably higher concentration of 79% (21). A further study by Du et al. demonstrated that preconditioning with 70% helium inhalation attenuated intestinal permeability after I/R in animals in-vivo (105), indicating a barrier protective effect of helium. However, this study evaluated the effect of helium on intestinal permeability and can, therefore, not be directly transferred to the endothelium.

The mean permeability of non-transfected, control gas treated HUVEC monolayers, assessed by FITC-BSA flow-through in 2 hours, was lower at 24 hours compared to 6 and 12 hours. These findings might be explained by the experimental set-up. As described in the methods section, the different timepoints (6, 12 and 24 hours) refer to the time span between helium or control gas treatment and the onset of assessing monolayer permeability by FITC-BSA flow-through in 2 hours. HUVEC monolayers used at the 24 hour timepoint were cultured on the microporous membranes for 18 hours longer compared to HUVEC monolayers used at the 6 hour timepoint and for 12 hours longer compared to HUVEC monolayers used at the 12 hour

timepoint. This might have led to a higher confluency and, thereby, less inter-endothelial space of the HUVEC monolayer, resulting in decreased FITC-BSA flow-through. However, this assumption does not explain why the permeability of Cav-1 siRNA transfected HUVEC monolayers increases over time independently of helium or control gas treatment. SiRNA transfection is cytotoxic and leads to cell death in HUVEC (133). This cytotoxicity might have led to increasing inter-endothelial gaps and therefore increasing FITC-BSA flow-through over time in Cav-1 siRNA transfected HUVEC monolayer.

In order to investigate whether Cav-1 is involved in the helium-mediated stabilization of the endothelial barrier, Cav-1 protein levels were significantly reduced by siRNA transfection. Utilizing a Cav-1 knock-down model, we showed that reduced protein levels of Cav-1 abrogated the protective effect of helium on the endothelial barrier function. These findings suggest a key role of Cav-1 in the pathway that finally leads to a decreased endothelial permeability after helium treatment.

The role of Cavs in anesthetic-induced organ protection is well described (68,82,86). Reports have shown that the Cav isoforms Cav-1 and 3 are critically involved in pharmacological conditioning by volatile anesthetics (68,82,86). Patel et al. have demonstrated that infarct size reduction induced by isoflurane preconditioning was abrogated in Cav-1-null mice (82). Experiments in Cav-3-null mice revealed that also the absence of Cav-3 abrogated the protective effect of the volatile anesthetic isoflurane on the infarct size reduction and, furthermore, abrogated the protective effect on troponin release (68). A similar study, published in 2015, recently confirmed the abrogation of the protective isoflurane effect in Cav-3 knock-out mice and additionally showed in wild type mice that pharmacological preconditioning with isoflurane led to increased Cav-3 levels in the mice hearts (86). These findings suggest a key role of Cavs in anesthetic-induced conditioning. The main difference between the present study and the mentioned preconditioning studies is that in the present study no ischemia model was applied and that purely the effect of helium on the endothelium was investigated.

Jiang et al. have recently shown that hypoxic trophoblast conditioned medium increased Cav-1 protein levels as well as the permeability for albumin of HUVEC monolayers (84). In the mentioned report, high cellular protein levels of Cav-1 were associated with high permeability for albumin (84). This correlation between Cav-1 levels and HUVEC permeability matches the findings of our laboratory that helium leads to decreased intra-cellular protein levels of Cav-1 and a decreased permeability for albumin of HUVEC monolayers (43). Jiang et al. have, furthermore, found that the increase in permeability induced by hypoxic trophoblast conditioned medium was abrogated by a Cav-1 knock-down in endothelial cells (84). These results are once more in line with our results, revealing that the helium-induced attenuation of permeability of HUVEC monolayers is abrogated by a Cav-1 knock-down.



In mouse brain microvascular endothelial cells deprivation of oxygen and glucose led to an increase in dextran flow-through measured after 2 hours, indicating a disruption of the endothelial barrier function (131). A Cav-1 knock-down by siRNA significantly reduced the permeability of the endothelial monolayer treated with a deprivation of oxygen and glucose (131), indicating that Cav-1 is also involved in regulating endothelial barrier function in response to other stimuli than helium. The same study further revealed that the TJ protein claudin-5 is regulated by an interaction with Cav-1 (131). Deprivation of oxygen and glucose in endothelial cells led to increased protein levels of claudin-5 in the cytosolic, membrane and organelle fraction and a decrease in the levels in the actin cytoskeleton fraction (131). This dissociation of claudin-5 from the cytosolic framework was abrogated in Cav-1 siRNA transfected cells (131). These findings of Liu et al. demonstrated that Cav-1 is involved in the regulation of the TJ protein claudin-5 in endothelial cells (131). However, the mentioned study was performed using a different endothelial cell line and a different treatment was applied compared to the present study. Nevertheless, by demonstrating the association of Cav-1 to the regulation of a protein of the inter-endothelial junction, this study points in the same direction as the present study.

Western blot analyses revealed that 20 min of helium treatment led to significantly elevated protein levels of VE-cadherin and Cx43 in HUVEC and a Cav-1 protein knock-down completely abrogated this effect.

VE-cadherin, the main molecule to form AJ, is essential in maintaining endothelial barrier function (47,54). Recent studies have shown that VE-cadherin is also involved in pharmacological conditioning: Anesthetic-induced preconditioning by sevoflurane preserves endothelial barrier dysfunction by maintaining VE-cadherin levels (72), angiotensin-1 preserves vascular leakage through a regulation of VE-cadherin phosphorylation (73) and the herbal medicine extract tongxinluo, which is also known to induce preconditioning, leads to an upregulation of VE-cadherin (74).

Cx43, the protein forming GJ, enables inter-cellular communication in endothelial cells and, thereby, regulates vascular tone and endothelial function (75). Alterations in the Cx43 levels are associated with myocardial diseases such as heart failure, hypertrophic cardiomyopathy and ischemia (75). Cx43 is critically involved in ischemic preconditioning of the heart (87,134,135) and experiments have shown that the organ protecting effect of ischemic preconditioning was abrogated in Cx43 deficient mice (87) as well as after using the pharmacological GJ uncoupler heptanol (134). Furthermore, augmented levels of Cx43 were found in ischemically preconditioned rabbit hearts (135).

As the mentioned pharmacological and ischemic conditioning agents lead to elevated levels of VE-cadherin and/or Cx43, these findings are in line with our finding that helium leads to

augmented protein levels of VE-cadherin and Cx43 in the endothelium. Since VE-cadherin maintains endothelial barrier function (47,54,136) and Cx43 is known to modulate endothelial function (75), the increased levels of these junctional molecules could be involved in the underlying mechanism of helium-mediated attenuation of the endothelial barrier function.

Findings of a study investigating the effect of the ginkgo leaf extract ginkgolide B in HUVEC, stand in contrast to our findings (137). This study showed that ginkgolide B leads to decreased expressions of the junctional molecules VE-cadherin and Cx43 and decreased monocyte transigrations, indicating enhanced endothelial barrier function (137). The same study further showed decreased monocyte migrations after knock-down of VE-cadherin and Cx43 by siRNA. Experiments of our laboratory, however, showed that helium leads to a decreased permeability of the endothelium (43) and increased protein levels of VE-cadherin and Cx43 after helium treatment. The contrary correlation between the VE-cadherin or Cx43 levels and endothelial permeability might be explained by the different experimental set-ups used to assess permeability. In the studies performed in our laboratory, permeability was measured by albumin flow-through in 2 hours (43), whereas in the study testing the effect of ginkgolide B, a monocyte flow-through in 24 hours was used to evaluate permeability (137). Monocytes can actively secrete proteases and thereby have a direct influence on cell-cell adhesions (138). The active secretion of proteases, the difference in the size of FITC-BSA and monocytes as well as the difference in the timespan (2 hours for FITC-BSA vs. 24 hours for monocytes) might explain the deviating findings.

We demonstrated that Cav-1 plays a possible role in helium-mediated attenuation of endothelial permeability and since an interaction of Cav-1 with VE-cadherin (139,140) and Cx43 (79,141–144) has been described in literature, this prompted us to investigate whether Cav-1 is also involved in the helium-induced increase of the protein levels of VE-cadherin and Cx43.

Our results showed that helium significantly increased the VE-cadherin and Cx43 levels in wild type HUVEC. However, this effect was completely abrogated in a Cav-1 knock-down model of the endothelium. These findings enabled us to conclude that Cav-1 is essential in mediating the helium-induced alterations in the junctional molecules VE-cadherin and Cx43, which might finally lead to a decreased vascular permeability.

A study by Kronstein et al. supports our results, showing that Cav-1 plays a key role in VE-cadherin dependent cell adhesion and the regulation of the barrier function (139). The authors determined that Cav-1 forms complexes with VE-cadherin and catenin in HUVEC and demonstrated that this association is essential for the decreased barrier function in response to the pro-inflammatory mediator thrombin (139). Our findings are further supported by the

findings that Cx43 binds to the scaffolding domain of caveolin (CSD) and might, therefore, be regulated by Cav-1 (79).

Interestingly, in Cav-1 siRNA transfected HUVEC we found a time dependent decrease in protein levels of Cx43 independently of helium or control gas treatment. To our knowledge this time dependent decrease of the Cx43 protein levels in Cav-1 siRNA transfected HUVEC has not been described before. Cx43 has a relatively short half-life of a few hours (145), most likely to enable a regulation of the number of GJs in response to physiological requirements (146). Since Cx43 is regulated by Cav-1 (79,142), the time dependent decrease in the protein levels of Cx43 might be explained by lower rates of protein synthesis in absence of Cav-1. This in combination with the relatively short half-life of Cx43 could lead to a time dependent decrease of the protein levels of Cx43 in Cav-1 knock-down cells. Langlois et al. have transfected rat epidermal keratinocytes with Cav-1 siRNA to reduce Cav-1 protein levels; the expression of Cx43 were not affected by the Cav-1 knock-down (142). However, the authors did not assess Cx43 protein levels at different timepoints. Therefore, a time dependent decrease could not be ruled out.

Taken together, our results in combination with current literature suggest that helium attenuates endothelial permeability by increasing protein levels of VE-cadherin and Cx43, most likely through an interaction with Cav-1.

#### 4.4 Limitations of the study

There are several limitations of this study that need to be considered:

(i) This study was performed using an in-vitro model of the human endothelium. Therefore, the results cannot be directly transferred to the in-vivo situation. For a possible translation of this data to a clinical setting, further in-vivo research is necessary.

(ii) No flow or shear stress was applied to endothelial cells in the present study. It is known that endothelial cells are sensitive to flow and shear stress and caveolae are known to sense flow, shear stress and stretch and control membrane tension (98,147,148). Therefore, it cannot be excluded that the absence of flow, shear stress and stretch has influenced the results.

(iii) In the present study siRNA was used to accomplish a functional knock-down of Cav-1. This technique was used in HUVEC in various publications (84,95–101). However, there are some limitations to this technique. Although siRNA for Cav-1 was used to induce a post-transcriptional gene silencing, the Cav-1 protein was not completely absent: in the present study 34.82% of the protein levels were shown to remain in the cell and might have influenced the results.

(iv) It cannot be excluded that off-target effects of the siRNA have influenced the results. Furthermore, the use of a transfection reagent (133) or “serum starving” prior to the transfection might have influenced the results.

(v) The final objective of the present study in combination with previous and future studies is the use of helium to prevent organs from the I/R-injury in various clinical settings. In the present study, however, only the effect of helium on the human endothelium was investigated and no damage model was applied to induce an I/R-injury. Further in-vitro and in-vivo studies have to reveal whether helium pre- and postconditioning is potent enough to maintain the endothelial barrier function after an I/R-injury.

#### 4.5 Conclusion

Our findings suggest that Cav-1 is involved in (i) the helium-mediated attenuation of permeability of HUVEC monolayers and in (ii) the helium-mediated increase in protein expression of the junctional molecules VE-cadherin and Cx43.

This study in combination with further translational trials could help to implement helium into organ protective strategies in humans.

## 4.6 Further perspectives

There are numerous clinical situations of ischemia and reperfusion in which patients could profit from pre- or postconditioning by the non-anesthetic noble gas helium. Possible clinical applications of helium preconditioning are predictable events of ischemia and reperfusion, including situations like heart-, vascular- or transplantation surgery. Possible clinical applications for helium postconditioning include more unpredictable events like infarctions of the coronaries (acute myocardial infarction, AMI), infarctions of intestinal blood vessels (acute mesenteric ischemia) or any other type of arterial occlusion (e.g. acute peripheral arterial occlusion).

Based on the results of this study further scientific questions arose:

(i) Results of the present study revealed that helium attenuates permeability of HUVEC monolayers possibly via Cav-1. Since the final perspective is the use of helium to protect organs in various clinical I/R situation, further animal experiments should be performed in combination with the application of an ischemia model.

(ii) Since helium leads to decreased intra-cellular protein levels of Cav-1 and elevated extra-cellular levels as well as a strengthened endothelial barrier function that is abrogated in a Cav-1 knock-down model, further studies should investigate whether externally added Cav-1 can mimic the protective effect of helium on the endothelial barrier function.

(iii) In the present study a Cav-1 knock-down model in HUVEC was used. Further experiments have to reveal in what way a knock-down of other proteins like eNOS, also known to be involved in helium-induced organ protection (32), would affect the helium-induced attenuation of permeability of HUVEC monolayers.

(iv) Patients who could profit from the protective effects of helium conditioning in a clinical setting are patients undergoing coronary artery bypass graft surgery for example (21). These patients, however, are often of advanced age and have comorbidities, such as hypertension, diabetes mellitus and atherosclerosis (149). Therefore, further studies in cell models, animal models and humans are needed to investigate the protective effect of helium on the endothelium also in combination with an advanced age and comorbidities.

## 5. Summary

### **Background:**

Protective effects of ischemic conditioning on the heart have been described in numerous publications. Recent studies suggested that the noble gas helium is also able to protect the heart against ischemia/reperfusion injury (I/R-injury). Mechanistically, helium can mimic the early and late effects of ischemic preconditioning and as helium lacks anesthetic and other side effects, it might be a potential conditioning agent. We have recently shown that (i) helium attenuates the permeability of monolayers of human umbilical vein endothelial cells (HUVEC) and that (ii) helium leads to decreased intra-cellular protein levels of caveolin-1 (Cav-1). Nevertheless, the underlying cellular and molecular mechanisms and the precise role of Cav-1 in helium-mediated regulation of endothelial barrier integrity are still unclear. Employing in-vitro cultured primary HUVEC and small interfering RNA (siRNA) transfection experiments, we therefore investigated (i) whether Cav-1 is involved in helium-mediated stabilization of the endothelial barrier and (ii) whether the expression of the key junctional molecules vascular endothelial - cadherin (VE-cadherin) and connexin-43 (Cx43) is regulated by helium and might be involved in the effects of helium on endothelial barrier function.

### **Methods:**

HUVEC were obtained from umbilical cords, pooled and grown to confluency. Cells were either exposed to 20 min of helium (5% CO<sub>2</sub>, 25% O<sub>2</sub>, 70% He) or control gas (5% CO<sub>2</sub>, 25% O<sub>2</sub>, 70% N<sub>2</sub>) in a specialized gas chamber. Permeability was measured using confluent monolayers of HUVEC on microporous membranes and fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA). To investigate if the effects of helium on the endothelial barrier function are related to Cav-1, HUVEC were transfected with Cav-1 siRNA prior to helium application in order to knock down the Cav-1 expression. Expression of VE-cadherin and Cx43 was analyzed by western blotting in non-transfected and Cav-1 siRNA transfected HUVEC.

### **Results:**

HUVEC transfected with siRNA for Cav-1 showed significantly lower amounts of Cav-1 protein compared to controls (34.82±20.07% vs. 100.00%, p<0.05), confirming successful knock-down of Cav-1.

While helium attenuated the permeability of HUVEC monolayers in non-transfected HUVEC (p<0.05), effects of helium on cell permeability were abrogated in Cav-1 siRNA transfected

HUVEC ( $p > 0.05$ ), suggesting a key role of Cav-1 in endothelial permeability. In the helium group, protein analyses in cell lysates showed a significantly higher expression of VE-cadherin and Cx43 at 6 and 12 hours (VE-cadherin in helium treated cells at 6h:  $1.19 \pm 0.28$  and 12h:  $1.14 \pm 0.37$ . VE-cadherin in control cells at 6h:  $0.87 \pm 0.11$  and 12h:  $0.85 \pm 0.14$ . Cx43 in helium treated cells at 6h:  $1.31 \pm 0.39$  and 12h:  $1.27 \pm 0.37$ . Cx43 in control cells at 6h:  $0.90 \pm 0.32$  and 12h:  $0.82 \pm 0.26$ . Helium 6h and 12h vs. control 6h and 12h:  $p < 0.05$  for all groups). Transfecting HUVEC with Cav-1 siRNA abolished the effect of helium on VE-cadherin and Cx43 expression ( $p > 0.05$  for all time points), pointing towards a role of Cav-1 in the helium-induced alteration of junctional molecule expression.

### **Conclusion:**

Our findings suggest that Cav-1 is involved in (i) the helium-mediated attenuation of HUVEC permeability and in (ii) the helium-mediated increase in the expression of the junctional molecules VE-cadherin and Cx43. This study in combination with further translational trials could help to implement helium into organ protective strategies in humans.

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## 7. Appendix

### 7.1 List of manufacturers

Abcam, UK; [www.abcam.com](http://www.abcam.com)

Ambion, Germany; [www.thermofisher.com](http://www.thermofisher.com)

Appli-Chem, Germany; [www.applichem.com](http://www.applichem.com)

BD Diagnostic system, USA; [www.bdbiosciences.com](http://www.bdbiosciences.com)

Bio-Rad, USA; [www.bio-rad.com](http://www.bio-rad.com)

BioTek, USA; [www.biotek.com](http://www.biotek.com)

BioTek Instruments, USA; [www.biotek.com](http://www.biotek.com)

Cell Signaling Technology, USA; [www.cellsignal.com](http://www.cellsignal.com)

Corning Costar, USA; [www.corning.com](http://www.corning.com)

Corning Life sciences, USA; [www.corning.com](http://www.corning.com)

Datex, USA; [www.dremed.com](http://www.dremed.com)

Eppendorf, Germany; [www.eppendorf.com](http://www.eppendorf.com)

Fisher Scientific, USA; [www.fishersci.com](http://www.fishersci.com)

Greiner Bio One, Austria; [www.greinerbioone.com](http://www.greinerbioone.com)

Invitrogen, USA; [www.lifetechnologies.com](http://www.lifetechnologies.com)

Leica Microsystems, Germany; [www.leica-microsystems.com](http://www.leica-microsystems.com)

LI-COR Biosciences, The Netherlands; [www.westburg.eu](http://www.westburg.eu)

Life technologies, USA; [www.thermofisher.com](http://www.thermofisher.com)

Linde Gas Benelux, The Netherlands; [www.linde-gas.nl](http://www.linde-gas.nl)

Merck, Germany; [www.merck.de](http://www.merck.de)

MERCK Millipore, Germany; [www.merckmillipore.de](http://www.merckmillipore.de)

MFG by Henry Troemner, USA; [www.troemner.com](http://www.troemner.com)

PAA, Germany; [www.gelifesciences.com](http://www.gelifesciences.com)

Packard BioScience, USA; [www.packardbioscience.com](http://www.packardbioscience.com)

PAN, Germany; [www.pan-biotech.com](http://www.pan-biotech.com)

Promocell, Germany; [www.promocell.com](http://www.promocell.com)

Roche, Switzerland; [www.roche-diagnostics.ch](http://www.roche-diagnostics.ch)

Sigma-Aldrich, The Netherlands; [www.sigmaaldrich.com](http://www.sigmaaldrich.com)

Telstar Laboratory Equipment, The Netherlands; [www.telstar-lifesciences.com](http://www.telstar-lifesciences.com)

Thermo Scientific, USA; [www.thermoscientific.com](http://www.thermoscientific.com)

## 7.2 Molecular formulas

$C_2H_6OS$	2-Sulfanylethan-1-ol (2-Mercaptoethanol)
$C_7H_{15}NO_4S$	3-(N-morpholino)propanesulfonic acid (MOPS)
$CaCl_2$	Calcium chloride
$CO_2$	Carbon dioxide
$CuSO_4 \times H_2O$	Copper(II) sulfate
He	Helium
$KH_2PO_4$	Potassium dihydroxidodioxidophosphate
$KNaC_4H_4O_6 \times 4H_2O$	KNa-Tartrate
$MgCl_2$	Magnesium chloride
$N_2$	Nitrogen
$Na_2CO_3$	Sodium carbonate
$Na_2HPO_4$	Sodium hydrogen phosphate
$Na_3VO_4$	Sodium vanadate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
$O_2$	Oxygen

## 8. Cooperation

In cooperation with:

The Laboratory of Experimental Intensive Care and Anesthesiology (L.E.I.C.A.)

Department of Anesthesiology

(Chair: Prof. Dr. med. Dr. rer. nat. Markus W. Hollmann, D.E.A.A.)

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### School education

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Cerrahpasa Faculty of Medicine, Istanbul, Turkey  
11/2014 – 03/2015 Clinical rotation in Surgery, Hospital San Gabriel, Teaching  
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since 10/2018 Resident, University Hospital Femme-Mère-Enfant (HFME) of  
Lyon, Department of Pediatric Orthopedic Surgery, Bron,  
France



## Publications

- 03/2017 Nederlof R\*, Guerel-Gurevin E\*, Eerbeek O, Xie C, Deijs GS, Konkel M, Hu J, Weber NC, Schumacher CA, Baartscheer A, Mik EG, Hollmann MW, Akar FG, Zuurbier CJ. Reducing mitochondrial bound hexokinase II mediates transition from non-injurious into injurious ischemia/reperfusion of the intact heart. \*Both authors contributed equally to the present publication, J Physiol Biochem. (2017);73(3):323–33.
- 04/2017 Konkel M, Smit KF, Hollmann MW, Patel HH, Albrecht M, Weber NC. Helium mediated regulation of endothelial permeability: Involvement of connexin 43, VE-cadherin and caveolin-1. FASEB J. (2017);31:1065.5.  
Poster presentation: EB2017, Experimental Biology Congress 2017, Chicago, IL, USA.
- 03/2018 Smit KF\*, Konkel M\*, Kerindongo R, Landau MA, Zuurbier CJ, Hollmann MW, Preckel B, Nieuwland R, Albrecht M, Weber NC. Helium alters the cytoskeleton and decreases permeability in endothelial cells cultured in vitro through a pathway involving caveolin-1. \*Both authors contributed equally to the present publication, Sci Rep. (2018);8(1):4768.
- 06/2019 Smit K, Oei G, Konkel M, Augustijn Q, Hollmann MW, Preckel B, Patel HH, Weber NC. Plasma from volunteers breathing helium reduces hypoxia induced cell damage in human endothelial cells - Mechanisms of remote protection against hypoxia by helium. Cardiovasc Drugs Ther. (2019);33(3):297–306

## 11. Publications

Parts of this work have been published:

Published in:

The FASEB Journal; 2017

Title:

Helium Mediated Regulation of Endothelial Permeability: Involvement of Connexin 43, VE-Cadherin and Caveolin-1

Published in:

Scientific Reports; 2018

Title:

Helium alters the cytoskeleton and decreases permeability in endothelial cells cultured in vitro through a pathway involving Caveolin-1