Aus der

Berufsgenossenschaftlichen Unfallklinik

Klinik für Hand-, Plastische, Rekonstruktive und
Verbrennungschirurgie an der Universität Tübingen

The Influence of Saturation-Guided Protocols on the Remote Ischemic Conditioning Effect

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von

Mayer, Konrad Frederik

2023

Dekan:	Professor Dr. B. Pichler
1. Berichterstatter:	Professor Dr. J. Kolbenschlag
2. Berichterstatter:	Professor Dr. B. Balletshofer
Tag der Disputation:	04.10.2023

Dedication

My family, Annemarie, Manfred, and friends.

Table of Contents

List of figures

List of tables

List of abbreviations

AMP	adenosine monophosphate
ATL	antero-lateral thigh
ATP	adenosine triphosphate
BAK	Bcl-2-antagonist/killer
BAX	Bcl-2-associated X protein
BMI	body mass index
Ca^{2+}	calcium
CASP3	caspase-3
cGMP	cyclic guanosine monophosphate
CVC	cutaneous vascular conductance
CYC	cytochrome C
DNA	deoxyribonucleic acid
H^{+}	hydrogen
I1	ischemia 1
I2	ischemia 2
I3	ischemia 3
IRI	ischemia-reperfusion injury
K^{+}	potassium
MLKL	mixed-lineage kinase domain-like pseudokinase
n/a	not applicable
Na^{+}	sodium
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NCX	Na^{+}/Ca^{2+} exchanger
NHE	Na^{+}/H^{+} exchanger
NKA	Na^{+}/K^{+} ATPase
NO	nitric oxide
NPRS	Numeric Pain Rating Scale
O2C	oxygen to see
OMM	outer mitochondrial membrane
OR	operating room
P1	protocol 1
P2	protocol 2

P3	protocol 3
P4	protocol 4
pH	potential of hydrogen
PMCA	plasma membrane Ca^{2+}-ATPase
PS	phosphatidylserin
PY	pack year
R1	reperfusion 1
R2	reperfusion 2
R3	reperfusion 3
rHb	relative hemoglobin
RIC	remote ischemic conditioning
RIPK1	receptor-interacting protein kinase-1
RIPK3	receptor-interacting protein kinase-3
SO2	oxygen saturation

1. Introduction

Plastic surgery is a specialty regularly consulted in cases assigned to other departments since it deals with and treats tissue as such. Not only do plastic surgeons treat primary post-traumatic defects, but because the surgery itself is a trauma for the body's tissue, they are called upon by other specialties to secondarily treat wound healing disorders that can occur after every procedure. More often than not, the tissue of interest is the connective and epithelial tissues of the skin. A widespread and necessary procedure is covering skin defects to reinstate an intact integument. Only a non-defective skin layer, with its accompanying microbial and chemical properties, can function as the necessary boundary of human bodies to the outside world. Unfortunately, some defects are so significant that wound healing by primary or secondary intention is insufficient. Wound healing by primary intention occurs when wound edges heal together without forming granulation tissue, for example, when adjacent wound edges are sutured. Wound healing by secondary intention is the body's process of cutaneous healing when primary wound healing is impossible. It entails the formation of granulation tissue and wound contraction. When these methods are insufficient, it is necessary to look further up the reconstructive ladder, or as some authors would say: "somewhere else in the reconstructive matrix" (1).

The selected approach ideally not only covers the defect sufficiently but also restores normal form and function. Surgeons can choose from a manifold of options, each accompanied by its benefits and drawbacks. The free flap is the best option in many cases, especially for more significant defects. For this reason, there has been an increase in the popularity of various free flaps. A 2002 study published by Wei et al. shows the versatility and broad application of the anterolateral thigh flap (ALT) (2). Furthermore, the study demonstrated the level of confidence in the free flap technique to the extent that the majority of failed flaps were replaced by another free flap (2). In 2016 Chaput et al. showed that especially the younger generation of microvascular surgeons prefers to use the free anterolateral thigh flap (3). It is, therefore, necessary to investigate methods that can increase the survival rate of these flaps and thus reduce failure and complication rates. Not only is failure and following salvage of a free flap additional time in the operating room (OR) and an increased risk for the patient but also a financial strain on the healthcare system and the patient (4). Setala et

al. quantified the financial cost of salvaging a failed flap and concluded that it was almost double the original cost (4).

In every procedure involving a flap, one of the main goals is to avoid flap failure. Failure is often attributed to microcirculatory complications, most of which are venous, mainly venous insufficiency (5). Common complications are partial flap or tip necrosis and, in some cases, complete flap loss. Studies find necrosis can occur in up to 36 % of patients (6). There have been countless attempts to find a solution to this problem. Many of these have proven ineffective in animal testing stages (7, 8). Others that have proven more effective, such as extracorporeal shock wave therapy or preconditioning the flap with hypothermia, have not found a widespread clinical application (9, 10). The search continues for a feasible clinically applied method to significantly increase flap survival. The method has to be effective, non-invasive, cheap and ideally easy to administer to be clinically feasible.

Remote Ischemic Conditioning (RIC) has the potential to fulfill all the criteria above. It has been proven to enhance systemic cutaneous microcirculation and thus harbors expectations to increase flap survival in the clinical setting (11). Previous studies have shown the RIC effect's effectiveness in flap survival (12). Although the exact mechanisms of the RIC effect are still the subject of research, it ultimately aims at protecting tissue from the effects of ischemia and ischemia-reperfusion injury.

At the heart of increasing flap survival rate lies the question of establishing flap-supporting blood flow after an ischemic event. Ischemia harms tissue, as does the following reperfusion coining the term ischemia-reperfusion injury (IRI). Turning away from plastic surgery and looking at other specialties, many address similar problems, such as cardiologists dealing with heart attacks due to occlusion of coronary arteries and neurologists dealing with strokes due to ischemia of brain areas. The most similar problems to that of the plastic surgeon are perhaps faced by transplant surgeons, such as during kidney and liver transplants. Covering a defect with a free flap is essentially an autologous transplant. These specialties have pioneered the principles of ischemic conditioning to avert the disastrous effects of ischemia and ischemia-reperfusion injury. Before diving deeper into what happens during ischemia and reperfusion a paragraph looking into the subject affected by these should be inserted.

1.1. Skin

As mentioned before, one main reason for the consultation of a plastic surgeon is a skin or soft tissue defect that requires more attention than primary wound closure. Skin is the human's largest organ with 1.5 to 2 m^2 of surface area and accounting for approximately 15 % of the body's weight (13). It serves as the body's barrier and boundary and as an organ that plays a significant role in signaling the body's current status. Its barrier function is, on the one hand, mechanical on the other hand, skin is also home to a wide-ranging microbiome that inhabits the various parts of the human skin. The microbiome usually consists of resident microorganisms that are not harmful and, in some cases, through a symbiotic relationship beneficial to the human body (14). The individual's microbiome depends on various factors such as sex, occupation and geographical location (14). Furthermore, pathobiological conditions such as an existing diabetic condition influence the microbiome's composition (14). While many exogenous factors themselves and via the microbiome affect skin functionality, this project focuses on manipulating blood circulation and observing its effects.

1.1.1. Skin Anatomy

The skin consists of three different layers. From superficial to deep, the epidermis is above the dermis, followed by the subcutaneous fat (15). Each layer plays a role in the overall function of the skin.

The epidermis consists of many layers as well. The deepest layer, the stratum basale, consists of multipotent stem cells that mainly produce keratinocytes that travel upwards. On their journey, they become part of the strata spinosum and corneum. Eventually, they fall off after reaching the most superficial layer of the stratum corneum. The epidermis is a dynamic, constantly self-replenishing layer of keratinocytes that fully replaces itself every two weeks (16). Furthermore, it is home to some cells that belong to the body's immune system, such as the Langerhans cells, and some sensory cells, i.e., the Merkel cells (15).

Deep to the epidermis the dermis can be found. Together with the epidermis, it forms the cutis. The dermis consists mainly of connective tissue that can be further split into the papillary stratum and the reticular stratum, although there is

no boundary between these. While the papillary stratum is rather loose and thin, the reticular stratum is dense and thick (15). The dermis's primary function is mechanical as its connective tissues provide the skin with the mechanical resistance and flexibility it needs to border a moving organism such as the human body to the outside world. However, the dermis's microvasculature is of utmost importance to this project. In addition, it contains structures such as sweat glands, hair follicles, and sensory neurons.

Beneath the dermis lies the subcutaneous fat, also known as the hypodermis or subcutis. While it also contains important vasculature in its septae, it mostly consists of adipose lobules (15). Its primary purpose is isolation. Like the dermis, it also contains sensory neurons and hair follicles.

The human skin's general structural anatomy can vary depending on its region on the body. For example, the epidermis on the fingertips contains far more sensory bodies than the skin on the back. On the other hand, the skin of the palms and soles does not contain hair follicles. These differences must be considered when moving skin from one area to another. The survival of skin after being moved to a different region of the body depends on the reaction and function of the microvasculature of the skin flap. Below, a more in-depth look is taken at the aforementioned system.

1.1.2. Microvasculature of the skin

When plastic surgeons autologously transplant cutaneous flaps, one of the main reasons the procedure fails is an insufficient microvascular system, which leads to ischemia and nutrient deprivation, or if only temporary, to ischemia-reperfusion-injury (17). Before we dive deeper into the heart of this project which investigates procedures that manipulate this microvascular supply, it is important to understand its anatomy.

Cutaneous microvasculature can be structured into two horizontal plexuses (18). While the deep plexus can be found in the dermal reticular stratum near the dermal-subcutis border, the superficial plexus is located in the dermal papillary stratum. The dermal capillary loops arise from this superficial horizontal plexus and lie in the dermal papillae (18).

The deep plexus is fed by perforating vessels from underlying tissues and muscles. It provides nutrients to the dermal reticular stratum and feeds into the

superficial plexus. The connecting vessels give off branches to local hair follicles and other dermal structures. In the dermal papillary stratum, the superficial plexus with its capillary loops nurture itself and, by diffusion, the epidermis above. While the cutaneous microvasculature is responsible for the cutaneous blood supply, it also plays a crucial role in the body's thermoregulation, immune system, and wound healing.

1.2. Microcirculation

End-organ microvasculature is the final destination of the cardiovascular system (19). This is where the oxygen and nutrients are transferred to the end-organ cells that require these to function correctly. The microvasculature can be separated into three consecutive parts. As the blood flows, arterioles feed capillaries and are drained by venules. In addition, there are terminal lymphatic vessels.

The blood vessel segments can be differentiated by looking at the structure and size of the vessels. A common structural element of all segments is the lining with endothelial cells (19). The outside diameter of the arterioles can vary from 17 to 26 μm (18). This endothelial tube is typically surrounded by two layers of smooth muscle cells (18). Once the vessels are less than 15 μm in diameter the smooth muscle cells are gradually replaced by pericytes (18). Pericytes do not have the contractile capabilities that smooth muscle cells have. Once the vessels have an outside diameter range of 10 to 12 μm they are classified as capillaries (18). The following segment is that of the post-capillary venules. 80 % of their endothelial tube is surrounded by pericytes (18). As the architectural compositions are different, each segment's role also differs. The endothelial cells are the primary regulators of the microvasculature function. The substances the endothelial cells produce can affect the amount of coagulation, inflammation, and blood flow. Creating an almost impermeable tube also functions as the controlling unit over transport from blood to tissue.

When it comes to affecting blood flow, there are a variety of substances the endothelium produces. One essential regulator regarding microcirculation is nitric oxide (NO) (20). Other vaso-modulating substances that the endothelium produces are prostaglandins which are enzymatically produced from arachidonic acid. These prostaglandins can have both vasoconstrictive and vasodilatory effects.

While an underlying sympathetic neuronal firing causes vasoconstriction, NO counters that with its vasodilatory effect. NO causes a rise in cyclic guanosine monophosphate (cGMP), which leads to vasodilation and inhibits platelet aggregation (20). Thus, NO results in increased blood flow. Many substances essential to adequate microcirculation are produced in the local endothelium. Therefore, any deviance from normal endothelial function can have detrimental effects on microcirculation. While many diseases can alter the endothelium, i.e., diabetes and atherosclerosis, ischemia and reperfusion have also been shown to harm regular endothelium function (20). As autologous transplantation goes hand in hand with ischemia and reperfusion, it is necessary to understand the effects of these on the endothelium.

1.3. Ischemia and Ischemia-Reperfusion Injury

In all fields of surgery, ischemia and reperfusion are omnipresent topics relevant to every surgery's outcome. Ischemia describes a drastic reduction of blood flow to the affected tissue, resulting in an insufficient supply of oxygen and nutrients. A steady blood supply is essential to the cell and tissue's survival. The absence of this supply, in other words, an insufficient supply of oxygen and nutrients, results in ischemia-derived cell damage. Reperfusion describes the ischemic state's cessation by reestablishing blood flow to the tissue. Reperfusion forms the central pillar of treating ischemia; however, it is also the cause of exacerbation of ischemia-related damage. This led to the coining of the term ischemia-reperfusion injury (IRI). IRI can result in different types of cell death. The following three types of cell death are the prevalent modes of cell death caused by ischemia and ischemia-reperfusion injury.

1.3.1. Cell Death through Ischemia and Ischemia-Reperfusion Injury

Generally speaking, there are two ways by which cells can die. They can undergo Programmed Cell Death (PCD) or die unphysiologically via necrosis. Programmed cell death includes necroptosis, apoptosis, and autophagy. While the nature of autophagy is beneficial to tissue and development as it mainly selects damaged or pathogenic properties, it may play a role in IRI when it reaches an uncontrolled state (21).

1.3.1.1. Necrosis

Necrosis is the most common form of non-regulated uncontrolled cell death. While it used to be presumed the leading cause of cell death resulting from IRI, it no longer plays a sole significant role. Current research suggests that necroptosis is a major player in IRI, the aforementioned controlled form of necrosis. Necrosis itself results from the inability of a cell to cope with the physiological damage resulting from stress created by its environment. Necrosis is characterized by cell membrane continuity loss and consequent leakage of cellular matrix. There is no defined intracellular messaging pathway that regulates necrosis, despite multiple partial cascades contributing to necrosis (22). Cells undergoing necrosis characteristically undergo swelling as a whole or only their organelles. The tensioned membranes rupture and the cells disintegrate, culminating in an inflammatory response. While necrotic cells show characteristics similar to other more regulated forms of cell death, the main characteristic of necrosis is its procession's uncontrollable and unregulated nature.

1.3.1.2. Necroptosis

As mentioned above, research is beginning to suggest that there are forms of necrosis mediated by controllable signal pathways and, thus, no longer an uncontrollable death. Therefore the need for new terms arose, and one such pathway that has been proven to play a role in IRI is necroptosis (23). Linkermann et al. and other authors such as Galluzzi et al. have defined necroptosis as a necrotic cell death mediated by receptor-interacting protein kinase-1 and 3 (RIPK1 and RIPK3) as well as mixed-lineage kinase domain-like protein (MLKL) (24, 25). RIPK1 hereby activates RIPK3 by physically interacting and forming the necrosome to which MLKL is a substrate (26). Activated MLKL is responsible for both an influx in Calcium-ions (Ca^{2+}) as well as phosphatidylserine (PS) exposure which results in cell death (27).

1.3.1.3. Apoptosis

The most significant pathway of apoptosis resulting from IRI, intrinsic apoptosis, is due to cytochrome-C (CYC) leakage from mitochondria. This is induced by BAX and BAK proteins of the BCL2 family, which form pores across the outer mitochondrial membrane (OMM) (24). These pores cause the OMM to be

permeable and for CYC leakage from the intermembrane space into the cytosol. Loss of CYC, an electron transporter, in the mitochondria results in loss of the mitochondrial membrane potential, which is necessary for the respiratory chain of the cell that produces adenosine triphosphate (ATP) to function. ATP production, therefore, diminishes. In the cytosol, CYC initiates the forming and is part of the apoptosome, which via mediators, activates so called executioner caspases, including CASP3. This results in cell demising deoxyribonucleic acid (DNA) fragmentation and a cell's reduced ability to communicate with its environment in the form of, e.g., disrupted protein import (24). Not only are the internal workings of the cell malfunctioning, but CASP3 also activates enzymes known as scramblases that are usually in balance with flippases. This causes phosphatidylserine exposure, an "eat-me" signal characteristic of apoptotic cells, and leads to phagotization by immune cells (28).

1.3.1.4. Autophagy

Autophagy is the fourth and most controversial form of cell death IRI causes. The process of autophagy is not malevolent but rather benevolent. Its fundamentals have been clear for a long time. Autophagy occurs in two main instances. The first is damaged or malfunctioning organelles, e.g., mitochondria, and the second is when the cell is starving and needs nutrients, e.g., fatty acids. The process of autophagy generally describes the envelopment of the cell's own structures with a bilipid layer creating an autophagosome that then fuses with a lysosome, after which the contents are degraded.

Autophagy can be induced by energy (ATP) deficiency. Hydrolysis of ATP releases energy and results in adenosine monophosphate (AMP). When a cell is in an energy-deprived state, it has a high AMP/ATP ratio. This imbalance towards AMP activates the AMP-activated protein kinase (AMPK) and thus functions as a metabolic sensor of the cell (29). Inoki et al. found that AMPK phosphorylates TSC2 and enhances its activity when the cell is in an energy-deprived state, causing inhibition of mTOR activity and thus leading to autophagy (30).

Hypoxia is also capable of inducing autophagic cell death. In a study, Azad et al. were able to show that by inhibiting autophagy, hypoxia-induced cell death was reduced while hypoxia upregulates BNIP3 and its overexpression led to an increase in autophagy (31). A specific form of autophagy has been discovered,

called autosis, that has, amongst other causes, been linked to hypoxia. It is regulated by the sodium (Na^+)-potassium (K^+) ATPase (NKA), shows increased numbers of autophagosomes, and uniquely presents with nuclear convolution and perinuclear swelling (32). There is a wide variety of autophagy-triggering effects. The research still needs to be done in this field, and it remains a controversial subject.

1.3.2. IRI Mechanism

Just as blood is not responsible for only delivering one substance or molecule to the cells, IRI cannot be pinpointed to a single malfunctioning mechanism or missing molecule. However, the combination of multiple factors missing and going wrong results in IRI. When a certain level of IRI is inflicted, the result is cell death by the afore-described modes. Studies in other tissues have shown that the intensity of the metabolic stress can determine the pathway to eventual cell death (33). Several pathways and processes contributing to IRI, which will be considered in the following paragraphs.

1.3.2.1. Calcium Overload

Perhaps the most important process that contributes to IRI is the intracellular Ca^{2+} overload. The physiological cell has multiple ion-exchanging channels and transporters integrated within its lipid-bilayer membrane. Relevant for the balance of intra- and extracellular Ca^{2+} are the Na^+/hydrogen (H^+) exchanger (NHE), the Na^+/Ca^{2+} exchanger (NCX), NKA, plasma membrane Ca^{2+}-ATPase (PMCA), Ca^{2+} channel, Na^+ channel. Noteworthy is that all Ca^{2+} mobility towards extracellular is contingent on either an exchange with Na^+ or costs ATP.

When ischemia occurs, ATP is produced via anaerobic glycolysis. This process produces lactate and protons (H^+), lowering the cytosolic potential of hydrogen (pH) level from its ideal of around 7.2, varying between different cell types. The abnormally low pH results in abnormal cell functioning as processes such as protein biosynthesis require a certain pH level to be maintained. All human cells, skin included, show the presence of NHE. They have been shown to correct cytosolic pH levels by removing excess protons taking advantage of the sodium concentration difference as the driving factor (34). pH correction partially resolves the transmembrane sodium difference.

Because anaerobic glycolysis is far less effective than aerobic glycolysis, it results in a lack of ATP. This, and the fact that there is less sodium extracellularly, reduces activity of the NCX, which leads to an increased intracellular Ca^{2+} concentration. The lack of ATP also results in a less effective NKA so that the sodium concentration difference across the plasma membrane remains reduced, which also lowers the NCX's activity. The endoplasmic reticulum acts as the main Ca^{2+} storage site within the cell. Its uptake of Ca^{2+}, however, is also ATP-dependent and therefore decreased. While calcium uptake is limited, the endoplasmic reticulum continues to release Ca^{2+} via the ryanodine receptors (35).

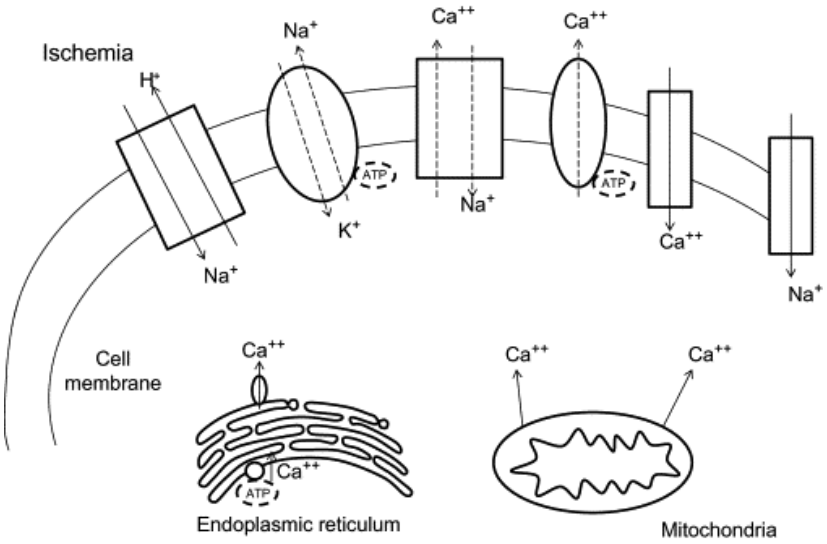

Figure 1 Ion traffic during ischemia results in increased intracellular Ca2+ (21).

Finally, reperfusion exacerbates this effect because it washes away the protons creating a steeper proton gradient and thus increasing the NHE activity resulting in a high intracellular sodium concentration. This causes the NCX to go into reverse mode, increasing the intracellular Ca^{2+} overload (36). Ca^{2+} enters the mitochondria electrophoretically via the mitochondrial Ca^{2+} uniporter (37). This eventually causes mitochondrial Ca^{2+} overload. The uptick in sodium after reperfusion increases the activity of the mitochondrial NCXs, releasing mitochondrial Ca^{2+} into the cell's cytoplasm and leading to even more Ca^{2+} in the cytosol (38). This method of Ca^{2+} cycling within the cell allows for cytosolic Ca^{2+} changes to be relayed to the mitochondrial matrix (37). Increased mitochondrial levels of Ca^{2+} eliminate the original transmembrane potential – negative inside – which coincides with the irreversible opening of the permeability transition pore

(PTP) (38, 39). The PTP opens in response to increased calcium levels when the mitochondria do not produce sufficient ATP and undergo oxidative stress (37). The PTP is large enough to allow a nearly unregulated influx of metabolites into the mitochondria, leading to further collapse of the transmembrane potential (37). The inner membrane of the mitochondria is folded extensively and thus has a much larger surface area than the outer membrane. Therefore, when the mitochondria swell, the outer membrane ruptures at a certain point exposing the cell to the contents of the mitochondrial intermembrane space, such as apoptosis-inducing factor (AIF) and CYC resulting in the aforementioned types of cell death mediated in part by protease activation(37).

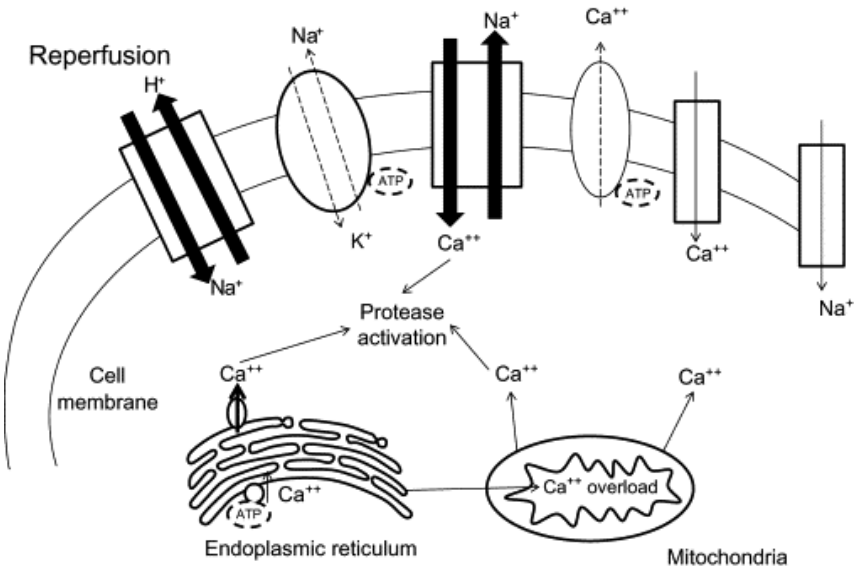

Figure 2 Ion traffic during reperfusion exacerbating intracellular Ca2+ results in cell death (21).

1.3.2.2. Reactive Oxygen Species

The many characteristics of cells undergoing IRI cannot be isolated. The following paragraph talks about the factor of reactive oxygen species (ROS) mainly created by reperfusion but enabled in part by the Ca^{2+} overload. Increased intracellular Ca^{2+} can induce change in the tertiary structure of certain enzymes. For example, the enzymes xanthine dehydrogenase and xanthine oxidase catalyze the same reactions: hypoxanthine to xanthine and xanthine to uric acid. Ca^{2+} overload resulting from prolonged ischemia plays a role in converting xanthine dehydrogenase to xanthine oxidase (39).

Engerson et al. compared xanthine dehydrogenase to its ischemia-induced oxidase form and found that the oxidase is a direct proteolytic and irreversible

product of the dehydrogenase (40). Chambers et al. showed a significant increase in the concentration of xanthine oxidase post-ischemia (41). While the dehydrogenase uses oxidized nicotinamide adenine dinucleotide (NAD) as a cofactor, the oxidase uses oxygen provided during reperfusion and creates the radical anion superoxide (39). Zweier et al. showed the quantity distribution of adenine nucleotides in human endothelial cells pre and post anoxia. They discovered that anoxia caused a two-thirds decrease (13.5 to 4.1) in ATP, while the concentration of hypoxanthine increased tenfold from 0.9 to 9.9 (42). While the control cells showed a concentration ratio of 10:1 of oxidase to dehydrogenase, they also reported no significant change to either enzyme concentration in control cells versus cells that had undergone anoxia and reoxygenation (42). They concluded that ROS production during reoxygenation is thus substrate and not enzyme-concentration-dependent (42). The concentration and ratio of the enzymes xanthine oxidase and xanthine dehydrogenase vary between different tissues. Nevertheless, xanthine oxidase is responsible for producing ROS in the reoxygenated cells accompanying reperfusion.

Another source of ROS is the mitochondrial electron transport chain (ETC). Under physiologic conditions, the NADH dehydrogenase complex, which is complex 1 of the ETC, generates superoxide from 1-2 % of the electrons that pass through the ETC (43). During ischemia, however, the rate of ATP production decreases, resulting in an increased NADH/NAD^+ ratio. This ratio determines the rate of superoxide production from the ETC (44). Complex 3 of the ETC also increases superoxide production as a result of ischemia (43).

CYC also plays a role in the production of ROS. An increase in the reduced form of CYC and the reduction of functional cytochrome oxidase, which is necessary to replenish the pool of oxidized CYC, is another source of increased superoxide (43). In the non-ischemic cell, CYC is a superoxide scavenger.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase also produces superoxide. The most significant superoxide production can be the respiratory burst, where phagocytic leucocytes produce immense amounts. While IRI does have the potential to recruit these immune cells because it is an inflammatory event, relevant superoxide production by the NADPH-oxidase occurs by the endothelium. This endothelium-based NADPH-oxidase is always active to some

degree, in comparison to the leucocyte type, which needs to be activated, and under conditions of extreme stress as such can be in I/R, can also produce significant amounts of superoxide (21).

All the sources of superoxide and, thus, ROS cumulate in ROS production that the cell cannot handle. Its protective mechanisms are overwhelmed. Superoxide is so relevant because it is the parent of the ROS family. ROS evoke an inflammatory response by causing the release of pro-inflammatory mediators that lead to cell injury and death by compromisingly reacting with various organelles and structures (45). Superoxide itself is not considered very toxic in vivo because it mostly and very quickly dismutates with the help of the superoxide dismutase (SOD) enzyme that can be found in the mitochondria, cytoplasm, and extracellularly (21). Human SOD contains metals, e.g., copper, that can be a monovalent or divalent cation making it a metalloenzyme. Depending on the cation charge, SOD reacts with superoxide to create oxygen, if monovalent, and hydrogen peroxide, if divalent (43). Hydrogen peroxide is also a ROS and can inflict damage through oxidation to the cell. Cells are equipped with a defense system consisting of catalase and glutathione peroxidase to reduce hydrogen peroxide to water (46).

Not only are there reactive oxygen species but also reactive nitrogen species (RNS) that play a role in IRI, especially in combination with ROS (21). The superoxide of the nitrogen species is the molecule NO. It, too, has a very short half-life and is a highly reactive molecule. NO can diffuse across cell membranes and thus functions as a vasodilative and anti-adherent signal molecule in the vasculature, but can also react with many molecules (47, 48).

During a phase of ischemia, cells produce more metabolic waste products, such as reactive oxygen species, than can be compensated. Their accumulation leads to premature cell death. Paradoxically reperfusion exacerbates the inflammatory response leading to reperfusion injury.

1.3.2.3. Leucocyte adhesion

Studies with knockout mice have shown that ischemia stimulates the expression of leucocyte molecules of adhesion on endothelial cells and that this correlates with an increased area of necrosis following ischemia and reperfusion (49). Harlan et al. described that adherent leucocytes could enhance the local

inflammatory response by injuring the endothelium. This can eventually lead to endothelium denudation which allows free access to the subendothelial tissue for the inflammatory substances (50). Reduced endothelial integrity can result in forming of a platelet thrombus that obstructs blood flow downstream (50). Independent from platelet formation due to leucocyte adhesion, obstruction of blood flow is a complication of ischemia. Prolonged ischemia can lead to incomplete reperfusion due to a phenomenon called no-reflow, which means that despite reperfusion, the downstream tissue is still subject to ischemia (51).
As ischemia and reperfusion are unavoidable in certain scenarios, the question is how to reduce the extent of IRI. While there have been many different approaches to solving this problem, RIC has established itself as a practice that has the potential to be a feasible clinical solution to minimize IRI. The following section will take an in-depth look at RIC, including its evolution and discovered mechanisms to date.

1.4. Remote-ischemic conditioning

Ischemia and IRI are important topics in many fields of medicine. Any tissue can be affected by this. However, some are more sensitive than others. Because ischemia is an omnipresent problem and topic in medicine, there has been much of research on this topic. Most of the research originates from the interest in the kidney, heart, and brain. This research has shown that tissue treated with ischemic conditioning is more resilient toward future episodes of ischemia and IRI. The idea behind remote-ischemic conditioning is that the conditioning does not have to occur in the affected tissue but can take place remotely from the tissue that should be protected.

1.4.1. History

In 1986 Murry et al., motivated by their findings that brief ischemic periods slowed the rate of ATP depletion during subsequent ischemia, published a study showing that the infarct area in dog hearts could be reduced if the area was preconditioned with multiple rounds of ischemia (52). While they showed that the area could be reduced to 25% of the area without preconditioning, they also showed that the procedure was time sensitive. In the group that underwent 40 minutes of ischemia, the area was reduced to 25% compared to the control group. In a

separate group that underwent 3 hours of ischemia, there was no difference between the control and preconditioned groups.

In the following years, many more research groups continued building on this success. They tried to advance, and further the application of preconditioning as a method to protect against future sustained ischemia. In 1992 Mounsey et al. described their laboratory transferring the preconditioning method away from the myocardium to skeletal muscle. Here again, time was a dependent factor (53). The protocol with 30 minutes of ischemia followed by 30 minutes of reperfusion before sustained ischemia showed a 20% increase in muscle survival compared to the control group (53). The following year of 1993, marked the publication by Przyklenk et al. that first demonstrated that coronary occlusion in the form of preconditioning of one area not only increased survival in the occluded area but in all areas subjected to following sustained ischemia (54). While Przyklenk et al.'s work was confined to one organ, Gho et al. published a study in 1996 that showed the preconditioning effect was not exclusive to the preconditioned organ. Gho et al. concluded that remote preconditioning was just as effective as direct preconditioning (55). This assumes that the RIC effect is based on signals or substances that spread throughout the body after regional ischemia.

The first publications of RIC in plastic surgery appeared in 2002. Kuntscher et al. published a study showing that RIC did not significantly differ from direct ischemic conditioning in a muscle flap (56). In a further study published in the journal "Plastic and Reconstructive Surgery," Kuntscher et al. applied the technique that would be continued in many studies that merely used a tourniquet to induce ischemia in a remote limb. This study showed a significant reduction of flap necrosis area in the tourniquet and remote clamping in comparison to the control group that underwent no preconditioning (57). Finally, Kharbanda applied the knowledge of RIC to the human body and successfully introduced the protocol, which became a foundation in RIC experiments of three cycles of 5 minutes of tourniquet-induced contralateral ischemia at 200mmHg (58). This study is important because it shows that a noninvasive method can protect remote endothelial function.

While this evolving research path has led to the discovery of ever more feasible clinical application methods, many questions have arisen regarding the mechanism of the RIC effect. The evolution from direct ischemic conditioning to

RIC assumes that regional ischemia produces a global effect. How this global effect travels from the ischemic area to the rest of the body is still the subject of research. In the following section, an eye is turned to the state of research on this topic.

1.4.2. Mechanism

The discovery that the protective effect of preconditioning cycles of ischemia provides inter-organ protection, as mentioned above, has created more questions than answers regarding the exact mechanism of the RIC effect (59). Despite so much being unclarified, a consensus exists that it is the combination of many different pathways. It is assumed that the immediate protective reaction to local ischemia, which was discovered first, somehow spreads throughout the body using different signaling pathways and channels. Amongst the discussed signaling pathways are the neural pathway, the humoral pathway, and a systemic response (59). Findings in the past decade have shown that while there are multiple pathways, they might not be separable but rather dependent on each other (60).

While the general mechanisms are still being researched, preliminary understandings and results must be evaluated and appreciated carefully, for the question of inter-organ transferability of mechanisms remains. The production of signals that local ischemia causes and the signaling pathway are likely less specific than the reaction of the tissues that are supposed to be protected. Most research to date is focused on the protection of the myocardium. It can be assumed that the myocardium will react differently to remote signals than a myocutaneous flap. More research is necessary to understand the different end organ responses to RIC fully.

1.4.2.1. Neural Pathway

The neural pathway has been thought to play a role in the RIC pathway for a while. Its importance has been deducted from findings demonstrating a less pronounced RIC effect when the neural connection was not intact, or antagonists to neural signaling molecules were added to the equation.

In 1996 Gho et al. not only, as mentioned above, showed that the remoteness of the ischemic conditioning expanded beyond the organ that was then subjected to

sustained ischemia but also showed neural involvement in precisely this finding (55). To analyze the role of the neural pathway, they conducted their studies a second time after adding the ganglion blocker hexamethonium. In the group that underwent normal ischemic conditioning by coronary occlusion, no significant difference of the following myocardium infarct size was detected between the groups with and without hexamethonium. A significant difference, however, was detected in the group that underwent RIC by occlusion of a mesenteric artery before sustained coronary occlusion. Gho et al. wrote that the cardioprotection previously experienced by mesenteric arterial occlusion to precondition the myocardium was completely absent in the group that received the ganglion blocker, thus suggesting some critical role of the neural pathway, specifically in the RIC method (55).

Amongst the transmitter substances that play an important role in the RIC effect are adenosine and bradykinin. Adenosine is assumed to play a role since Pell et al. were able to eliminate any cardioprotection in the rat that underwent RIC by administering a nonselective adenosine receptor antagonist (8-SPT) before RIC (61). Ding et al. in 2001 elaborated on Pell's discovery. Firstly they showed that RIC applied to the renal artery as the remote stimulus had no cardioprotective effect when the renal nerve was severed (62). Secondly, they measured the afferent renal nerve discharge, which increased during RIC. Once they added the nonselective adenosine receptor antagonist, the discharge rate decreased markedly, and the cardioprotective effect disappeared. This permits the assumption that ischemic kidney tissue releases adenosine which stimulates the renal afferent, which contributes to the RIC that leads to cardioprotection (62). Adenosine's role was further solidified by Liem et al., who found that RIC at the mesenteric artery was as effective as infusing adenosine into the mesenteric artery in protecting the myocardium (63). Finally, Dong et al. in 2004 showed that the release of adenosine was not unique to mesenterial preconditioning but also existed in limb preconditioning (64). The femoral nerve section abolished the limb RIC effect, and adenosine injection into the femoral artery was cardioprotective comparable to the adenosine-related research done in the mesenterial area.

Similar to the findings on adenosine's contribution to the RIC effect, research was conducted to determine the extent of bradykinin's contribution to the RIC effect. It turns out that the findings were similar. Schoemaker et al. investigated the effect

of bradykinin in rats that underwent coronary artery occlusion with previous remote ischemic preconditioning. They discovered that applying a bradykinin receptor antagonist could significantly undermine preconditioning efforts and mesenteric infusion of bradykinin mimicked the effect of mesenteric artery occlusion (65).

1.4.2.2. Humoral Pathway

As mentioned above, the RIC effect is not reliant on a single pathway but rather a combination of many different ones. Most recently, in 2020, Ederer et al. published an article that concluded that RIC is not only dependent on neuronal signaling, as they compared groups that received anesthesia to the remote extremity with a non-anesthetized control group (66). The humoral pathway has been assumed to contribute to the RIC effect for decades. The assumption is that the organ used to precondition produces signaling molecules that, upon reperfusion, spread throughout the rest of the body and provide protection against future ischemia. Dickson et al. showed that some humoral substance must be released during preconditioning as they were able to reduce infarct area in a non-preconditioned rabbit heart by transfusing effluent from a rabbit that had undergone preconditioning (67). The humoral pathway was solidified as a hypothesis when in 2005, Konstantinov et al. were able to show a RIC effect in pigs that had received a denervated donor heart, thus excluding any neural pathway involvement (68). The first study demonstrating humoral involvement in humans was published in 2016 by Kolbenschlag et al. and showed that RIC improved blood flow and oxygen saturation in both pedicled and free flaps (11). Flee flaps, representing denervated tissue, benefiting from RIC inferred that the humoral pathway plays a role in the RIC effect within the target tissue (11). While there is agreement over the existence of humoral mediators of the RIC effect, the specific substances and their roles remain the focus of current research. Amongst the many humoral substances that constitute the humoral pathway are microRNA-144, calcitonin gene-related peptide, circulating nitrite, SDF-1, and interleukin-10 (69-73). These substances contribute to the RIC effect through vasodilatory, anti-inflammatory, and anti-apoptotic mechanisms.

1.4.2.3. Systemic response

Besides the specific neuronal and humoral pathways, remote ischemic preconditioning has also produced a systemic response that protects against future ischemic events. For example, in 2004, Konstantinov et al. used a microarray method to demonstrate a change in proinflammatory gene expression in circulating leucocytes after a standard RIC protocol (74). The expression of genes encoding proteins involved in apoptosis was suppressed as early as 15 minutes after the RIC stimulus and continued to be suppressed 24 hours later (74).

1.4.2.4. Target organ response to RIC

Most research in the field of ischemia and ischemia-reperfusion injury is conducted in the context of cardiac ischemic events commonly known as heart attacks. The question, therefore, is to what extent this research and its findings translate to other organs. The organ of relevance for this project is the skin and the mechanisms of the RIC effect on the skin's microvasculature. Generally speaking, RIC elicits several responses on a variety of signaling pathways. Whether neuronal or humoral, the signals released by RIC reach the target organ and achieve a protective reaction via not yet fully understood intracellular signaling cascades. In the cutaneous or myocutaneous flap, the molecule nitric oxide (NO) plays an important role. One of its primary functions in the body is vasodilation. Because lack of blood supply is the main reason for flap failure, vasodilation is a significant step toward flap survival.

Early research into NO production as a result of ischemia concluded that ischemic events could increase endothelial NO-synthase's (eNOS) function, resulting in the activation of protein kinase C (75). Furthermore, activated protein kinase C increased the transcription of immunologic NO synthase (iNOS) (75).

Küntscher et al. were then interested in NO's role in cutaneous flaps. They found that NO played a significant role in RIC, as the administration of the unspecific NO-synthesis blocker L-NAME eliminated the reducing effect of RIC on the flap necrosis rate. When the unspecific NO-synthesis blocker L-NAME blocked endogenous NO-synthesis, exogenous NO application was insufficient in ischemia-reperfusion injury protection. Without L-NAME exogenous NO given before ischemia simulated the effect of RIC. They concluded, based on these

findings and previous research, it is very likely, that endogenous NO production via eNOS and iNOS is essential to RIC (76, 77).

In 2016 Lambert et al. produced confirming findings in their research, which applied RIC to a limb in healthy humans. They could not only show that RIC had an effect on NO availability but also examined sympathetic nervous system activation, which results in vasoconstriction, and oxidative stress markers. While NO availability dropped after reperfusion in the control group, the RIC group maintained their pre-reperfusion NO availability (78). Furthermore, sympathetic nerve activity increased significantly less in the group that underwent RIC, which is in harmony with the observation that the RIC group showed a significant vasodilatory response, whereas the control group did not (78). Also, Lambert et al. found that their RIC group did not produce significantly more GSH, an erythrocyte marker for oxidative stress, showing that RIC reduced oxidative stress (78).

1.4.2.5. Duration of RIC Effect

Many of the above-mentioned mechanisms and released substances end in signal cascades that lead to increased or decreased gene transcription. This could explain why RIC offers immediate and late protection (79). The initial phase of protection has been reported to last up to 4 hours, and the second phase begins after 24 hours and lasts for up to 48 hours (79). Most of the initial response is probably produced by the immediate release of molecules and signaling substances, while Loukogeorgakis et al. said the second window of protection is consistent with the altered gene expression in the vessel walls that endogenously mimic the initial response (79).

1.4.3. Clinical application

Clinicians are ready for a method to reduce IRI. For any method to be applied widely, it must fulfill the criteria of easy application and good cost-effectiveness. The easy application implies it is non-invasive. While a good amount of research on the application in various medical fields exists, RIC's application in plastic surgery is still in the early stages. In plastic surgery, RIC is a technique that will hopefully increase flap survival rates and reduce microcirculatory complications due to IRI. A study containing information on 189 consecutive free flaps

determined the average ischemia time as 2 hours and 6 minutes (80). This is a significant amount of time, leading to a complete ischemic phase followed by reperfusion. However, a link between ischemia time, to the extent that it is needed to perform the procedures, and complications does not seem to exist. Thus Shaw et al. concluded that concern over complications such as no-reflow should not interfere with taking the time to perform good surgery (80).

The discovery that remote conditioning is just as effective as direct conditioning was a significant step toward achieving clinical application (55). Kolbenschlag et al. showed that using the upper extremity as the remote ischemic site is superior to the lower extremity (81). This is another step towards clinical application, as all that is needed to create extremity ischemia is a tourniquet commonly used to measure a patient's blood pressure. Most clinical studies have adopted this method.

Ever since ischemic conditioning has been around, the question has been when it is best to perform in relation to the ischemic event. Depending on the situation, the options can be limited. In theory, conditioning can occur before, during, or after the ischemic event. The only option in cardiology and neurology, where ischemic events occur unannounced, is postconditioning. Plastic surgery and specifically defect treatments with flaps offer the opportunity to precondition. Keskin et al. conducted a study that compared these three options in rats, ranking them by various parameters that included the nitric oxide levels in blood samples and a histopathological damage score to the muscle flap. Remote perconditioning, conditioning during the ischemic period of the target, proved to have the least histopathological damage (82). Additionally, remote perconditioning resulted in the highest levels of NO, followed by preconditioning and then postconditioning (82). All forms of conditioning outperformed the control group that did not undergo any remote conditioning. Since all forms of conditioning are effective to a significant extent, a clinical application is, in theory, always possible.

The other aspect of remote conditioning in the clinical setting is which protocol is best. The goal is to find a long enough protocol to have an effect but short enough to increase patient compliance, as an ischemic extremity is an uncomfortable experience. Traditionally thus far, protocols have mainly consisted of three cycles of ischemia, each followed by a reperfusion period. Kolbenschlag et al. conducted

a study to determine the effectiveness of different durations of ischemia during preconditioning. They concluded that three cycles of 10 minutes of ischemia were superior to any other length of the preconditioning cycles investigated (83). In 2021 Sogorski et al. published that three cycles of ischemia were the optimal clinical protocol (84). While the search for the optimal protocol continues, the approach of RIC is promising and is under current investigation.

This study aims to extrapolate the current knowledge of RIC and determine the best RIC protocol for clinical application. While we know there is a RIC effect, we do not fully understand what factors contribute to its effectiveness. Therefore, this study focuses on the two factors of deoxygenation and time to deoxygenation. Different protocols concerning the level of deoxygenation and time to deoxygenation are compared to differentiate these factors' degree of contribution. The question to be answered by this study is: Which RIC protocol produces the most significant difference of Flow, oxygen saturation (SO2) and relative hemoglobin (rHB) compared to baseline? As a result, this study aims to elucidate which factors determine the strength of the RIC effect.

2. Material & Methods

This study was approved by the ethics committee of the Eberhard Karls University of Tuebingen with project number: 439/2019BO2. It is a prospective clinical study conducted with healthy subjects. All devices used were provided by the BG Unfallklinik Tuebingen.

2.1. Subject population

Subjects were selected upon replying to a form that was posted on the local hospital notice boards of the university hospitals of Tuebingen, in various university social media groups of the University of Tuebingen, and various locations throughout the city of Tuebingen, Germany. All measurements were conducted from January to March 2020. All subjects were at least 18 years old. After thoroughly explaining of the study, answering all subjects' questions, and obtaining informed consent, they could begin the physical clearance phase. This phase determined the subject's suitability for the study. The age range was kept to 19-37 to minimize age-related differences in cutaneous blood flow (85, 86). The subject group was evenly split between the biological sexes, with 12 male

and 12 female subjects. All subjects had to be generally healthy, not requiring any prescribed medications, and healthy on the days of measurements. Any condition prohibiting a subject from having a tourniquet placed on their upper arm was an exclusion factor. Pregnancy amongst female participants was an exclusion factor. Any medical conditions that do not require medication, but affect circulation, such as Raynaud-Syndrome, were excluded. The dominant thenar eminence and the contralateral anterolateral thigh had to be free of open wounds, scratches, or irritations.

With the help of a standardized questionnaire, height (according to ID), weight (measured on the first day of study), age, physical activity, method of contraception, alcohol consumption, nicotine consumption, drug consumption, allergies, and health conditions were recorded. All subjects selected did not have any significant adiposity classifiable as obesity to preemptively eliminate any effect above-average subcutaneous fat could have on microcirculation (87). After they were physically cleared to participate in the study, written and oral consent was given prior to the first measurement.

Participants were offered a sum of 80 Euros to participate in all four measurements, while if any subject were to drop out, they would receive 15 Euros per measurement. The additional 20 Euros, if all four measurements were completed, were given to incentivize subjects to undergo all measurements to be able to conduct an intraindividual analysis.

2.2. Measurement procedure

All measurements were conducted in the Hand-, Plastic-, Reconstructive-, and Burn Surgery department examination room. Depending on the transportation the subject chose to take to the hospital, they would wait for the subject to be relaxed and at ease. The examiner waited for signs of increased microcirculation, such as hyperemia in the face and hyperhidrosis, to subside before commencing any measurements. The same examiner conducted all measurements. Subjects were told in advance to wear a shirt that can be rolled up or is sleeveless so that the tourniquet could be correctly placed around the upper arm. Further, they were instructed to wear short pants, preferentially lose running shorts, so that a sensor could be placed on the upper anterolateral thigh. These instructions were given in advance to avoid any subjects showing up with inconvenient clothing that

would have had to be removed not to influence the measurement. The idea was to avoid any nervousness from being under-clothed. Because of the thorough conversations before the first measurement and the physical clearance phase, all subjects had already had multiple conversations with the examiner to avoid any awkwardness or nervousness that could potentially influence cutaneous microcirculation or heart rate or saturation and breathing frequency.

The temperature in the room itself was constant, and subjects were asked whether they were cold or warm, and it was always ensured that subjects felt neither cold nor hot. The subjects would sit in a chair with adjustable armrests and an adjustable chair height. Their dominant arm was relaxed onto an armrest. Chair height was selected, so the feet were placed entirely on the ground. No active positioning with muscle activity was necessary for the subjects to remain in the same comfortable position for the measurement duration. All measurements were conducted with the “Oxygen to See” (O2C) device (serial number: 314-155-18), a product of the LEA Medizintechnik GmbH. This O2C uses two LFx-33 sensors with a horizontal separation of 3 mm between light and laser source and detection area. The estimated light and laser penetrance is 2.1 mm. Throughout all measurements, Sensor “P1S” was placed on the thenar eminence of the dominant hand, while Sensor “P1D” was placed in the area of the anterolateral thigh of the contralateral lower extremity. Subjects and O2C were situated so the subject could not view the monitoring process to avoid any possibility of visual feedback influencing the RIC effect. Once everything was set up, the appropriate protocol was selected and started. The examiner was positioned in a chair across from the subject and could constantly monitor the protocol’s correct execution. This was especially important during the protocol that involved the subject repeatedly clenching the ischemic hand into a fist, as this act could have potentially dislodged a sensor due to its placement on the thenar eminence.

Due to the nature of the measurement and the tourniquet increasing and reducing pressure, the subjects were constantly aware if they were currently in an ischemia or reperfusion phase and thus not blind to the protocol. We assumed and later confirmed that some protocols are more uncomfortable than others. Because it is impossible to blind the patient to the protocol, each subject was subjected to

the protocols in the same order. The goal of this strategy was to prevent subjects from not completing all four measurements.

An important consideration to achieve the desired intraindividual comparison was to ensure that each measurement for a single subject was separated by at least one week. This is because of the aforementioned two phases of protection that a RIC protocol offers, according to the available literature thus far. The initial phase of protection has been reported to last up to 4 hours, and the second phase begins after 24 hours and lasts for up to 48 hours (79). Therefore, seven days were considered sufficient after a previous measurement to avoid interference by previous measurements and overlapping RIC effects.

2.3. Protocols

In total, all 24 subjects were subjected to four different RIC protocols. The protocols aim at differentiating RIC-effect-affecting factors. All four protocols include an initial ten-minute baseline measurement. The first 5 minutes of baseline measurement are discarded in the analysis as they are likely to include measurements from a subject that finds themselves in a new situation and is not yet completely relaxed. The second 5 minutes of baseline measurement will later be the baseline that functions as the reference point for any fluctuations throughout the protocol.

2.3.1. Protocol 1

Protocol 1 (P1) is the current standard RIC protocol. It is a solely time-based protocol. The initial baseline measurement is followed by three cycles of five minutes of ischemia and ten consecutive minutes of reperfusion. In total, this protocol has a duration of 55 minutes.

Baseline	Ischemia 1 (I1)	Reperfusion 1 (R1)	Ischemia 2 (I2)	Reperfusion 2 (R2)	Ischemia 3 (I3)	Reperfusion 3 (R3)
10 min	5 min	10 min	5 min	10 min	5 min	10 min

Figure 3 Protocol 1's composition.

2.3.2. Protocol 2

Protocol 2 (P2) is controlled by deoxygenation level in the ischemia-subjected upper extremity. The initial baseline measurement is followed by three cycles of

alternating ischemia until 10% oxygen saturation is achieved for three consecutive seconds and ten minutes of reperfusion.

Baseline	I1	R2	I2	R2	I3	R3
10 min	≤ 10%	10 min	≤ 10%	10 min	≤ 10%	10 min

Figure 4 Protocol 2's composition.

2.3.3. Protocol 3

Protocol 3 (P3) is also controlled by deoxygenation level in the ischemia-subjected upper extremity. The initial baseline measurement is followed by three cycles of alternating ischemia until 30% oxygen saturation is achieved for three consecutive seconds and ten minutes of reperfusion.

Baseline	I1	R1	I2	R2	I3	R3
10 min	≤ 30%	10 min	≤ 30%	10 min	≤ 30%	10 min

Figure 5 Protocol 3's composition.

2.3.4. Protocol 4

Protocol 4 (P4) is controlled by the deoxygenation level in the ischemia-subjected upper extremity. The initial baseline measurement is followed by three cycles of alternating ischemia until 10% oxygen saturation is achieved for three consecutive seconds and ten minutes of reperfusion. In addition to the tourniquet subjecting the dominant upper extremity to ischemia, the subjects pump their fist until the sensor on the dominant hand registers an oxygen saturation of 10% or below for three consecutive seconds. During reperfusion, the subject is still. To avoid variance in exertion, the examiner provided a rhythm by clapping or tapping with an approximate rate of 1 fist clench every 3 seconds. As time progressed and the ischemia increased, the subjects had a more challenging time clenching their fist. All subjects slowed down significantly and abandoned exact rhythm due to ischemia induced pain.

Baseline	I1	R1	I2	R2	I3	R3
10 min	≤ 10% + muscular effort	10 min	≤ 10% + muscular effort	10 min	≤ 10% + muscular effort	10 min

Figure 6 Protocol 4's composition.

2.4. Microcirculation Analysis

Consistent with the method of previous studies analyzing the RIC effect, this study made use of the O2C device by the LEA Medizintechnik GmbH. O2C enables non-invasive measuring of the three parameters blood flow, tissue hemoglobin oxygen saturation, and the relative hemoglobin amount. O2C uses laser doppler spectroscopy and white light spectroscopy. Two separate sensors can measure all parameters at two separate sites. Both sensors measure at a depth of 2.1 mm and have a detection range of 450 – 850 nm.

As seen in the graphic provided by the manufacturer below, laser doppler spectroscopy is used to determine the blood flow. In general, a doppler is used to determine movement relative to the observer, in this case, movement of erythrocytes relative to the sensor. The reflection wavelength of the laser varies depending on the position of the erythrocytes. This velocity measurement is calculated with the number of erythrocytes traveling at different speeds. It reveals how many erythrocytes are traveling at what speed and thus reveals the blood supply and state of microcirculation in the tissue. A high level of circulation is called hyperemia, while a decreased level is ischemia (88).

White light spectroscopy is used to determine the oxygen saturation of the hemoglobin by absorption spectroscopy. Depending on its saturation, the hemoglobin has a distinct color and, thus, a distinct white light absorption rate. Saturated hemoglobin, as found in the arteries, is redder than the deoxygenated hemoglobin found in the venous parts of circulation. This color distinction gives insight into the tissue's oxygen saturation. Since hemoglobin is a major cromophore of tissue, at its sensitive wavelength, white light spectroscopy gives feedback on the hemoglobin content of the targeted tissue volume. The characteristics and the quantity of the reflected light are thus both the object of analysis by the O2C device. These are presented as tissue hemoglobin oxygen saturation and relative hemoglobin amount, respectively. In contrast to regular oxygen saturation measurements used in hospitals to monitor patients, the O2C measures venous oxygen saturation since most of the hemoglobin in the microcirculation is found in the post capillary venous aspect. This allows us to judge the oxygenation of tissue preceding the venules and quantify potential local tissue hypoxia (88).

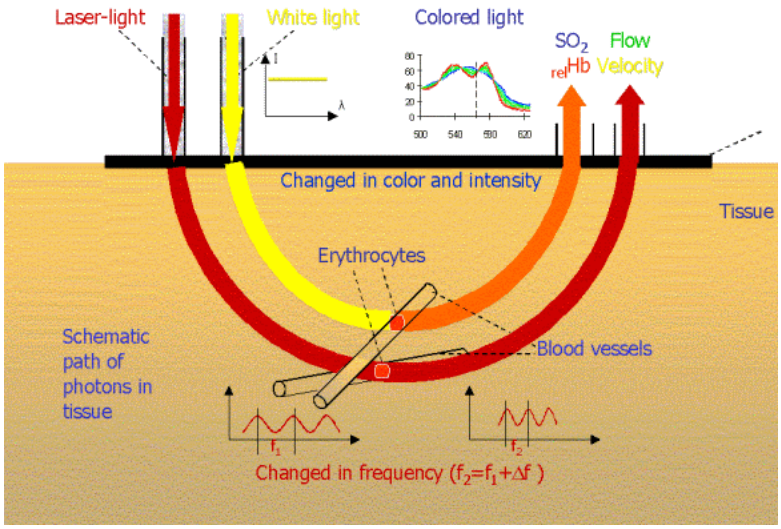

Figure 7 O2C technology: white light spectroscopy and laser doppler spectroscopy (89).

2.5. Statistical Analysis

Subject information was gathered using a questionnaire before measurements commenced to verify eligibility for inclusion in the study. All non-numerical data was transformed into a numerical code. The O2C device collected the relevant physical parameters (Flow, SO2 and rHB) throughout the measurement. The data was then exported and could be reviewed using the program O2CevaTime that the same manufacturer created. This platform enables a detailed review of the entire measurement. After reviewing the data and confirming that the data had been collected accordingly and the sensors were always receiving, the data was exported. One data point was registered every 1.000526 seconds (from now this timeframe is referred to as one second). This Excel file was viewed in Microsoft Office Excel 2007 and exported to IBM Statistics SPSS 26.

As there were a total of 24 subjects, each measured four times, and the goal was to compare the protocols, a repeated measures ANOVA (rmANOVA) was run on the P1D sensor data to compare the RIC effects. rmANOVA is robust to violations of normality in many studies, and corrections exist when sphericity is violated (90, 91). In addition to rmANOVA, differences to the baseline for each interval were individually calculated with paired t-tests. The paired t-test has been proven not to be sensitive to violations of normality, so Flow was not transformed (90). Having a value for every second resulted in a large number of data points per subject per measurement. That is why when comparing protocols, means for each interval were used. Each measurement consists of seven data points after

calculating a baseline and means for the remaining three cycles of ischemia and reperfusion. The baseline is the mean of the 300 seconds leading up to the beginning of the first ischemic phase. This represents seconds 300 to 600 of every measurement.

The O2C device measures blood flow (Flow), oxygen saturation (SO2), and relative hemoglobin content (rHb). Flow and rHb are measured in arbitrary units. SO2 is measured in percent. Flow and rHb are thus not comparable between subjects and within one subject between protocols. That is why all Flow and rHb values used in the statistical analysis were set in relation to the baseline of their measurement. The baseline hereby represents the value 1. While SO2 was measured in percent and thus is a comparable unit, each subject has a different baseline, and everyone's tissue deoxygenates differently. Hence, comparing the relative levels to the baseline makes sense when comparing interindividually.

An essential factor in running a rmANOVA is the sphericity of the data set. Mauchly's test of sphericity requires normally distributed data. When the raw data is not normally distributed, a transformation is required to achieve sphericity to run a rmANOVA. In the case of Flow, the logarithm of data corrected this violation, although the rmANOVA has been proven robust against violations of normality (91). When Mauchly's test of sphericity was significant, a correction was needed. Dependent on the Epsilon factor of the Greenhouse-Geisser correction, either the Greenhouse-Geisser correction or the Huynh-Feldt correction is used. Girden defined the Epsilon factor cutoff to be 0.75 (92). The Greenhouse-Geisser correction is used when Epsilon is less than 0.75. The Huynh-Feldt correction is used when Epsilon is greater than 0.75.

3. Results

3.1. Descriptive Statistics

This study was conducted on 24 healthy subjects who underwent all four protocols with at least a week between measurements. The biological sexes were split evenly, so that there were 12 male and 12 female participants. The average age of all participants was 24.67 years (± 3.52), with no significant difference between the genders ($p = 0.06$). The average height was 1.75 m (± 0.09). There was a significant difference between males and females ($p = 0.026$). Males had an average height of 1.80 m (± 0.05), and females had an average height of 1.70

m (± 0.089). All participants' average body mass index (BMI) was 22.22 kg/m^2, ranging from 19.23 kg/m^2 to 25.78 kg/m^2, and no significant difference ($p = 0.842$) between males and females.

The questionnaire registered various substance consumption. The average nicotine consumption was 0.07 pack years (PY). No participant smoked regularly, but a few smoked once in a while during festivities. No participant smoked the day of or the week before the measurement. To adapt the total nicotine consumption to age, pack years were divided by individual's age. This calculation produced a number of 0 pack years. The mean alcohol consumption was 3705.00 ml over the past year. For this calculation, the average alcohol contents of drinks were used as described by Stinson et al., which assigned 4.5% alcohol to a beer and 12.9% alcohol to a wine (93). According to Dawson et al., this puts the average participant at the bottom of the moderate drinker range (94). Caffeine consumption questioning produced an average number of 38409.80 mg/a. Participants were asked to refrain from consuming caffeinated products on the day of measurement if their appointment was in the morning or midday. If their appointment was after 5 p.m., they were asked not to drink any caffeinated beverage after their morning coffee or tea between 7 a.m. and 9 a.m.

Every participant was physically active during the week, so the average duration of physical activity per week was 3.08 h (± 1.77). Physical activity was considered to be any form of training, whether a soccer practice, climbing session, or jogging. While male subjects participated in 3.75 (± 2.05) physical activities weekly, females participated in 2.42 (± 1.18) physical activities weekly. The difference was significant at $p = 0.045$.

Regarding women's contraception, 5 of 12 reported hormonal contraception for an average of 3.8 years. The average menarche was 10.67 years ago. While the sexes were not paired, the only significant differences between the sexes were their height, which disappeared once converted to BMI ($p = 0.842$), and the amount of physical activity ($p = 0.045$). No participant consumed any regular medication or had any underlying health condition.

Table 1 Descriptive statistics with total means and standard deviations, further differentiated by biological sex and compared by independent t-test with p < 0.05 (*).

	total	Male (n=12)	Female (n=12)	p-value (Female vs. Male)
Age (a)	24.67 (± 3.52)	25.25 (± 4.35)	24.08 (± 2.503)	0.060
Height (m)	1.75 (± 0.09)	1.80 (± 0.05)	1.70 (± 0.089)	0.026*
Weight (kg)	68.63 (± 10.73)	75.92 (± 8.67)	61.33 (± 7.04)	0.625
BMI (kg/m^2)	22.22 (± 2.04)	23.30 (± 1.71)	21.15 (± 1.81)	0.842
Nicotine in PY	0.07 (± 0.23)	0.08 (± 0.29)	0.06 (± 0.16)	0.532
PY/a	0.00 (± 0.01)	0.00 (± 0.01)	0.00 (± 0.01)	0.850
Alcohol QF past year (ml)	3705.00 (± 2634.77)	3845.83 (± 2665.05)	3564.17 (± 2714.65)	1.000
Caffeine (mg/a)	38409.80 (± 39375.91)	41080.00 (± 44841.05)	35739.60 (± 34865.46)	0.387
Physical Activity/week in h	3.08 (± 1.77)	3.7500 (± 2.05)	2.42 (± 1.18)	0.045*
Hormonal Contraception (yes/no)	n/a	n/a	5/7	n/a
Duration of Hormonal Contraception (a)	n/a	n/a	3.80 (± 2.51)	n/a
Years since Menarche (a)	n/a	n/a	10.67 (± 2.15)	n/a

3.2. Microcirculation

3.2.1. Oxygen Saturation

White light spectroscopy was used to determine the oxygen saturation of the hemoglobin by absorption spectroscopy. This was measured for the entire duration of all protocols. Table 2 contains the average oxygen saturation of each interval within each protocol as a relative number toward the baseline. The following chapter will focus on differences within a single protocol and differences between protocols to determine when and by how much a protocol results in a higher oxygen saturation.

Table 2 Means and standard deviations of SO2 relative to baseline throughout protocols for each interval.

Interval	P 1	P 2	P 3	P 4
baseline	1	1	1	1
I1	1.107 (± 0.103)	1.089 (± 0.171)	1.005 (± 0.136)	1.342(± 0.261)
R1	0.986 (± 0.089)	0,996 (± 0.098)	0.935 (± 0.100)	1.027 (± 0.126)
I2	0.982 (± 0.120)	0.995 (± 0.142)	0.920 (± 0.158)	1.151 (± 0.282)
R2	0.924 (± 0.118)	0.953 (± 0.122)	0.894 (± 0.130)	0.988 (± 0.153)
I3	0.951 (± 0.132)	0.962 (± 0.121)	0.904 (± 0.222)	1.082 (± 0.275)
R3	0.913 (± 0.146)	0.904 (± 0.114)	0.849 (± 0.158)	0.971 (± 0.207)

Interval 1

Table 3 SO2 mean relative to baseline during Interval 1 for each protocol, p < 0.05 significant (*).

	P1	P2	P3	P4

Difference to baseline	0.107* (± 0.103)	0.089* (± 0.171)	0.005 (± 0.136)	0.342* (± 0.261)
p	0.00	0.02	0.86	0.00

Table 3 shows the differences from the baseline during the first ischemic interval. The biggest significant difference from baseline was produced by protocol 4. An equally significant, however, smaller difference was produced by protocol 1. Protocol 2 resulted in a less significant and even smaller difference from the baseline.

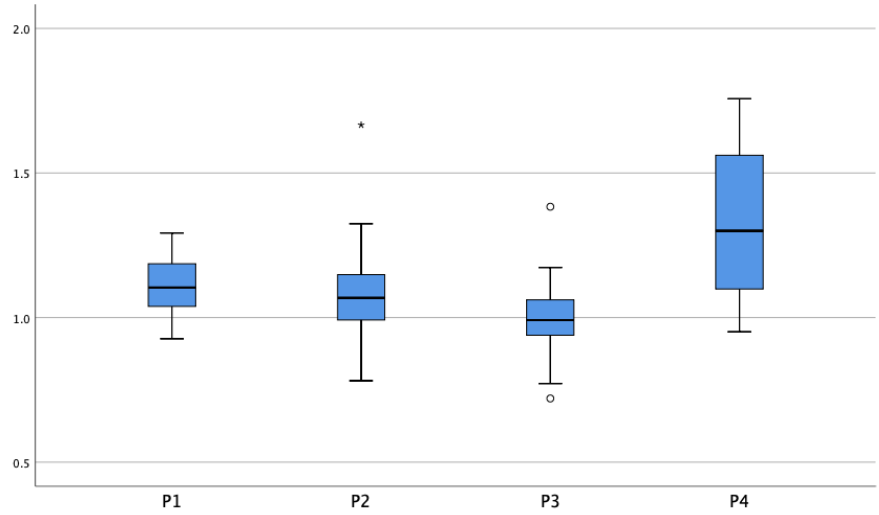

Figure 8 Mean SO2 relative to baseline during Interval 1 for each protocol.

Since Mauchly's test of sphericity was significant at p = 0.002 and Epsilon was 0.625, the Greenhouse-Geisser correction was used to correct the violations to sphericity. With Greenhouse-Geisser correction's significance at p < 0.001, the rmANOVA determined a statistically significant difference between measurements.

The pairwise comparisons in the Bonferroni-adjusted post-hoc analysis revealed significantly higher oxygen saturation in protocol 4 compared to all other protocols. Protocol 4 was significantly better than protocols 1, 2, and 3, with significances of p = 0.009, p = 0.005, and p < 0.001, respectively. There was no statistically significant difference between protocols 1, 2, and 3.

Table 4 Pairwise comparisons of SO2 means relative to baseline during Interval 1 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	0.018	1.000
P1 vs. P3	0.102	0.066
P1 vs. P4	- 0.235*	0.009
P2 vs. P3	0.084	0.188
P2 vs. P4	- 0.253*	0.005
P3 vs. P4	- 0.337*	0.000

Interval 2

Table 5 SO2 mean relative to baseline during Interval 2 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	- 0.014 (± 0.089)	- 0.004 (± 0.171)	- 0.066* (± 0.100)	0.027 (± 0.126)
p	0.442	0.842	0.004	0.298

Table 5 shows the differences from the baseline during the first interval of reperfusion. The only significant difference was a decrease in oxygen saturation in protocol 3. Protocol 4 showed a slight increase from baseline; however not statistically significant.

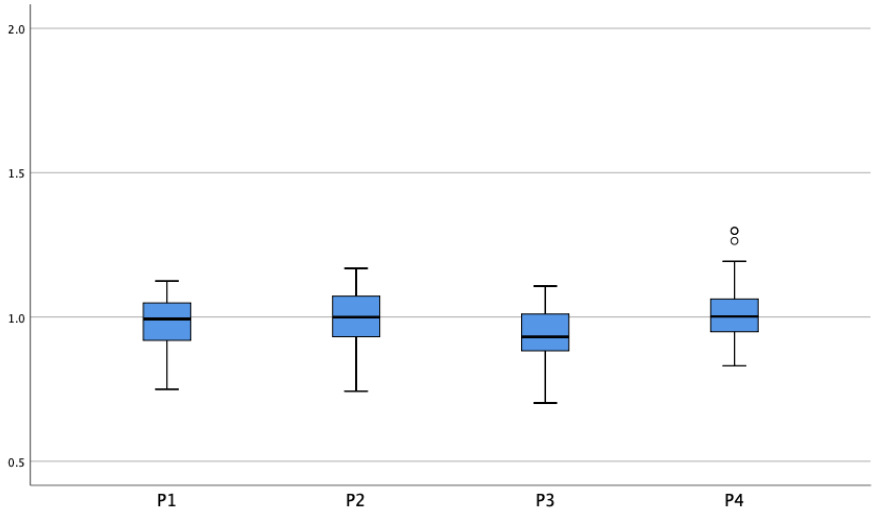

Figure 9 Mean SO2 relative to baseline during Interval 2 for each protocol.

Since Mauchly's test of sphericity was not significant with p = 0.486, sphericity is present, and therefore no significant differences could be assumed. Protocol 4 performed superior towards all other protocols in post-hoc analysis. Protocol 2 performed superior to protocols 1 and 3. All differences were not statistically significant.

Table 6 Pairwise comparisons of SO2 means relative to baseline during Interval 2 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.010	1.000
P1 vs. P3	0.051	0.380
P1 vs. P4	- 0.042	1.000
P2 vs. P3	0.061	0.103
P2 vs. P4	- 0.031	1.000
P3 vs. P4	- 0.093	0.066

Interval 3

Table 7 SO2 mean relative to baseline during Interval 3 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	- 0.018 (± 0.120)	- 0.005 (± 0.143)	- 0.081* (± 0.158)	0.151* (± 0.282)
p	0.463	0.862	0.020	0.015

Table 7 shows the differences from the baseline during the second interval of ischemia. Protocols 1 and 2 were statistically insignificantly below the baseline. Protocol 3 was significantly below baseline. Protocol 4 was significantly above the baseline by 15.1% with a significance of p = 0.015.

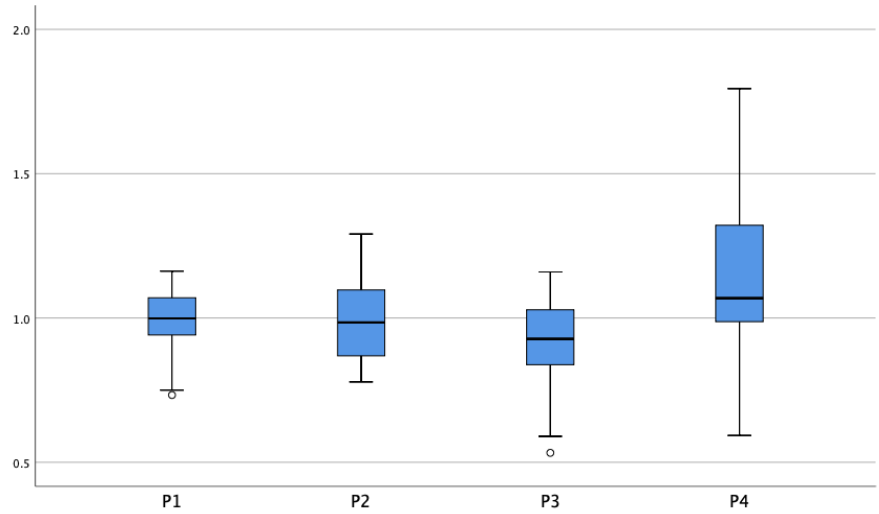

Figure 10 Mean SO2 relative to baseline during Interval 3 for each protocol.

Mauchly's test of sphericity was significant at p = 0.007, so sphericity is violated, and a significant difference between protocols was expected. With Greenhouse-Geisser epsilon at 0.681, the Greenhouse-Geisser correction was used and confirmed significant differences between protocols with p = 0.002.

Protocol 4 performed significantly superior in post-hoc analysis towards protocol 3 and statistically insignificantly superior towards protocols 1 and 2. Protocol 2 outperformed protocols 1 and 3.

Table 8 Pairwise comparisons of SO2 means relative to baseline during Interval 3 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.013	1.000
P1 vs. P3	0.062	0.968
P1 vs. P4	- 0.170	0.102
P2 vs. P3	0.075	0.223
P2 vs. P4	- 0.156	0.061
P3 vs. P4	- 0.232*	0.010

Interval 4

Table 9 SO2 mean relative to baseline during Interval 4 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4

Difference to baseline	- 0.076* (± 0.118)	- 0.047 (± 0.122)	- 0.106* (± 0.130)	- 0.012 (± 0.153)
p	0.005	0.074	0.001	0.708

The differences to the baseline during the second interval of reperfusion can be seen in table 9. All protocols were below the baseline. Protocols 1 and 3 were significantly below the baseline, while protocols 2 and 4 show a statistically insignificant decrease compared to the baseline.

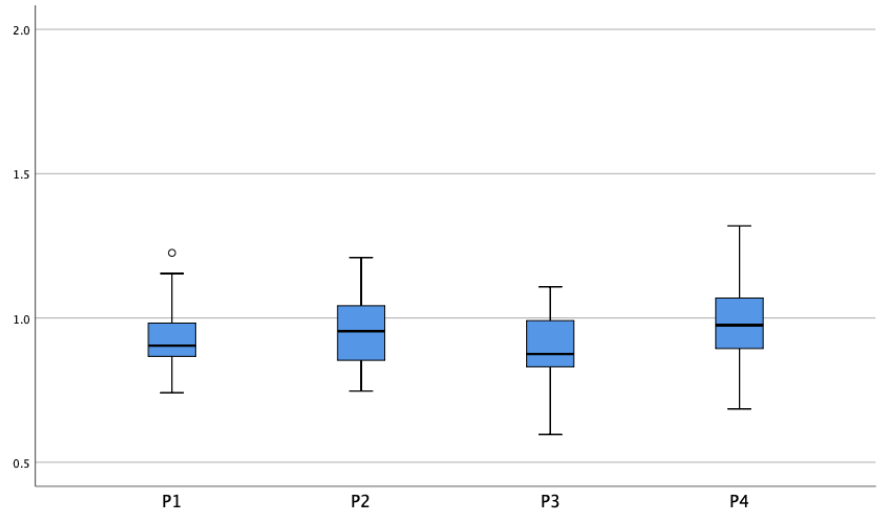

Figure 11 Mean SO2 relative to baseline during Interval 4 for each protocol.

As Mauchly's test of sphericity was insignificant (p = 0.592), no statistically significant differences existed between protocols. The post-hoc analysis showed protocol 4 outperforming all other protocols, with 2 following behind, which is in concordance with the significant differences to the baseline mentioned above.

Table 10 Pairwise comparisons of SO2 means relative to baseline during Interval 4 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.029	1.000
P1 vs. P3	0.031	1.000
P1 vs. P4	- 0.064	0.726
P2 vs. P3	0.060	0.374
P2 vs. P4	- 0.035	1.000
P3 vs. P4	- 0.094	0.129

Interval 5

Table 11 SO2 mean relative to baseline during Interval 5 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	- 0.049 (± 0.132)	- 0.038 (± 0.121)	- 0.096* (± 0.222)	0.082 (± 0.275)
p	0.082	0.136	0.044	0.159

The differences to the baseline during the third interval of ischemia can be seen in table 11. Protocols 1 and 2 were statistically insignificantly below the baseline, while protocol 4 was statistically insignificantly above the baseline. Protocol 3 was significantly below baseline by 0.096 (p = 0.044).

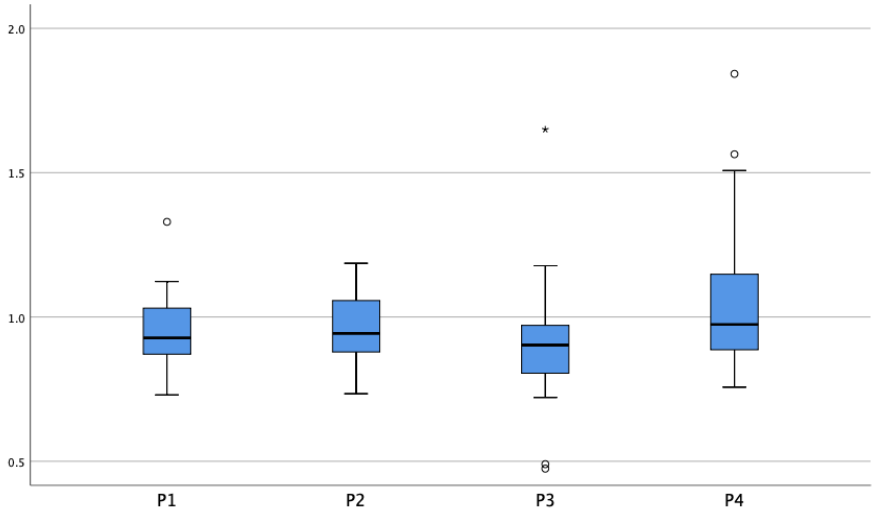

Figure 12 Mean SO2 relative to baseline during Interval 5 for each protocol.

Mauchly's test of sphericity indicates that sphericity was violated with a significance of p = 0.029. Greenhouse-Geisser epsilon was 0.762, so the Huynh-Feldt correction was used. This produced significance with p = 0.025. However, in the pairwise comparisons of the rmANOVA, no protocol was statistically significantly different from another. Comparisons of protocols 1, 2, and 3 to protocol 4 were the closest to achieving statistical significance. Protocol 1's saturation was 13% below protocol 4's (p = 0.317), protocol 2's saturation was

12% below protocol 4's (p = 0.256), and protocol 3's saturation was 17.8% below protocol 4's (p = 0.138).

Table 12 Pairwise comparisons of SO2 means relative to baseline during Interval 5 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.011	1.000
P1 vs. P3	0.048	1.000
P1 vs. P4	- 0.130	0.317
P2 vs. P3	0.058	1.000
P2 vs. P4	- 0.120	0.256
P3 vs. P4	- 0.178	0.138

Interval 6

Table 13 SO2 mean relative to baseline during Interval 6 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	- 0.087* (± 0.146)	- 0.096* (± 0.114)	- 0.151* (± 0.158)	- 0.029 (± 0.207)
p	0.008	< 0.001	< 0.001	0.498

The differences from the baseline during the third and final interval of reperfusion can be seen in table 13. Protocols 1, 2, and 3 were significantly below baseline, while protocol 4 was statistically insignificantly below baseline, meaning protocol 4 was the only protocol that, at the end of the protocol, had not dropped below the baseline. Protocol 1 dropped by 8.7% (p = 0.008), protocol 2 by 9.6% (p < 0.001), and protocol 3 by 15.1% (p < 0.001) below the baseline. Protocol 4 dropped by a statistically insignificant 2.9% (p = 0.498).

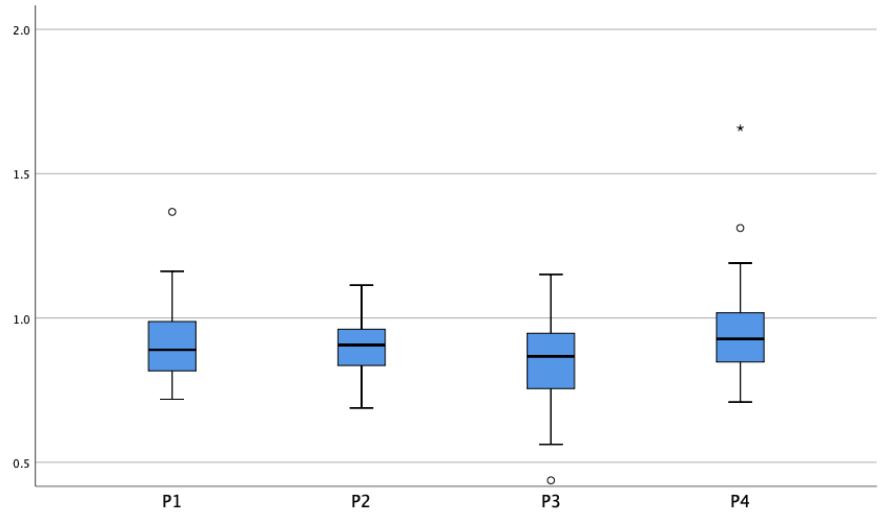

Figure 13 Mean SO2 relative to baseline during Interval 6 for each protocol.

Mauchly's test of sphericity was insignificant at p = 0.155, and thus sphericity was not violated, which infers that there was no significant difference between protocols in this interval. The post-hoc analysis comparing the protocols offered the results shown in the table below. The biggest difference between two protocols was protocol 4, which resulted in a saturation 12.2% higher than protocol 3 (p = 0.093).

Table 14 Pairwise comparisons of SO2 means relative to baseline during Interval 6 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	0.009	1.000
P1 vs. P3	0.064	0.954
P1 vs. P4	- 0.058	1.000
P2 vs. P3	0.055	0.406
P2 vs. P4	- 0.067	0.638
P3 vs. P4	- 0.122	0.093

3.2.2. rHB

Table 15 Means and standard deviations of rHb relative to baseline throughout protocols by interval.

Interval	P 1	P 2	P 3	P 4

baseline	1	1	1	1
I1	1.014 (± 0.019)	1.010 (± 0.016)	1.005 (± 0.014)	1.032 (± 0.035)
R1	0.996 (± 0.030)	0.997 (± 0.019)	0.999 (± 0.010)	0.996 (± 0.026)
I2	1.001 (± 0.038)	1.003 (± 0.027)	0.998 (± 0.017)	1.023 (± 0.036)
R2	0.995 (± 0.044)	0.997 (± 0.025)	0.994 (± 0.014)	0.998 (± 0.036)
I3	1.002 (± 0.054)	1.004 (± 0.030)	1,000 (± 0.020)	1.024 (± 0.050)
R3	0.998 (± 0.061)	1.002 (± 0.031)	0.997 (± 0.018)	1.007 (± 0.051)

Interval 1

Table 16 Difference of means of rHb to baseline during Interval 1 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	0.014* (± 0.020)	0.010* (± 0.016)	0.005 (± 0.014)	0.032* (± 0.035)
p	0.001	0.006	0.101	< 0.001

Table 16 shows the differences from the baseline during the first ischemic interval. The biggest significant difference to baseline was produced by protocol 4 with 3.2% ($p < 0.001$). A nearly equally significant, however, smaller difference was produced by protocol 1 with 1.4% increase from the baseline ($p = 0.001$). Protocol 2 resulted in a less significant and even smaller difference from the baseline. Protocol 3's difference from the baseline was small and statistically insignificant.

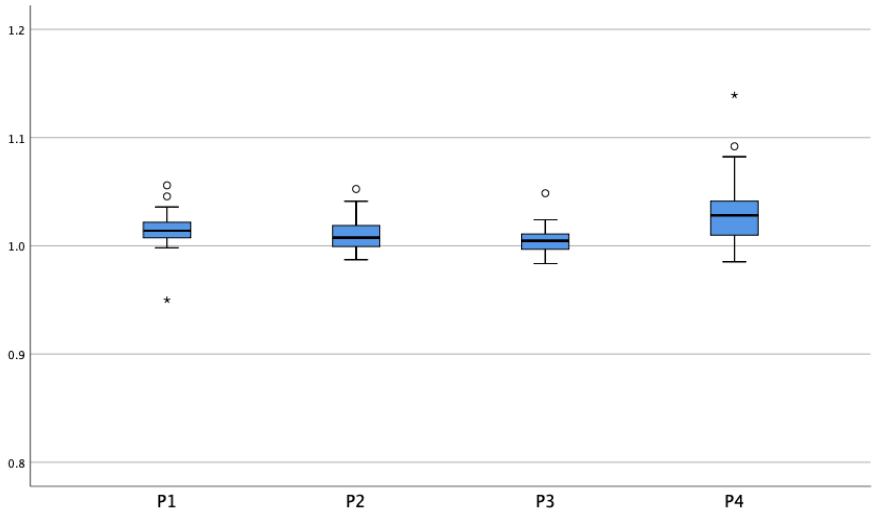

Figure 14 Mean rHb relative to baseline during Interval 1 for each protocol.

Mauchly's test of sphericity was significant at $p < 0.001$, so that sphericity was violated. Greenhouse-Geisser epsilon being 0.600, the Greenhouse-Geisser correction was used, which was significant at $p = 0.002$. The post-hoc analysis comparing the protocols offered the results shown in table 17. Protocol 4 was significantly better than protocol 2 by 2.2% ($p = 0.046$) as well as better than protocol 3 by 2.7% ($p = 0.007$). Protocol 1 was also better than protocols 2 and 3 but 1.8% less than protocol 4 ($p = 0.135$).

Table 17 Pairwise comparisons of rHb means relative to baseline during Interval 1 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	0.004	1.000
P1 vs. P3	0.010	0.243
P1 vs. P4	- 0.018	0.135
P2 vs. P3	0.005	0.531
P2 vs. P4	- 0.022*	0.046
P3 vs. P4	- 0.027*	0.007

Interval 2

Table 18 Difference of means of rHb to baseline during Interval 2 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	- 0.004 (± 0.030)	- 0.003 (± 0.019)	- 0.002 (± 0.010)	- 0.004 (± 0.026)
p	0.504	0.380	0.458	0.452

Table 18 shows the differences from the baseline during the first interval of reperfusion. There were no significant differences from the baseline. All protocols were statistically insignificantly below baseline by 0.2% - 0.4%.

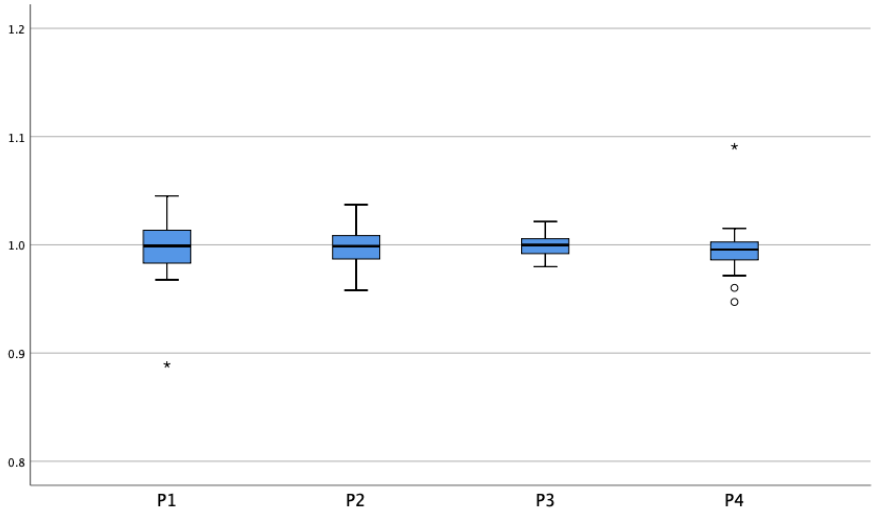

Figure 15 Mean rHb relative to baseline during Interval 2 for each protocol.

Mauchly's test of sphericity was significant at p = 0.025. With sphericity assumed, however, the significance was p = 0.958. Thus, no significant differences between protocols can be reported. Protocols 1 and 4 performed equally. Protocols 2 and 3 were insignificantly better than 1 and 4.

Table 19 Pairwise comparisons of rHb means relative to baseline during Interval 2 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.001	1.000
P1 vs. P3	- 0.003	1.000
P1 vs. P4	0.000	1.000
P2 vs. P3	- 0.002	1.000

P2 vs. P4	0.001	1.000
P3 vs. P4	0.003	1.000

Interval 3

Table 20 Difference of means of rHb to baseline during Interval 3 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	0.001 (± 0.038)	0.003 (± 0.027)	- 0.002 (± 0.017)	0.023* (± 0.036)
p	0.916	0.615	0.489	0.005

Table 20 shows the differences from the baseline during the second interval of ischemia. Protocol 4 produced a significant difference from the baseline by 2.3% (p = 0.005). Protocols 1 and 2 were statistically insignificantly above baseline, while protocol 3 was statistically insignificantly below baseline.

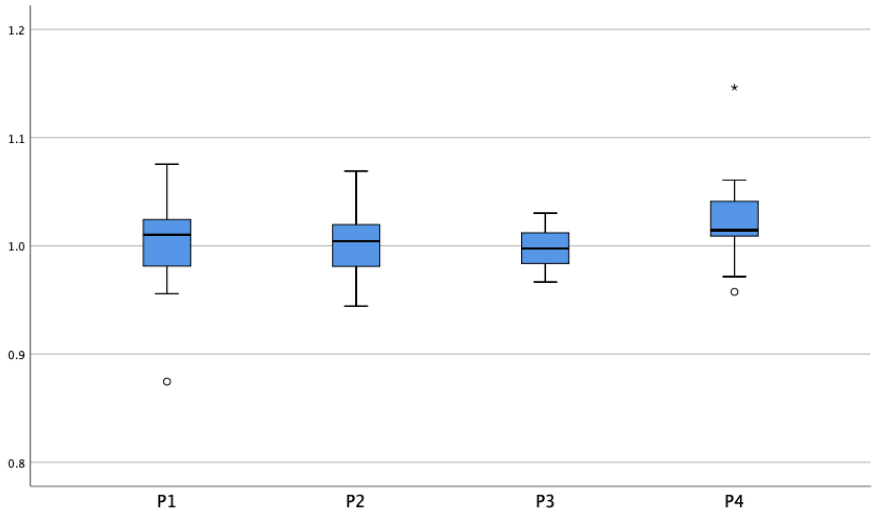

Figure 16 Mean rHb relative to baseline during Interval 3 for each protocol.

Mauchly's test of sphericity was significant at p = 0.009. With Greenhouse-Geisser epsilon at 0.77, the Huynh-Feldt correction was used to correct the violated sphericity. Huynh-Feldt correction offered significance at p = 0.004. In the post-hoc analysis, protocol 4 was more effective than protocols 1, 2, and 3. Protocols 1 and 2 were statistically insignificantly less by 2.2% (p = 0.200) and

2.0% (p = 0.094), respectively. Protocol 4 was significantly better than protocol 3 by 2.5% (p = 0.020).

Table 21 Pairwise comparisons of rHb means relative to baseline during Interval 3 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.002	1.000
P1 vs. P3	0.003	1.000
P1 vs. P4	- 0.022	0.200
P2 vs. P3	0.005	1.000
P2 vs. P4	- 0.020	0.094
P3 vs. P4	- 0.025*	0.020

Interval 4

Table 22 Difference of means of rHb to baseline during Interval 4 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	- 0.005 (± 0.044)	- 0.003 (± 0.025)	- 0.006 (± 0.014)	- 0.002 (± 0.036)
p	0.566	0.575	0.059	0.806

Table 22 shows the differences from the baseline during the second interval of reperfusion. All protocols were below the baseline. The closest protocol to being significantly below baseline was protocol 3. It was below baseline by 0.6% (p = 0.059). Protocols 1 and 2 were similarly statistically insignificantly below baseline be 0.5% (p = 0.566) and 0.3% (p = 0.059), respectively. Protocol 4 was statistically insignificantly below baseline by 0.2% (p = 0.806).

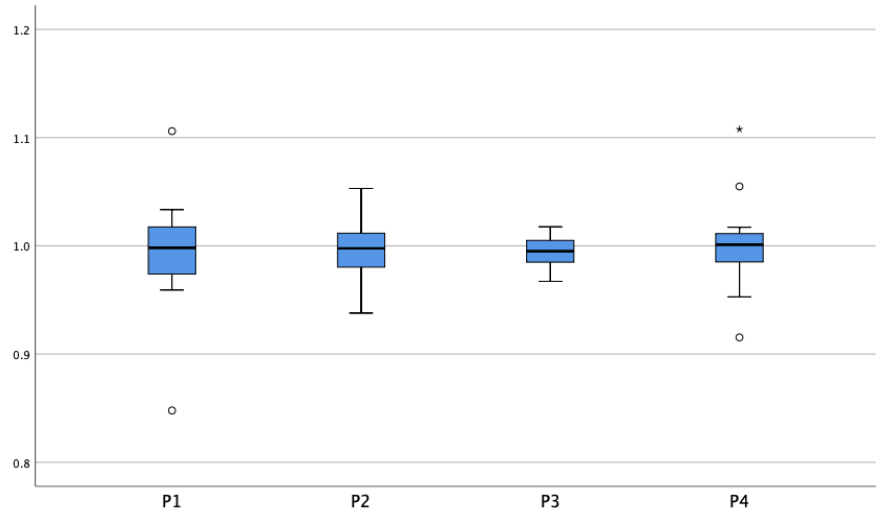

Figure 17 Mean rHb relative to baseline during Interval 4 for each protocol.

Mauchly's test of sphericity was significant at p = 0.001. With Greenhouse-Geisser epsilon at 0.625, the Greenhouse-Geisser correction was used to correct the violated sphericity. Greenhouse-Geisser, however, was at p = 0.893 and thus statistically insignificant. Accordingly, there were no significant differences between protocols.

Table 23 Pairwise comparisons of rHb means relative to baseline during Interval 4 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.002	1.000
P1 vs. P3	0.000	1.000
P1 vs. P4	- 0.003	1.000
P2 vs. P3	0.003	1.000
P2 vs. P4	- 0.001	1.000
P3 vs. P4	- 0.004	1.000

Interval 5

Table 24 Difference of means of rHb to baseline during Interval 5 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	0.002 (± 0.054)	0.004 (± 0.030)	0.000 (± 0.020)	0.024* (± 0.050)

p	0.825	0.517	0.930	0.027

Table 24 shows the differences from the baseline during the third interval of ischemia. All protocols were above or equal to the baseline. Protocol 4 was the only protocol that was significantly above baseline by 2.4% (p = 0.027). Protocol 3 showed no mean difference in this paired t-test. Protocols 1 and 2 showed minimal mean differences with a 0.2% (p = 0.825) and a 0.4% (p = 0.517) increase to baseline.

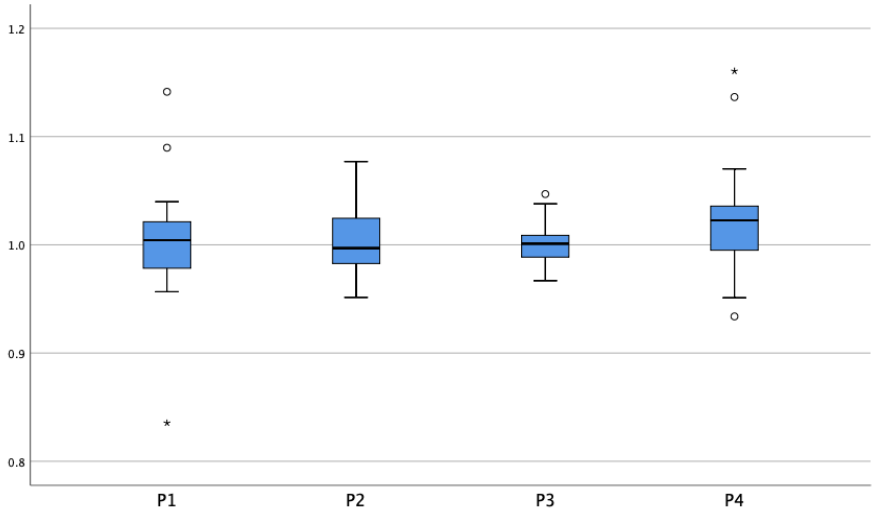

Figure 18 Mean rHb relative to baseline during Interval 5 for each protocol.

Mauchly's test of sphericity was significant at p = 0.003. With Greenhouse-Geisser epsilon at 0.724, the Greenhouse-Geisser correction was used. It was insignificant with p = 0.129. All post-hoc comparisons were statistically insignificant. The biggest difference was between protocol 3 and 4. Protocol 4 was 2.5% more effective than protocol 3 in this interval (p = 0.184).

Table 25 Pairwise comparisons of rHb means relative to baseline during Interval 5 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.002	1.000
P1 vs. P3	0.003	1.000
P1 vs. P4	- 0.022	0.869
P2 vs. P3	0.004	1.000

P2 vs. P4	- 0.020	0.363
P3 vs. P4	- 0.025	0.184

Interval 6

Table 26 Difference of means of rHb to baseline during Interval 6 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	- 0.002 (± 0.061)	0.002 (± 0.031)	- 0.003 (± 0.004)	0.007 (± 0.051)
p	0.852	0.769	0.389	0.507

Table 26 shows the differences from the baseline during the third and final reperfusion interval. Protocols 1 and 3 were statistically insignificantly below baseline by 0.2% (p = 0.852) and 0.3% (p = 0.0389), respectively. Protocols 2 and 4 were above baseline by 0.2% (p = 0.769) and 0.7% (p = 0.507), respectively.

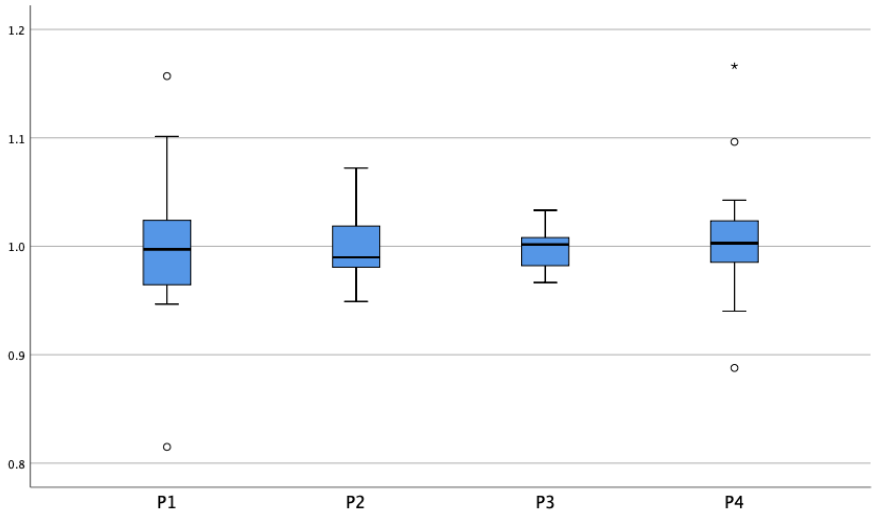

Figure 19 Mean rHb relative to baseline during Interval 6 for each protocol.

Mauchly's test of sphericity was significant at p < 0.001. Due to a Greenhouse-Geisser epsilon of 0.642, the Greenhouse-Geisser correction was used to correct violated sphericity. Greenhous-Geisser was not statistically significant, with p = 0.705. All protocols showed no statistically significant difference from each other.

Statistically insignificantly protocol 4 was the better protocol for rHB in the last reperfusion interval.

Table 27 Pairwise comparisons of rHb means relative to baseline during Interval 6 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.004	1.000
P1 vs. P3	0.001	1.000
P1 vs. P4	- 0.009	1.000
P2 vs. P3	0.005	1.000
P2 vs. P4	- 0.005	1.000
P3 vs. P4	- 0.010	1.000

3.2.3. Flow

Table 28 contains raw Flow data. Table 29 shows the transformed Flow data to achieve normality within the data to provide more accurate sphericity although it has, as previously mentioned, been proven that rmANOVA is robust against violations of sphericity and normality (90, 91).

Table 28 Means and standard deviations of Flow compared to baseline throughout protocols by interval.

Interval	P 1	P 2	P 3	P 4
baseline	1	1	1	1
I1	1.202 (± 0.237)	1.117 (± 0.261)	1.026 (± 0.209)	1.981 (± 0.992)
R1	0.976 (± 0.094)	1.000 (± 0.116)	0.961 (± 0.095)	1.080 (± 0.292)
I2	0.991 (± 0.129)	1.030 (± 0.179)	1.039 (± 0.159)	1.573 (± 0.757)

R2	0.895 (± 0.168)	0.946 (± 0.132)	0.946 (± 0.114)	1.025 (± 0.205)
I3	0.935 (± 0.232)	0.965 (± 0.125)	0.943 (± 0.157)	1.406 (± 0.688)
R3	0.869 (± 0.186)	0.904 (± 0.125)	0.903 (± 0.142)	0.989 (± 0.206)

Table 29 Transformed means and standard deviations of Flow compared to baseline throughout protocols by interval.

Interval	P 1	P 2	P 3	P 4
baseline	1	1	1	1
I1	0.074 (±.073)	0.039 (± 0.086)	0.004 (± 0.077)	0.251 (± 0.199)
R1	- 0.014 (± 0.042)	- 0.003 (± 0.050)	- 0.019 (± 0.041)	0.022 (± 0.093)
I2	- 0.008 (± 0.056)	0.006 (± 0.075)	0.012 (± 0.064)	0.158 (± 0.181)
R2	- 0.056 (± 0.082)	- 0.029 (± 0.063)	- 0.027 (± 0.051)	0.003 (± 0.082)
I3	- 0.042 (± 0.107)	- 0.019 (± 0.058)	- 0.031 (± 0.073)	0.110 (± 0.176)
R3	- 0.071 (± 0.100)	- 0.048 (± 0.062)	- 0.049 (± 0.066)	- 0.013 (± 0.086)

Interval 1

Table 30 Difference of means of Flow to baseline during Interval 1 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	0.202* (± 0.237)	0.117* (± 0.261)	0.026 (± 0.209)	0.981* (± 0.992)
p	< 0.001	0.038	0.553	< 0.001

Table 30 shows the differences to the baseline during the first interval of ischemia. All protocols' means were above the baseline, and all but protocol 3's was statistically significantly above the baseline. Protocol 2 was 11.7% (p = 0.038) above the baseline. Protocols 1 and 4 were equally significantly above the baseline with p < 0.001. Protocol 1 was 20.2%, and protocol 4 was 98.1% above the baseline.

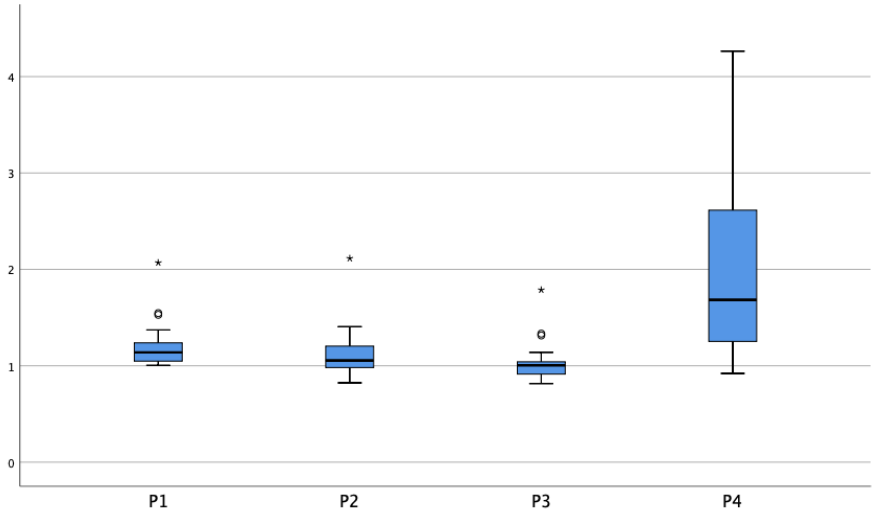

Figure 20 Mean Flow relative to baseline during Interval 1 for each protocol.

Mauchly's test of sphericity was significant at p < 0.001, and Greenhouse-Geisser epsilon was 0.517, so the Greenhouse-Geisser correction was used to correct violated sphericity. Greenhouse-Geisser was significant at p < 0.001, so a difference between protocols was expected.

Table 31 shows the post-hoc analysis of the rmANOVA. Protocol 1 was significantly better than protocol 3, with p = 0.003. Protocol 4 was significantly better than all other protocols in direct comparison. Protocol 4 was better than protocols 1 and 2 with p = 0.001 and better than protocol 3 with p < 0.001.

Table 31 Pairwise comparisons of transformed Flow means relative to baseline during Interval 1 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	0.035	0.634
P1 vs. P3	0.070*	0.003
P1 vs. P4	- 0.177*	0.001
P2 vs. P3	0.035	0.585
P2 vs. P4	- 0.212*	0.001
P3 vs. P4	- 0.247*	0.000

Interval 2

Table 32 Difference of means of Flow to baseline during Interval 2 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	- 0.024 (± 0.094)	0.000 (± 0.116)	- 0.039 (± 0.095)	0.079 (± 0.292)
p	0.227	0.994	0.057	0.197

Table 32 shows the differences from the baseline during the first interval of reperfusion. Protocols 1 and 3 were below baseline by 2.4% (p = 0.227) and 3.9% (p = 0.057), respectively. Protocol 2 showed no mean difference from baseline at p = 0.994. Finally, protocol 4 was the only protocol above baseline by 7.9%; however statistically insignificant with p = 0.197.

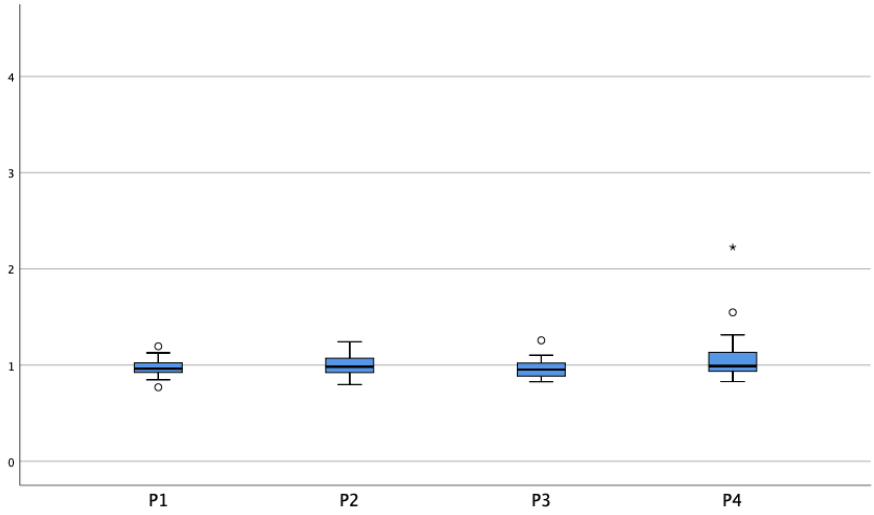

Figure 21 Mean Flow during Interval 2 relative to baseline for each protocol.

Mauchly's test of sphericity was significant at $p < 0.001$ and Greenhouse-Geisser epsilon at 0.569, so the Greenhouse-Geisser correction was used. Greenhouse-Geisser was insignificant at $p = 0.160$. Accordingly, no significant differences in post-hoc analysis were expected. The closest difference to achieving statistical significance was protocol 4 outperforming protocol 3 with $p = 0.525$.

Table 33 Pairwise comparisons of transformed Flow means relative to baseline during Interval 2 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.010	1.000
P1 vs. P3	0.007	1.000
P1 vs. P4	- 0.034	0.596
P2 vs. P3	0.016	0.878
P2 vs. P4	- 0.025	1.000
P3 vs. P4	- 0.041	0.525

Interval 3

Table 34 Difference of means of Flow to baseline during Interval 3 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	- 0.009 (± 0.129)	0.030 (± 0.179)	0.039 (± 0.159)	0.573* (± 0.757)
p	0.733	0.427	0.247	0.001

Table 34 shows the differences from the baseline during the second interval of ischemia. Protocol 1 was statistically insignificantly below the baseline by 0.9% ($p = 0.733$). Protocols 2 and 3 were statistically insignificantly above the baseline by 3% ($p = 0.427$) and 3.9% ($p = 0.247$), respectively. Protocol 4 was significantly above the baseline by, on average, 57.3% ($p = 0.001$).

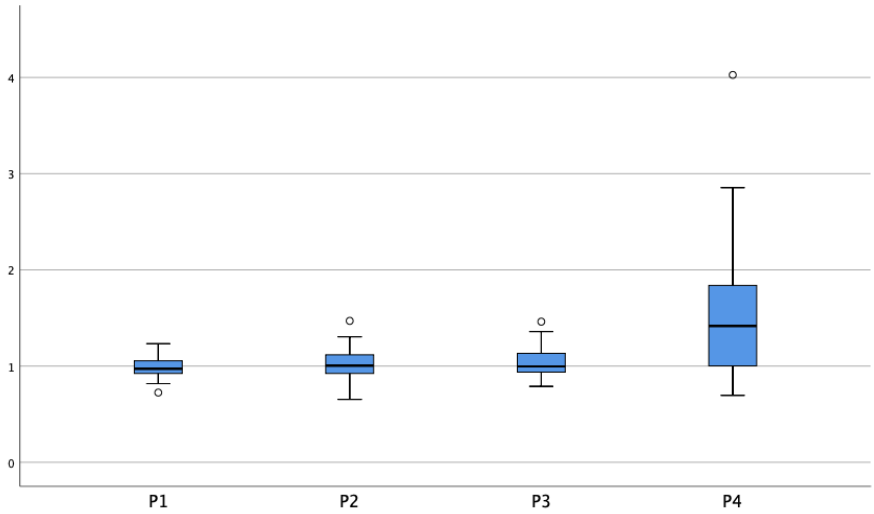

Figure 22 Mean Flow relative to baseline during Interval 3 for each protocol.

Mauchly's test of sphericity was significant at $p < 0.001$. Greenhouse-Geisser epsilon was 0.551, so the Greenhouse-Geisser correction was used. This was significant at $p < 0.001$. In a post-hoc analysis, the protocols performed significantly differently well.

Protocol 4 was significantly different from all other protocols. Post-hoc comparisons between all other protocols delivered a difference with significance $p = 1.000$. Protocol 4 was better than protocol 1 with $p = 0.002$, better than protocol 2 with $p=0.005$, and better than protocol 3 with $p = 0.009$.

Table 35 Pairwise comparisons of transformed Flow means relative to baseline during Interval 3 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.014	1.000
P1 vs. P3	- 0.019	1.000
P1 vs. P4	- 0.165*	0.002
P2 vs. P3	- 0.005	1.000
P2 vs. P4	- 0.151*	0.005
P3 vs. P4	- 0.146*	0.009

Interval 4

Table 36 Difference of means of Flow to baseline during Interval 4 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	- 0.105* (± 0.168)	- 0.055 (± 0.132)	- 0.054* (± 0.114)	0.025 (± 0.205)
p	0.005	0.054	0.030	0.552

Table 36 shows the differences from the baseline during the second interval of reperfusion. Solely protocol 1 showed a statistically significant difference at 10.5% (p = 0.005) below the baseline. Protocols 2 and 3 were statistically insignificantly below the baseline by 5.5% (p = 0.054) and 5.4% (p = 0.030), respectively. Protocol 4 was statistically insignificantly above the baseline by 2.5% (p = 0.552).

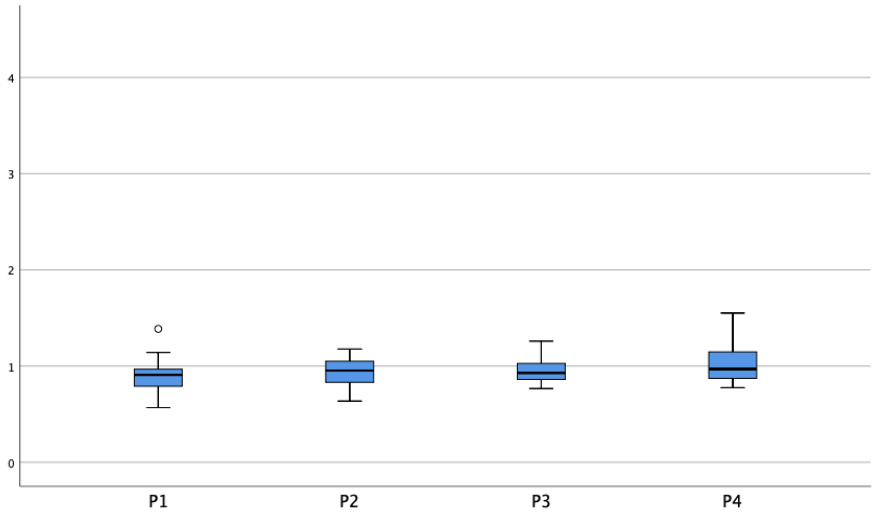

Figure 23 Mean Flow relative to baseline during Interval 4 for each protocol.

Mauchly's test of sphericity was significant at p = 0.004, and Greenhouse-Geisser epsilon was 0.674. The appropriate Greenhouse-Geisser correction was statistically insignificant at p = 0.094. The post-hoc analysis did not produce any significant differences between protocols which is in line with the aforementioned values. The pairwise comparisons showed that the difference closest to significance was protocol 1 versus protocol 4. Protocol 1 showed less flow than protocol 4, with p = 0.269.

Table 37 Pairwise comparisons of transformed Flow means relative to baseline during Interval 4 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.027	0.708
P1 vs. P3	- 0.029	0.738
P1 vs. P4	- 0.059	0.269
P2 vs. P3	- 0.002	1.000
P2 vs. P4	- 0.032	1.000
P3 vs. P4	- 0.030	1.000

Interval 5

Table 38 Difference of means of Flow to baseline during Interval 5 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	- 0.065 (± 0.232)	- 0.035 (± 0.125)	- 0.057 (± 0.157)	0.406* (± 0.688)
p	0.186	0.180	0.088	0.008

Table 38 shows the differences from the baseline during the third interval of ischemia. Protocols 1, 2, and 3 all averaged statistically insignificantly below the baseline. Protocol 2 was closest to baseline, with an average of 3.5% below baseline at p = 0.180. Protocol 4 was significantly above baseline by 40.6% (p = 0.008).

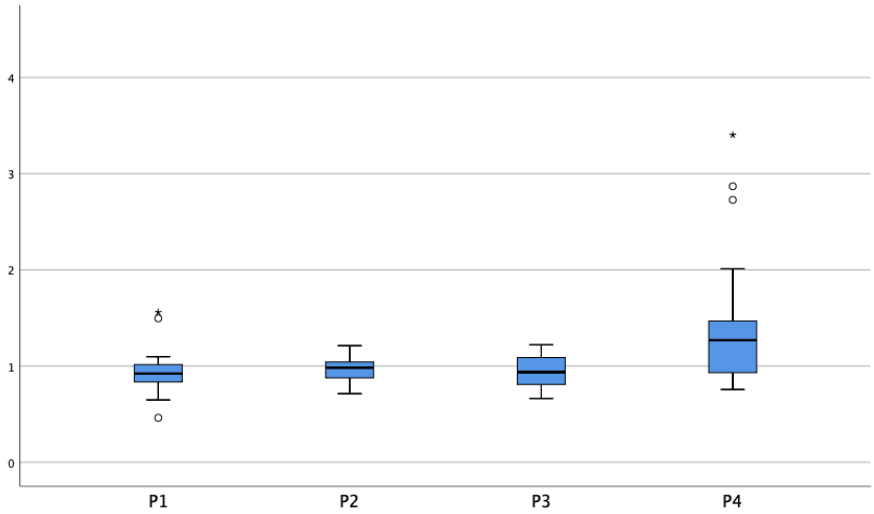

Figure 24 Mean Flow relative to baseline during Interval 5 for each protocol.

Mauchly's test of sphericity was significant at $p < 0.001$. Greenhouse-Geisser epsilon was at 0.475, resulting in the use of Greenhouse-Geisser correction. Greenhouse-Geisser correction was significant at $p = 0.003$. The post-hoc analysis was therefore expected to show significant differences between protocols. Protocol 4 significantly produced more Flow compared to the others. Protocol 4 was better than protocol 1 with $p = 0.044$, better than protocol 2 with $p = 0.022$, and better than protocol 3 with $p = 0.011$. Protocols 1, 2, and 3 had no significant differences as $p = 1.000$ for all inter-protocol comparisons.

Table 39 Pairwise comparisons of transformed Flow means relative to baseline during Interval 5 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.022	1.000
P1 vs. P3	- 0.010	1.000
P1 vs. P4	- 0.151*	0.044
P2 vs. P3	0.012	1.000
P2 vs. P4	- 0.129*	0.022
P3 vs. P4	- 0.141*	0.011

Interval 6

Table 40 Difference of means of Flow to baseline during Interval 6 by protocol, p < 0.05 significant (*).

	P1	P2	P3	P4
Difference to baseline	- 0.131* (± 0.186)	- 0.096* (± 0.125)	- 0.097* (± 0.142)	- 0.011 (± 0.205)
p	0.002	0.001	0.003	0.802

Table 40 shows the differences from the baseline during the third and final reperfusion interval. Protocols 1, 2, and 3 all averaged significantly below the baseline. Protocol 1 was 13.3% (p = 0.002), protocol 2 was 9.6% (p = 0.001), and protocol 3 was 9.7% (p = 0.003) below the baseline. Protocol 4 was statistically insignificantly below the baseline and thus closest to it out of all protocols. Protocol 4 was 1.1% (p = 0.802) below the baseline in the final interval of reperfusion.

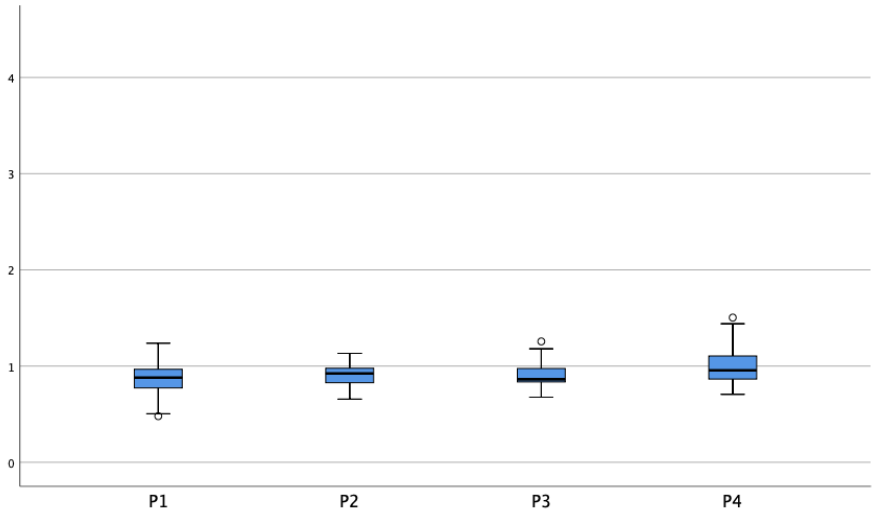

Figure 25 Mean Flow relative to baseline during Interval 6 for each protocol.

Mauchly's test of sphericity was significant at p = 0.17 and Greenhouse-Geisser epsilon was 0.699. The Greenhouse-Geisser correction was statistically insignificant at p = 0.126. Post-hoc analysis accordingly did not produce any significant differences between protocols. Closest to significance was the comparison of protocols 1 and 4 at p = 0.474, in which protocol 4 had the higher amount of Flow.

Table 41 Pairwise comparisons of transformed Flow means relative to baseline during Interval 6 by protocol, p < 0.05 significant (*).

	Mean Difference	Sig.
P1 vs. P2	- 0.023	1.000
P1 vs. P3	- 0.022	1.000
P1 vs. P4	- 0.058	0.474
P2 vs. P3	0.001	1.000
P2 vs. P4	- 0.035	1.000
P3 vs. P4	- 0.036	0.754

3.3. Protocol tolerance

Participants were asked to assign a level of comfort to each protocol immediately after completing a protocol. The Numeric Pain Rating Scale (NPRS) of 0-10 was used, with 0 being the highest level of comfort and 10 being the highest level of discomfort. The mean NRPS ratings for each protocol can be seen in table 42.

Table 42 Means of NRPS ratings for each protocol.

	P1	P2	P3	P4
Mean overall protocol-comfort level	5.79 (± 1.444)	3.88 (± 1.569)	2.71 (± 1.459)	6.71 (± 1.517)

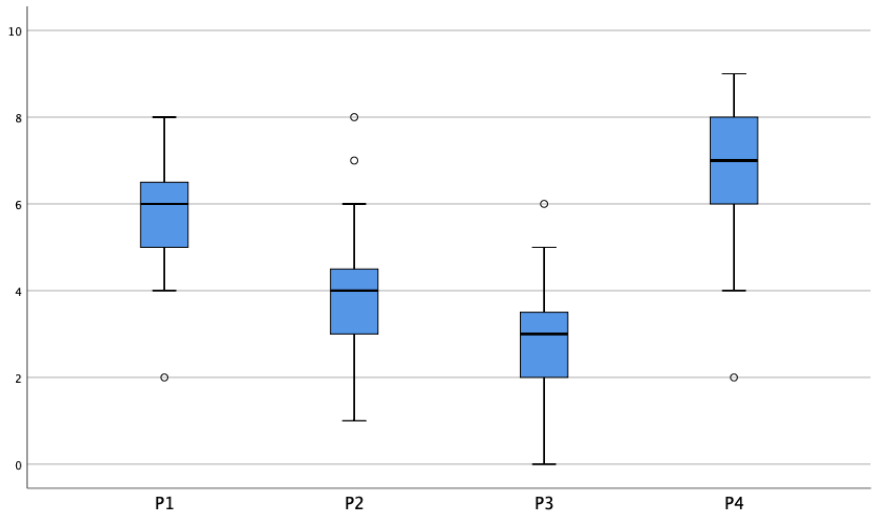

Figure 26 Means of comfort rating on NPRS for each protocol.

Mauchly's test of sphericity was insignificant at p = 0.324. Greenhouse-Geisser epsilon was 0.872, so Huynh-Feldt was used to correct violated sphericity. Huynh-Feldt was significant at p < 0.001. Post-hoc analysis delivered multiple significant comfort differences between protocols. Protocols 2 and 3 were significantly differently comfortable from protocols 1 and 4, with all differences' p-value at < 0.001. Protocols 2 and 3 were also significantly different from each other as protocol 2 was, on average, 1.167 points more uncomfortable (p = 0.001). Protocols 1 and 4 were statistically insignificantly different, as protocol 1 was 0.917 more comfortable than protocol 4 (p = 0.118).

Table 43 Pairwise comparisons of mean NRPS ratings.

	Mean Difference	Sig.
P1 vs. P2	1.917*	0.000
P1 vs. P3	3.083*	0.000
P1 vs. P4	- 0.917	0.118
P2 vs. P3	1.167*	0.001
P2 vs. P4	- 2.833*	0.000
P3 vs. P4	- 4.000*	0.000

3.4. Duration of Ischemia in protocols

Table 44 Mean duration of ischemia interval lengths by protocol.

	P1	P2	P3	P4
Mean overall ischemia interval length (s)	300.00 (± 0.00)	192.81 (± 67.70)	97.54 (± 48.66)	141.93 (± 59.31)

Table 44 shows the mean length of all ischemic intervals (intervals 1, 3, and 5) combined for each protocol. Protocol 1 was defined by 5 minutes of ischemia alternating with 10 minutes of reperfusion so that all ischemic intervals had the exact duration of 300 seconds. Protocols 2, 3, and 4, were saturation controlled and thus resulted in different durations of ischemia. During protocol 2, subjects took 192.81 seconds (± 67.70) to desaturate to a level of < 10% for three seconds. Protocol 3 ended ischemia after a level of < 30 % was detected for three seconds.

Subjects needed 97.54 seconds (± 48.66) on average. During protocol 4, which included opening and closing the ischemia-subjected hand, subjects needed 141.93 seconds (± 59.31) to desaturate to a level of < 10% for three seconds. Mauchly's test of sphericity was significant at p = 0.026, and Greenhouse-Geisser epsilon was 0.898. The Huyhn-Feldt correction was therefore used and was significant at p < 0.001. Post-hoc analysis showed that all protocols differed from each other in a statistically significant way. All comparisons were significant at p < 0.001.

Table 45 Pairwise comparisons of mean interval length of ischemia intervals.

	Mean Difference	Sig.
P1 vs. P2	107.19*	< 0.001
P1 vs. P3	202.46*	< 0.001
P1 vs. P4	158.07*	< 0.001
P2 vs. P3	95.26*	< 0.001
P2 vs. P4	50.88*	< 0.001
P3 vs. P4	- 44.39*	< 0.001

3.5. Summary of Results

The results of each parameter are summarized in the following paragraphs to provide a basis for the discussion. SO2 was initially significantly above the baseline during I1 in protocols 1, 2, and 4. Protocol 4 showed the greatest increase of 34% from the baseline at p < 0.001. Protocol 3 dropped significantly below baseline during R1 by 6.6% at p = 0.004, while the other protocols remained statistically insignificantly below baseline. During I2, protocol 4 was the only protocol to be significantly above the baseline by 15.5% and p = 0.015. Protocol 3 was significantly below the baseline, while protocols 1 and 2 were statistically insignificantly below the baseline. In R2 all protocols were below the baseline. Protocols 1 and 3 were significantly below the baseline, while protocols 2 and 4 were statistically insignificantly below the baseline. Protocol 4 was below baseline by 1.2% (p = 0.708), while protocol 3 was below baseline by 10.6% (p = 0.001). During I3, all but protocol 4 were below the baseline. Protocol 3 was significantly below the baseline by 9.6% (p = 0.044). Protocol 4 stayed above the baseline by 8.2% on average with p = 0.159. During final reperfusion in R3, all

protocols but protocol 4 were significantly below baseline. Protocol 1 was 8.7% ($p = 0.008$), protocol 2 was 9.6% ($p < 0.001$) and protocol 3 was 15.1% ($p < 0.001$) below the baseline. Protocol 4 averaged 2.9 % below the baseline with an insignificant $p = 0.498$.

In SO2 comparisons by protocol with rmANOVA, protocol 4 was significantly better than all other protocols during I1. The only other significant difference was protocol 4 outperforming protocol 3 in I2 by 23.2% ($p = 0.010$). All other intervals showed no significant differences in the rmANOVA comparisons.

The rHb significantly increased during I1 in protocols 1, 2, and 4. Protocol 1 increased it by 1.4% ($p = 0.001$), protocol 2 increased it by 1.0% ($p = 0.006$), and protocol 4 increased it most significantly by 3.2% ($p < 0.001$). During R1, all protocols' rHb dropped similarly statistically insignificantly below the baseline. I2 saw an increase above baseline in rHb in all but protocol 3. During R2, all protocols dropped statistically insignificantly below the baseline. In I3, protocol 4 was the only one to significantly raise rHb above baseline by 2.4% ($p = 0.027$). Protocols 1 and 2 showed an insignificant increase from baseline, while protocol 3 remained on average exactly at baseline. During R3, protocols 1 and 3 statistically insignificantly lowered rHb below the baseline, while protocols 2 and 4 statistically insignificantly raised it above the baseline.

In the rmANOVA comparisons protocol 4 yielded significantly higher results than protocols 2 and 3 in the first interval by 2.2% ($p = 0.046$) and 2.7% ($p = 0.007$), respectively. In I2, protocol 4 was increased rHB significantly compared to protocol 3 ($p = 0.20$). No other significant differences were recorded.

Flow began with the familiar pattern of protocols 1, 2, and 4 producing significantly more Flow compared to the baseline in I1. Protocol 1 produced 20.2% ($p < 0.001$), protocol 2 11.7% ($p = 0.038$), and protocol 4 98.1% ($p < 0.001$) more Flow. During R1, protocols 1 and 3 dropped insignificantly below the baseline, while protocol 2 remained at the baseline on average. Protocol 4 statistically insignificantly increased Flow by 7.9% ($p = 0.197$). In I2, protocol 1's Flow was, on average, below the baseline by 0.9% ($p = 0.733$), while protocol 2 and 3 raised Flow statistically insignificantly above the baseline. Protocol 4 produced a significant 57.3% ($p = 0.001$) increase in Flow. In R2, protocols 1 and 3s' Flow was significantly below baseline, while in protocol 2, Flow was statistically insignificantly below the baseline. On the other hand, protocol 4 was

the only protocol to elicit above-baseline Flow on average in R2 by increasing it statistically insignificantly by 2.5% ($p = 0.552$). During I3, protocols 1, 2, and 3 remained statistically insignificantly below the baseline. Protocol 4, however, was significantly above the baseline, producing an average 40.6% ($p = 0.008$) increase in Flow. R3 found protocols 1, 2, and 3 to remain significantly below the baseline on average, while protocol 4 was statistically insignificantly below the baseline by 1.1% ($p = 0.802$).

The rmANOVA comparisons showed a clear dominance of protocol 4 in I1, with it performing significantly better than protocol 1 by a transformed 0.177 ($p = 0.001$), better than protocol 2 by a transformed 0.212 ($p = 0.001$), and better than protocol 3 by a transformed 0.247 ($p < 0.001$). Protocol 1 also performed significantly better than protocol 3 by a transformed 0.070 ($p = 0.003$). This shows an apparent weakness of protocol 3. In I2 and I3 protocol 4 produced significantly higher Flow than all other protocols.

4. Discussion

This clinical study's purpose was to compare the effectiveness of various RIC protocols to further elucidate the RIC effects' mechanism and dependencies and advance the determination of the optimal RIC protocol for clinical application. Due to its microcirculatory benefits, the RIC effect has the potential to be a tool in the plastic surgeon's armamentarium. RIC's prospective application is in the care and treatment of any surgeries involving flaps. Baumeister et al. thoroughly analyzed the complication rate of flaps in a realistic multimorbid patient group and found that 59% experienced some complication (6). Furthermore, they described 36 %, the majority of the complications, were either partial or complete necrosis. Almost all complications required a revision surgery resulting in 54% of patients undergoing a second operation. As many of these complications can arise from poor microcirculation in the flap, any procedure that increases microcirculation and lowers complication rates is clinically indicated.

The desire to enhance tissue survival following ischemic events and protect tissue from potential future ischemia is part of many specialties of medicine. While there has been an enormous amount of research in cardiology, nephrology, and neurology, there has been, comparatively speaking, little research in plastic surgery and its unique ischemic events and demands. Many approaches to

improving the cutaneous microcirculation in flaps have failed in the stages of animal testing (7, 8). Some methods that have proven more effective, such as hypothermic preconditioning and extracorporeal shock wave treatment, are inconvenient and thus have not found a clinical application (9, 10). RIC is a promising method that fulfills the prerequisites for making clinical application likely: simplicity, effectivity, and low cost.

Ischemic conditioning research first began in the field of cardiology. In 1986 Murry et al. published a study showing that the infarct area in dog hearts could be reduced if the area was preconditioned with multiple rounds of ischemia (52). Przyklenk et al. in 1993 showed that the ischemic conditioning effect protected tissues outside of the conditioned area, and Gho et al. found protection outside of the conditioned organ in 1996 (54, 55). In addition, Gho et al. concluded that remote preconditioning was just as effective as direct preconditioning (55). This research laid the groundwork for the following adaptation into the field of plastic surgery. First, the protocols were tested in the animal model. Here studies showed that using a tourniquet to induce remote ischemia produced a RIC effect and resulted in less necrotic tissue following a future ischemic event (57). The translation to the human body occurred in 2002 when Kharbanda et al. used the now standard protocol of three cycles of 5 minutes of ischemia to produce extremity ischemia as the trigger for the RIC effect (58). The question of which extremity is best suited to provoke the RIC effect was answered by Kolbenschlag et al., as they concluded that the upper extremity performs superior to the lower extremity (81). They additionally continued the search for the optimal RIC protocol. In their study, a protocol with three cycles of ten minutes of ischemia was more effective than other protocols that contained shorter ischemia cycles (83). As their research was done on healthy individuals, the RIC effect was quantified by measuring saturation, blood flow, and relative hemoglobin in the region of the anterolateral thigh (83). This is in line with Kraemer et al.'s publication showing the acute RIC effect measurable at a remote cutaneous location (95). To conclude, this study's role in current literature is to judge protocols not solely dependent on time using the established methods of quantifying the acute RIC effect.

We continue searching for the optimal RIC protocol for clinical application and aim to shed further light on RIC mechanisms. To date, all RIC research has been

conducted with solely time-dependent protocols. The method of RIC using an extremity as the ischemia-tolerating tissue creates the opportunity to measure the level of ischemia of this extremity distally. It is possible to control how ischemic the extremity will become. Therefore, the question begging to be answered is whether the level of extremity ischemia is relevant to the RIC effect and, if so, what level needs to be achieved to produce a systemic effect. To our knowledge, this is the first study to use the saturation level of the remote extremity to control the RIC protocol.

The protocols were dependent on various factors. Protocol 1 was merely time dependent. Protocols 2 and 3 are saturation dependent. Protocol 4 is a mixture as it is controlled by the level of saturation and is designed to reach a specific level quicker than protocol 2. It thus is a combination of time and saturation.

4.1. Material & Methods

4.1.1. Subject selection

Going into the subject selection, the primary thought was to find as close to a homogenous group as possible to isolate the RIC effect from any age and health-related distractors. The subject group with a mean age of 24.67 years (± 3.52) is young in comparison to the average age of patients receiving myocutaneous flaps as treatment (96). It is very similar to Kraemer et al.'s study groups' age range when he described and quantified the acute RIC effect in cutaneous tissue (95). Aging affects cutaneous microcirculation significantly, and Roustit et al. mention it as the first factor to be aware of when conducting studies using laser spectroscopy to measure microcirculation (97). Munce et al. discovered a significant decrease in local skin blood flow response to capsaicin application starting at age 40 (86). No participant was, therefore, above the age of 40. It is thus the question if results of this subject group are transferable to the average skin flap patient. Nevertheless, a younger subject group was selected to avoid multimorbid patients with varying degrees of, e.g., hypertonic and subcutaneous fat adipose tissue (98). Adipose tissue blood flow is negatively correlated to BMI, so all subjects were supposed to have similar BMI to exclude BMI as a distracting factor (98).

The significant difference in heights between males and females is in line with the average heights of the German population. The average height in Germany was

recorded at 1.80 m for males and 1.66 m for females, showing a similar difference between the biological sexes (99).

Physical activity was a parameter amongst the collected data that showed a significant difference between males and females. While there have not been many studies regarding cutaneous microcirculation during exercise, there have been a few in the animal model and human skeletal and cardiac muscle. These studies, however, compared sedentary controls to professional cyclists or multiple training sessions a week (100, 101). While there is a difference between males and females in our study population, the difference is not comparable to the difference between the control and trained groups of the cited studies. Since these studies have, however, proven microcirculatory differences in physically active versus sedentary organisms, this information is relevant to cutaneous microcirculatory studies and should be considered when selecting subjects.

A category that should not go unmentioned is the topic of contraception in females. The hormones estrogen and progesterone, which are part of contraception methods and play a role in the female cycle, affect cutaneous microcirculation. This can be seen in patients with chronic liver disease where decompensated patients produce less sex hormone binding globulin, which results in an estrogen/testosterone disbalance favoring estrogen. This results in symptoms such as palmar erythema, which is linked to increased microcirculation. Studies have shown that estrogen and progesterone independently increase cutaneous vascular conductance (CVC), a measure for cutaneous microcirculation in response to thermal hyperemia without changing the CVC plateau (102). 5 of 12 females used hormonal contraception, and the average duration of hormonal contraception was 3.80 years (± 2.51). Further studies need to be conducted regarding hormonal contraception and its effects on the RIC effect.

Smoking is another factor that was assessed when selecting our subjects. The average pack years of subjects were 0.07 (± 0.23). No subjects reported regular smoking. Monfrecola et al. showed that smoking a single cigarette decreased cutaneous blood flow (103). Additionally, they concluded that cutaneous microcirculation recovered after 2 and 5 minutes in nonsmokers and smokers, respectively (103). As no subjects smoked the day of their measurement, the last

cigarette, even of the occasional smoker, was safely outside the reduced cutaneous microcirculatory window.

Caffeine consumption was another variable assessed that affects cutaneous microcirculation. Melik et al. have shown that caffeine ingestion reduces cutaneous post-occlusive reactive hyperemia (104). The acute effect on cutaneous microcirculation has been quantified at one-hour post-ingestion (105). No measurements were conducted first thing in the morning. All measurements were conducted in the afternoon or evening to avoid acute post-caffeine intake effects on microcirculation. The effect of caffeine on the RIC effect has yet to be studied.

Alcohol consumption is known to cause some people to develop flushing, which is an effect that facilitates cutaneous microvasculature. No subjects had ingested alcohol before any measurement on the day of their measurement. Any acute effects of increased microcirculation on the RIC effect were thus avoided.

In conclusion, the study population is a homogenous young group of males and females without comorbidities, taking no medication, and without current cutaneous issues. They indulged in no social habits with acute effects on cutaneous microcirculation, as Roustit et al. recommended (97). This is why they were accepted into the study and provided ideal and unaltered microcirculatory feedback.

4.1.2. Material

We used the same devices as previous research on this topic to record the acute RIC effects. In RIC research in humans, the O2C device by LEA Medizintechnik GmbH has been the standard device as it combines the techniques of laser doppler spectroscopy and white light spectroscopy into one sensor and one device. Many previous studies have established it as a reliable and eligible measuring device (11, 66, 81, 83, 95, 106). Like other authors, we were also confronted with handling arbitrary units. We applied relative units to the baseline to show increases and decreases (11). We transformed the oxygen saturation into relative units to avoid differences between individuals. This study aimed to find differences and quantify the delta, while the absolute saturation had no further meaning to the objective of this study.

Caution is advised when using these technologies to measure reactive microcirculation (97). According to Roustit et al., we recorded many factors that need to be accounted for and documented when conducting microcirculation studies (97). Any measurements using laser and white light spectroscopy are sensitive to ambient light changes. Our subjects were, for this reason, measured in the same setting with no light changes during or between measurements. Furthermore, all measurements were conducted by the same examiner to ensure consistent and correct equipment application and use.

4.1.3. Method

In this study, the same 24 subjects were to undergo four different protocols. This was the ideal way to compare these protocols, as the protocol would be the only changing variable. The basis for the comparison for these protocols was primarily the acute RIC effects. Depending on the results of this study and whether or not multiple protocols perform similarly, further studies can compare the longer-term differences between those protocols. The immediate RIC effects and the first phase of protection are reported to last up to 4 hours, and the second phase of protection is likely to begin after 24 hours and last for another 48 hours (79). Working with these time frames, a week between measurements was determined as a safe window. All subjects adhered to this timeframe.

The protocols themselves were based upon protocols thus far established in literature and modified to address this study's objective, which was to question the protocol guidance. All protocols started with a baseline which is common practice in past studies (11). Kraemer et al. also placed their patients in a resting position before starting baseline measurement (95). We decided to use the average of the five minutes leading up to the first ischemic phase as our baseline period as established by Kraemer et al. (95). The examiner attached the sensors five minutes prior to baseline measurement starting, so the subjects became used to the sensors by the time the baseline measurement started.

Protocol 1 is the standard RIC protocol used in most studies (11, 95). It is time controlled. It consists of three cycles of five minutes of ischemia followed by ten minutes of reperfusion. In other studies, this protocol was shown to be effective and have acute measurable RIC effects (11, 95). This study asks if other protocols are more or less effective than this protocol. There was no

randomization because all subjects underwent all protocols. All subjects completed all four protocols in the same order. Preliminary testing within the clinic revealed great discomfort differences between protocols. To prevent subjects from not following through with all four protocols, an order was selected that placed the probably most uncomfortable protocol in the last position. Feedback on NPRS from the participants later confirmed our preliminary discomfort findings, as seen in figure 26. Due to the nature of the study, it cannot be a blind study. However, the participants could not see the monitor as it was turned towards the examiner, who ensured the protocol was executed correctly.

Protocol 2 consisted of three cycles of ischemia and reperfusion. The ischemia was induced until the distal extremity recorded a saturation level of 10% or less for three consecutive seconds. This was expected to take less than 5 minutes, and this assumption was confirmed with a mean ischemia duration of 192.81 seconds (± 67.70). Protocol 3 consisted of three cycles of ischemia and reperfusion. The ischemia was induced until the distal extremity recorded a saturation level of 30 % or less for three consecutive seconds. This was also expected to take less than five minutes, and the resulting 97.54 seconds (± 48.66) confirmed this. Protocol 4 was designed to be an accelerated Protocol 2. This was confirmed with an average ischemia length of 141.93 seconds (± 59.31).

4.2. Microcirculation

The RIC protocol is principally comprised of baseline measurement followed by three cycles of alternating ischemia and reperfusion. These states are very different from each other and affect the rest of the body differently. Therefore, we decided, as in many previous studies, to dissect one protocol in multiple segments. The baseline measurement of five minutes is the first interval, Interval 0, and serves as the reference for every protocol's further intervals. Interval 1 is the first ischemic interval, and Interval 2 is the first reperfusion interval, and so on. As the purpose was not to show that there is a RIC effect but what protocol is more effective, we not only compared a start and an end point which would be partly inconclusive as shown in previous studies but looked at the protocols more closely over their whole duration (11). We compared all four protocols in all intervals in all three parameters measured. In the following paragraphs, we will analyze the differences and similarities between the protocols. We will begin with

the oxygen saturation, followed by the relative hemoglobin, and conclude with blood flow.

4.2.1. Oxygen saturation

From the beginning protocols 1, 2, and 4 produced significantly higher SO2 than the baseline. Protocol 4 set itself apart by an increase of 34% at $p < 0.001$ in Interval 1. It never let SO2 drop significantly below the baseline and intermittently pushed SO2 significantly above the baseline. Protocols 1 and 2 performed similarly by maintaining an average SO2 mostly statistically insignificantly below the baseline. In contrast Protocol 3 showed a statistically insignificant increase from the baseline in Interval 1 and dropped significantly below the baseline for the entire rest of the measurement.

To conclude, protocol 4 was the only protocol to attain above baseline SO2 during all intervals. While we reproduced the finding that there was no significant increase in the standard RIC protocol (protocol 1) when comparing the beginning and end points, we did find that the protocol produced some significantly higher oxygen saturation during the first ischemic interval (11). In rmANOVA comparisons, protocol 4 was the only protocol to significantly outperform other protocols in various intervals.

Currently the increase in SO2 is seen as part of the delayed second phase of the RIC effect as demonstrated by Kolbenschlag et al. (11). Protocol 4 already producing increases in SO2, especially significantly different to protocol 1, is a great promise for this protocol's future potential and warrants further investigation into its application to human surgical flaps. Protocol 3, only desaturating to 30%, is clearly not enough stimulus to elicit any acute RIC effect. Protocol 4, representing a combination of desaturation and time, shows that it is unnecessary to produce ischemia over five minutes to the extremity to produce a global SO2 response. According to our results, a quicker desaturation to 10% is more effective regarding global cutaneous SO2.

4.2.2. Relative hemoglobin

Just like the SO2, the rHb significantly increased during Interval 1 in protocols 1, 2, and 4. Protocol 1 increased it by 1.4% ($p = 0.001$), and protocol 2 increased it by 1.0% ($p = 0.006$). Protocol 4 increased it most significantly by 3.2% ($p < 0.001$).

Protocols 2 and 3 remained statistically indifferent from the baseline for the remainder of the measurements. In Interval 5, protocol 4 was the only one to significantly raise rHb above baseline by 2.4% ($p = 0.027$). All protocols resulted in statistically insignificant differences from the baseline at the end of the measurements.

In the rmANOVA comparisons protocol 4 yielded significantly higher rHB results than protocols 2 and 3 in Interval 1. In Interval 3, protocol 4 raised rHB significantly in comparison to protocol 3. No other significant differences were recorded. A significantly higher rHb which represents postcapillary filling and increased venous stasis is an undesired effect. In contrast to SO2 and Flow a significantly lower rHb would be preferred.

These findings match the end point results of the controls measured in Kolbenschlag et al.'s study published in 2016 (11). rmANOVA showed no significant difference between the standard protocol 1, which was used in Kolbenschlag et al.'s study, and the other protocols trialed in this study (11). In comparison Kraemer and Lorenzen et al. were able to produce significantly lower rHb using the standard protocol 1 at specific times during their measurement, not however in regards to the end point (95).

Protocols 2 and 3 are intermittently significantly better for postcapillary venous pressure compared to protocol 4. Future investigation of protocol 4 in surgical flap patients should bear it's temporarily significantly increased postcapillary venous pressure in mind since it increases its risk of venous stasis and thrombosis. Such a clinical study is very likely due to protocol 4's strongly significantly improvement in SO2 and Flow as demonstrated in this study.

4.2.3. Flow

Flow began with the familiar pattern of protocols 1, 2 and 4, producing significantly more Flow compared to the baseline in Interval 1. Protocol 4 almost doubled blood flow during the first interval with a 98.1% increase ($p < 0.001$). The starkest contrast existed towards protocol 3 which never produced any significantly elevated Flow throughout the entire measurement and ended significantly below baseline. Protocol 2 was the only other protocol to show statistically insignificantly elevated Flow in I2. It also ended in significantly reduced Flow in R3. During the standard protocol, protocol 1, Flow was

statistically insignificantly below the baseline and dropped to a significantly reduced level of Flow below the baseline in R3. Protocol 4 was the only protocol to consistently increase Flow from the baseline with intermittent significance. In I2 it averaged 57.3% above the baseline ($p = 0.001$) and in I3 40.6% above the baseline ($p = 0.008$). It is the only protocol to not end statistically significantly below baseline and finished statistically insignificantly below baseline by 1.1% ($p = 0.802$).

The rmANOVA comparisons showed a clear dominance of protocol 4 in Interval 1, with it performing significantly better than protocol 1, 2, and 3. Protocol 1 also performed significantly better than protocol 3. This shows an apparent weakness of protocol 3. Protocol 4 produced significantly elevated Flow in the following ischemic intervals in comparison to all other protocols.

While we could not reproduce the standard protocol's effects as described in current literature, we demonstrated that the new protocol 4 significantly outperformed this standard protocol (protocol 1) in multiple intervals by significantly increasing Flow globally (11, 95). This study confirms that it is unnecessary to induce ischemia for five minutes to elicit a global increased blood flow response. In fact, this study shows that it is significantly more effective to ask patients to contract muscles subjected to ischemia in a remote extremity resulting in shorter ischemic time to stimulate global cutaneous blood flow. Merely waiting for a certain level of desaturation or time to pass was significantly less effective. This warrants a further investigation into protocol's 4 application to the post- or pre-surgical flap patient as the significantly inferior protocol 1 was already proven to have an effect in the clinical setting (11).

4.3. Clinical Application

One aspect affecting the clinical application of RIC protocols is patient tolerance which impacts compliance. The tolerance described by subjects reflects the effectiveness of the individual protocols. Protocol 3, as the least effective protocol, was also considered the most tolerable protocol, with a mean pain level of 2.71 ($\pm$ 1.459). Protocol 4, on the other hand, as the most effective protocol, had a reported pain level of 6.71 ($\pm$ 1.517) on the NPRS. Protocol 1, which did produce RIC effects but significantly less than protocol 4, had a reported pain level of 5.79 ($\pm$ 1.444) on the NPRS, and protocol 2, which was less effective than

protocol 1, had a pain level reported of 3.88 (± 1.569). Protocols 1 and 4 were significantly more painful than protocols 2 and 3. Increased reported pain levels correlated with increased effectiveness. This observation questions whether the protocols are truly more effective or whether the increased parameters in the anterolateral thigh are sympathetic reactions to the pain to be understood as part of the fight or flight response or actual RIC triggered increases. While Jones et al. showed that peripheral cutaneous nociception alone initiates the cardioprotective effect of remote preconditioning, this study showed that certain RIC protocols are accompanied by certain pain (107).

Regarding clinical application, the importance of an exact distinction is questionable as it is impossible to induce ischemia in an easy manner without inducing pain, as ischemia itself is accompanied by pain. Merely in the setting of anesthesia would it be possible to apply RIC without experiencing pain. When comparing myocardial infarct size, Cho et al. discovered that the protective RIC effect disappeared in the anesthetized group (108). However, when Ederer et al. subjected patients to RIC with regional anesthesia, they found a significantly increased microcirculatory response (66). This microcirculatory response was less pronounced than in their control that did not receive anesthesia (66). To our knowledge, no studies exist that include a pain-subjected control group in which pain is elicited by a method that does not induce ischemia but has the same intensity as the ischemic pain demonstrated in this study.

4.4. Contingencies and pitfalls

This study was conducted on 24 healthy and young subjects. The foremost question is whether these results can translate to the older and comorbid patient, the average flap recipient. A patient that has been operated on is not in a physiologic physical state which could translate into a different RIC effect. In addition, the mean age in this study population of 24.67 years (± 3.52) is young compared to the average age of patients receiving flaps, 50 – 64 years (96, 109, 110). The combination of age and comorbidities poses a significant risk to the success and survival of flaps (109). Few studies have analyzed the various comorbidity's effect on the RIC effect. Engbersen et al., for example, found that while patients with type 1 diabetes mellitus tolerated ischemia-reperfusion injury better than healthy counterparts, the efficacy of ischemic conditioning was almost

nonexistent in the diabetic collective (111). Further research into the exact effects of comorbidities and age on the RIC effect is necessary to aid the translation of current data into the clinical setting. McCafferty et al. state that the difficulty of translation to the clinic because of age and comorbidities has been established (112). They appeal it is now time to research ways to circumnavigate these difficulties to bring the established benefits of RIC to patients (112).

While the method of measuring parameters with the O2C device is reliable and trusted, the nature of the protocol that includes muscle movement poses a risk to the reliability of these measurements. The person conducting the measurements ensured the sensor never left the thenar eminence. The effect of potential palmar sweating on the accuracy of measurements has yet to be discovered. The thenar sensor was used to detect the saturation level in the remote ischemic limb to ensure correct protocol compliance. It was thus not responsible for measuring the RIC effect, which is important to note.

The position of the subject while undergoing RIC has received little attention. In this study, all subjects were sitting. In other studies, such as Kolbenschlag et al.'s study in 2016, all subjects underwent RIC protocols lying down (11). The effect of a patient's body position on the RIC effect has not been determined to this date and could be the subject of further research.

Just as the mechanism of the RIC effect is a combination of multiple interlinking pathways, various factors contribute to the effectiveness of a RIC protocol. This study has shown that the rate of desaturation is a significant driving factor of the RIC effect. The main hurdle for future RIC protocol research is that the duration of ischemia, the level of ischemia, and the concomitant pain go hand in hand.

5. Summary

In plastic surgery and the use of flaps to cover defects, most complications are perfusion derived. The contributing processes are the result of ischemia and ischemia-reperfusion injury. Many other specialties, such as cardiology, dealt with similar complications and discovered that tissue could be conditioned to tolerate future ischemic events better. One effective way is to subject tissue to non-deleterious cycles of ischemia and reperfusion. While elucidating the mechanism of these observations, researchers found that these conditioning cycles of ischemia and reperfusion could be applied remotely to the target tissue of future ischemic insults. This was termed “remote ischemic conditioning” (RIC) and enabled ischemic conditioning to have an actual clinical application.

After establishing a RIC effect in myocutaneous tissue, it is time to determine the most effective RIC protocol for clinical use. To date, all tested protocols have been time-guided protocols. This study is the first to research the RIC effect of saturation-guided protocols in healthy subjects. The goal is to further decipher the contribution of different factors to the RIC effect’s strength.

This study included 24 healthy subjects that underwent four different protocols. All protocols were composed of initial baseline measurement followed by three cycles of alternating ischemia and reperfusion. Protocol 1 was the established time-guided protocol. Protocol 2 desaturated to 10%, protocol 3 desaturated to 30%, and protocol 4 desaturated to 10% while the ischemic limb exerted muscular effort. Each measurement was separated by a week to avoid overlapping RIC effects. Statistical analysis by rmANOVA was used to intraindividually compare the established time-guided protocol with saturation-guided protocols. The microcirculatory parameters SO2, rHb, and Flow, were measured with the established O2C device using laser doppler spectroscopy and white light spectroscopy. The question was whether the effect on cutaneous microcirculation differed significantly amongst the protocols.

Only Protocol 4 produced SO2 above baseline in all intervals with intermitting significance. SO2 increase was significantly different from the other protocols. Protocols 1, 2, and 3 did not differ significantly. Protocol 4 resulted in significant rHb increases in some intervals while differing significantly from the other protocols. Flow was significantly increased in protocol 4 compared to baseline and the other protocols. In all three metrics, protocol 4 performed significantly

better than the other protocols. The results demonstrate that the most effective protocol was defined by an increased velocity of desaturation, representing the contribution of both time and level of desaturation.

6. Deutsche Zusammenfassung

In der plastischen Chirurgie sind die meisten Komplikationen von Lappenplastiken perfusionsbedingt. Die bedeutendsten beitragende Faktoren sind die Ischämie und der folgende Ischämie-Reperfusionsschaden. Andere Bereiche der Medizin, wie die Transplantationschirurgie, sind ähnlicher Problematik ausgesetzt. Sie haben in wissenschaftlichen Arbeiten gezeigt, dass es möglich ist, Gewebe zu konditionieren, um zukünftige Ischämie Ereignisse mit anschließender Reperfusion besser zu tolerieren. Eine etablierte Methode ist das ischämische Konditionieren durch wiederholte Zyklen von Ischämie und darauffolgender Reperfusion. Weitere Forschung auf diesem Gebiet ergab, dass das begrenzte Konditionieren einer Region systemisch konditionierende Effekte hat. Diese Entdeckung ermöglicht das sogenannte „Remote Ischemic Conditioning“ um perfusionsbedingte Komplikationen zu minimieren.

Im muskulo-kutanen Modell haben aktuelle Studien mit der Anwendung verschiedener RIC-Protokolle an gesunden Probanden den RIC-Effekt am Menschen gezeigt. Ziel dieser Arbeit ist es, die treibenden Faktoren des RIC-Effektes zu differenzieren und die Protokolle daraufhin zu optimieren. Bisher verwendete Protokolle basieren auf zeitabhängigen Ischämie Phasen gefolgt von 10-minütiger Reperfusion. Es liegen keine Studien mit sättigungsabhängigen Ischämie Phasen vor. In dieser Arbeit wird das klassische zeitgesteuerte RIC Protokoll (Protokoll 1) mit mehreren sättigungsabhängigen Protokollen verglichen. Die untersuchten sättigungsabhängigen Protokolle definieren sich über eine Entsättigung auf 10% (Protokoll 2), eine Entsättigung auf 30% (Protokoll 3) und eine Entsättigung auf 10% bei gleichzeitiger Muskelarbeit (Protokoll 4).

Zu diesem Zweck wurden alle vier Protokolle an 24 Probanden/-innen durchgeführt. Die Parameter SO2, rHb, und Flow wurden mit dem etablierten O2C Gerät mittels Laser-Doppler-Spektroskopie und Weißlichtspektroskopie gemessen. Die Protokolle wurden hinsichtlich dieser Parameter durch das statistische Verfahren der rmANOVA miteinander verglichen. Allein in Protokoll 4 zeigte SO2, mit wechselhafter Signifikanz, eine Zunahme in allen Intervallen. Zudem lag ein signifikanter Unterschied im Vergleich zu den anderen Protokollen vor. Der Parameter rHb war in Protokoll 4 in manchen Intervallen signifikant erhöht und signifikant höher im Vergleich zu den anderen Protokollen. Eine

signifikante Flow Zunahme wurde teilweise ebenfalls im Protokoll 4 erzeugt. Der Vergleich des Flow zwischen den Protokollen lieferte positiv signifikante Unterschiede, vor allem des Protokoll 4 gegenüber den anderen Protokollen.
Die Ergebnisse zeigen, dass das effektivste Protokoll durch eine erhöhte Entsättigungsgeschwindigkeit gekennzeichnet ist, die die Beiträge sowohl der Zeit als auch des Entsättigungsgrades vereint.

7. Bibliography

1. Erba P, Orgill DP. Discussion. The new reconstructive ladder: modifications to the traditional model. Plast Reconstr Surg. 2011;127 Suppl 1:213S-4S.
2. Wei FC JV, Celik N, Chen HC, Chuang DC, Lin CH. Have we found an ideal soft-tissue flap? An experience with 672 anterolateral thigh flaps. Plast Reconstr Surg 2002.
3. Chaput B, Mojallal A, Bertheuil N, Carloni R, Grolleau JL, Sinna R, et al. Free-Flap Reconstruction: What Do Microsurgeons Prefer for Themselves? J Reconstr Microsurg. 2016;32(8):639-42.
4. Setala L, Koskenvuori H, Gudaviciene D, Berg L, Mustonen P. Cost analysis of 109 microsurgical reconstructions and flap monitoring with microdialysis. J Reconstr Microsurg. 2009;25(9):521-6.
5. Brown JS, Devine JC, Magennis P, Sillifant P, Rogers SN, Vaughan ED. Factors that influence the outcome of salvage in free tissue transfer. Br J Oral Maxillofac Surg. 2003;41(1):16-20.
6. Baumeister SP, Spierer R, Erdmann D, Sweis R, Levin LS, Germann GK. A realistic complication analysis of 70 sural artery flaps in a multimorbid patient group. Plast Reconstr Surg. 2003;112(1):129-40; discussion 41-2.
7. Basher AW, Novotny MJ, Hanna PE. Failure of oral taurine supplementation to influence skin-flap survival in rats. Plast Reconstr Surg. 1995;95(5):888-93.
8. Freedman AM, Hyde GL, Luce EA. Failure of pentoxifylline to enhance skin flap survival in the rat. Ann Plast Surg. 1989;23(1):31-4.
9. Reichenberger MA, Heimer S, Schaefer A, Lass U, Gebhard MM, Germann G, et al. Extracorporeal shock wave treatment protects skin flaps against ischemia-reperfusion injury. Injury. 2012;43(3):374-80.
10. Kubulus D, Amon M, Roesken F, Rucker M, Bauer I, Menger MD. Experimental cooling-induced preconditioning attenuates skin flap failure. Br J Surg. 2005;92(11):1432-8.
11. Kolbenschlag J, Sogorski A, Kapalschinski N, Harati K, Lehnhardt M, Daigeler A, et al. Remote Ischemic Conditioning Improves Blood Flow and Oxygen Saturation in Pedicled and Free Surgical Flaps. Plast Reconstr Surg. 2016;138(5):1089-97.
12. Kuntscher MV, Hartmann B, Germann G. Remote ischemic preconditioning of flaps: a review. Microsurgery. 2005;25(4):346-52.
13. Richardson M. Understanding the structure and function of the skin. Nurs Times. 2003;99(31):46-8.
14. Grice EA, Segre JA. The skin microbiome. Nat Rev Microbiol. 2011;9(4):244-53.
15. Yousef H, Alhajj M, Sharma S. Anatomy, Skin (Integument), Epidermis. StatPearls. Treasure Island (FL)2021.
16. Fuchs E, Raghavan S. Getting under the skin of epidermal morphogenesis. Nat Rev Genet. 2002;3(3):199-209.
17. Harder Y, Amon M, Laschke MW, Schramm R, Rucker M, Wettstein R, et al. An old dream revitalised: preconditioning strategies to protect surgical flaps from critical ischaemia and ischaemia-reperfusion injury. J Plast Reconstr Aesthet Surg. 2008;61(5):503-11.

18. Braverman IM. Ultrastructure and organization of the cutaneous microvasculature in normal and pathologic states. J Invest Dermatol. 1989;93(2 Suppl):2S-9S.
19. Guven G, Hilty MP, Ince C. Microcirculation: Physiology, Pathophysiology, and Clinical Application. Blood Purif. 2020;49(1-2):143-50.
20. Davies MG, Hagen PO. The vascular endothelium. A new horizon. Ann Surg. 1993;218(5):593-609.
21. Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell biology of ischemia/reperfusion injury. Int Rev Cell Mol Biol. 2012;298:229-317.
22. Nirmala JG, Lopus M. Cell death mechanisms in eukaryotes. Cell Biol Toxicol. 2020;36(2):145-64.
23. Linkermann A, Brasen JH, Himmerkus N, Liu S, Huber TB, Kunzendorf U, et al. Rip1 (receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. Kidney Int. 2012;81(8):751-61.
24. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death Differ. 2018;25(3):486-541.
25. Linkermann A, Green DR. Necroptosis. N Engl J Med. 2014;370(5):455-65.
26. Li J, McQuade T, Siemer AB, Napetschnig J, Moriwaki K, Hsiao YS, et al. The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell. 2012;150(2):339-50.
27. Gong YN, Guy C, Olauson H, Becker JU, Yang M, Fitzgerald P, et al. ESCRT-III Acts Downstream of MLKL to Regulate Necroptotic Cell Death and Its Consequences. Cell. 2017;169(2):286-300 e16.
28. Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death Differ. 1998;5(7):551-62.
29. Hardie DG, Carling D, Carlson M. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem. 1998;67:821-55.
30. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell. 2003;115(5):577-90.
31. Azad MB, Chen Y, Henson ES, Cizeau J, McMillan-Ward E, Israels SJ, et al. Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3. Autophagy. 2008;4(2):195-204.
32. Liu Y, Shoji-Kawata S, Sumpter RM, Jr., Wei Y, Ginet V, Zhang L, et al. Autosis is a Na+,K+-ATPase-regulated form of cell death triggered by autophagy-inducing peptides, starvation, and hypoxia-ischemia. Proc Natl Acad Sci U S A. 2013;110(51):20364-71.
33. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci U S A. 1995;92(16):7162-6.
34. Madshus IH. Regulation of intracellular pH in eukaryotic cells. Biochem J. 1988;250(1):1-8.
35. Raffaello A, Mammucari C, Gherardi G, Rizzuto R. Calcium at the Center of Cell Signaling: Interplay between Endoplasmic Reticulum, Mitochondria, and Lysosomes. Trends Biochem Sci. 2016;41(12):1035-49.

36. Sanada S, Komuro I, Kitakaze M. Pathophysiology of myocardial reperfusion injury: preconditioning, postconditioning, and translational aspects of protective measures. Am J Physiol Heart Circ Physiol. 2011;301(5):H1723-41.
37. Crompton M. The mitochondrial permeability transition pore and its role in cell death. Biochem J. 1999;341 (Pt 2):233-49.
38. Contreras L, Drago I, Zampese E, Pozzan T. Mitochondria: the calcium connection. Biochim Biophys Acta. 2010;1797(6-7):607-18.
39. Gourdin MJ, Bree B, De Kock M. The impact of ischaemia-reperfusion on the blood vessel. Eur J Anaesthesiol. 2009;26(7):537-47.
40. Engerson TD, McKelvey TG, Rhyne DB, Boggio EB, Snyder SJ, Jones HP. Conversion of xanthine dehydrogenase to oxidase in ischemic rat tissues. J Clin Invest. 1987;79(6):1564-70.
41. Chambers DE, Parks DA, Patterson G, Roy R, McCord JM, Yoshida S, et al. Xanthine oxidase as a source of free radical damage in myocardial ischemia. J Mol Cell Cardiol. 1985;17(2):145-52.
42. Zweier JL, Broderick R, Kuppusamy P, Thompson-Gorman S, Lutty GA. Determination of the mechanism of free radical generation in human aortic endothelial cells exposed to anoxia and reoxygenation. J Biol Chem. 1994;269(39):24156-62.
43. Raedschelders K, Ansley DM, Chen DD. The cellular and molecular origin of reactive oxygen species generation during myocardial ischemia and reperfusion. Pharmacol Ther. 2012;133(2):230-55.
44. Kussmaul L, Hirst J. The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. Proc Natl Acad Sci U S A. 2006;103(20):7607-12.
45. Jordan JE, Zhao ZQ, Vinten-Johansen J. The role of neutrophils in myocardial ischemia-reperfusion injury. Cardiovasc Res. 1999;43(4):860-78.
46. Battin EE, Brumaghim JL. Antioxidant activity of sulfur and selenium: a review of reactive oxygen species scavenging, glutathione peroxidase, and metal-binding antioxidant mechanisms. Cell Biochem Biophys. 2009;55(1):1-23.
47. Kubes P, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci U S A. 1991;88(11):4651-5.
48. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci U S A. 1987;84(24):9265-9.
49. Kakkar AK, Lefer DJ. Leukocyte and endothelial adhesion molecule studies in knockout mice. Curr Opin Pharmacol. 2004;4(2):154-8.
50. Harlan JM, Killen PD, Harker LA, Striker GE, Wright DG. Neutrophil-mediated endothelial injury in vitro mechanisms of cell detachment. J Clin Invest. 1981;68(6):1394-403.
51. Loerakker S, Oomens CW, Manders E, Schakel T, Bader DL, Baaijens FP, et al. Ischemia-reperfusion injury in rat skeletal muscle assessed with T2-weighted and dynamic contrast-enhanced MRI. Magn Reson Med. 2011;66(2):528-37.
52. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation. 1986;74(5):1124-36.

53. Mounsey RA, Pang CY, Forrest C. Preconditioning: a new technique for improved muscle flap survival. Otolaryngol Head Neck Surg. 1992;107(4):549-52.
54. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. Circulation. 1993;87(3):893-9.
55. Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. Circulation. 1996;94(9):2193-200.
56. Kuntscher MV, Kastell T, Sauerbier M, Nobiling R, Gebhard MM, Germann G. Acute remote ischemic preconditioning on a rat cremasteric muscle flap model. Microsurgery. 2002;22(6):221-6.
57. Kuntscher MV, Schirmbeck EU, Menke H, Klar E, Gebhard MM, Germann G. Ischemic preconditioning by brief extremity ischemia before flap ischemia in a rat model. Plast Reconstr Surg. 2002;109(7):2398-404.
58. Kharbanda RK, Mortensen UM, White PA, Kristiansen SB, Schmidt MR, Hoschtitzky JA, et al. Transient limb ischemia induces remote ischemic preconditioning in vivo. Circulation. 2002;106(23):2881-3.
59. Hausenloy DJ, Yellon DM. Remote ischaemic preconditioning: underlying mechanisms and clinical application. Cardiovasc Res. 2008;79(3):377-86.
60. Jensen RV, Stottrup NB, Kristiansen SB, Botker HE. Release of a humoral circulating cardioprotective factor by remote ischemic preconditioning is dependent on preserved neural pathways in diabetic patients. Basic Res Cardiol. 2012;107(5):285.
61. Pell TJ, Baxter GF, Yellon DM, Drew GM. Renal ischemia preconditions myocardium: role of adenosine receptors and ATP-sensitive potassium channels. Am J Physiol. 1998;275(5):H1542-7.
62. Ding YF, Zhang MM, He RR. Role of renal nerve in cardioprotection provided by renal ischemic preconditioning in anesthetized rabbits. Sheng Li Xue Bao. 2001;53(1):7-12.
63. Liem DA, Verdouw PD, Ploeg H, Kazim S, Duncker DJ. Sites of action of adenosine in interorgan preconditioning of the heart. Am J Physiol Heart Circ Physiol. 2002;283(1):H29-37.
64. Dong JH, Liu YX, Ji ES, He RR. [Limb ischemic preconditioning reduces infarct size following myocardial ischemia-reperfusion in rats]. Sheng Li Xue Bao. 2004;56(1):41-6.
65. Schoemaker RG, van Heijningen CL. Bradykinin mediates cardiac preconditioning at a distance. Am J Physiol Heart Circ Physiol. 2000;278(5):H1571-6.
66. Ederer IA, Goertz O, Bosselmann T, Sogorski A, Zahn PK, Lehnhardt M, et al. Anesthesia of the conditioned limb does not abolish the remote ischemic conditioning stimulus on cutaneous microcirculation in humans. Clin Hemorheol Microcirc. 2020;74(2):155-66.
67. Dickson EW, Lorbar M, Porcaro WA, Fenton RA, Reinhardt CP, Gysembergh A, et al. Rabbit heart can be "preconditioned" via transfer of coronary effluent. Am J Physiol. 1999;277(6):H2451-7.
68. Konstantinov IE, Li J, Cheung MM, Shimizu M, Stokoe J, Kharbanda RK, et al. Remote ischemic preconditioning of the recipient reduces myocardial ischemia-reperfusion injury of the denervated donor heart via a Katp channel-dependent mechanism. Transplantation. 2005;79(12):1691-5.

69. Cai ZP, Parajuli N, Zheng X, Becker L. Remote ischemic preconditioning confers late protection against myocardial ischemia-reperfusion injury in mice by upregulating interleukin-10. Basic Res Cardiol. 2012;107(4):277.
70. Davidson SM, Selvaraj P, He D, Boi-Doku C, Yellon RL, Vicencio JM, et al. Remote ischaemic preconditioning involves signalling through the SDF-1alpha/CXCR4 signalling axis. Basic Res Cardiol. 2013;108(5):377.
71. Rassaf T, Totzeck M, Hendgen-Cotta UB, Shiva S, Heusch G, Kelm M. Circulating nitrite contributes to cardioprotection by remote ischemic preconditioning. Circ Res. 2014;114(10):1601-10.
72. Tang ZL, Dai W, Li YJ, Deng HW. Involvement of capsaicin-sensitive sensory nerves in early and delayed cardioprotection induced by a brief ischaemia of the small intestine. Naunyn Schmiedebergs Arch Pharmacol. 1999;359(3):243-7.
73. Li J, Rohailla S, Gelber N, Rutka J, Sabah N, Gladstone RA, et al. MicroRNA-144 is a circulating effector of remote ischemic preconditioning. Basic Res Cardiol. 2014;109(5):423.
74. Konstantinov IE, Arab S, Kharbanda RK, Li J, Cheung MM, Cherepanov V, et al. The remote ischemic preconditioning stimulus modifies inflammatory gene expression in humans. Physiol Genomics. 2004;19(1):143-50.
75. Jones WK, Flaherty MP, Tang XL, Takano H, Qiu Y, Banerjee S, et al. Ischemic preconditioning increases iNOS transcript levels in conscious rabbits via a nitric oxide-dependent mechanism. J Mol Cell Cardiol. 1999;31(8):1469-81.
76. Kuntscher MV, Juran S, Altmann J, Menke H, Gebhard MM, Germann G. Role of nitric oxide in the mechanism of preclamping and remote ischemic preconditioning of adipocutaneous flaps in a rat model. J Reconstr Microsurg. 2003;19(1):55-60.
77. Kuntscher MV, Kastell T, Altmann J, Menke H, Gebhard MM, Germann G. Acute remote ischemic preconditioning II: the role of nitric oxide. Microsurgery. 2002;22(6):227-31.
78. Lambert EA, Thomas CJ, Hemmes R, Eikelis N, Pathak A, Schlaich MP, et al. Sympathetic nervous response to ischemia-reperfusion injury in humans is altered with remote ischemic preconditioning. Am J Physiol Heart Circ Physiol. 2016;311(2):H364-70.
79. Loukogeorgakis SP, Panagiotidou AT, Broadhead MW, Donald A, Deanfield JE, MacAllister RJ. Remote ischemic preconditioning provides early and late protection against endothelial ischemia-reperfusion injury in humans: role of the autonomic nervous system. J Am Coll Cardiol. 2005;46(3):450-6.
80. Shaw WW, Ko CY, Ahn CY, Markowitz BL. Safe ischemia time in free-flap surgery: a clinical study of contact-surface cooling. J Reconstr Microsurg. 1996;12(7):421-4.
81. Kolbenschlag J, Sogorski A, Harati K, Daigeler A, Wiebalck A, Lehnhardt M, et al. Upper extremity ischemia is superior to lower extremity ischemia for remote ischemic conditioning of antero-lateral thigh cutaneous blood flow. Microsurgery. 2015;35(3):211-7.
82. Keskin D, Unlu RE, Orhan E, Erkilinc G, Bogdaycioglu N, Yilmaz FM. Effects of Remote Ischemic Conditioning Methods on Ischemia-Reperfusion Injury in Muscle Flaps: An Experimental Study in Rats. Arch Plast Surg. 2017;44(5):384-9.

83. Kolbenschlag J, Sogorski A, Timmermann C, Harati K, Daigeler A, Hirsch T, et al. Ten minutes of ischemia is superior to shorter intervals for the remote ischemic conditioning of human microcirculation. Clin Hemorheol Microcirc. 2017;66(3):239-48.
84. Sogorski A, Spindler S, Wallner C, Dadras M, Wagner JM, Behr B, et al. Optimizing remote ischemic conditioning (RIC) of cutaneous microcirculation in humans: Number of cycles and duration of acute effects. J Plast Reconstr Aesthet Surg. 2021;74(4):819-27.
85. Li L, Mac-Mary S, Sainthillier JM, Nouveau S, de Lacharriere O, Humbert P. Age-related changes of the cutaneous microcirculation in vivo. Gerontology. 2006;52(3):142-53.
86. Munce TA, Kenney WL. Age-specific skin blood flow responses to acute capsaicin. J Gerontol A Biol Sci Med Sci. 2003;58(4):304-10.
87. Andreieva IO, Riznyk OI, Myrnyi SP, Surmylo NN. State of Cutaneous Microcirculation in Patients with Obesity. Wiad Lek. 2021;74(9 cz 1):2039-43.
88. O2C LMG. Blut- und Sauerstoffversorgung des Gewebes 2016 [Available from: http://www.lea.de/deu/fro2chd.htm.
89. Medizintechnik L. Messprinzip Giessen: LEA Medizintechnik Giessen; [Available from: http://www.lea.de/deu/fro2chd.htm.
90. Pagano RR. Understanding statistics in the behavioral sciences. Belmont, CA: Wadsworth/Cengage Learning; 2010.
91. Berkovits I, Hancock, G. R., Nevitt, J. Bootstrap Resampling Approaches for Repeated Measure Designs: Relative Robustness to Sphericity and Normality Violations. Educational and Psychological Measurement. 2000; 60(6):877-92.
92. Girden ER. ANOVA: Repeated measures. Newbury Park, Calif.: Sage Publications; 1992.
93. Stinson FS. US Apparent Consumption of Alcoholic Beverages: Based on State Sales, Taxation, Or Receipt Data: National Institutes of Health, National Institute on Alcohol Abuse and …; 1997.
94. Dawson D, Grant B, Chou S. Gender differences in alcohol intake. Monograph on: Stress, Gender, and Alcohol-seeking Behavior. Rockville, MD, National Institute on Alcohol Abuse and Alcoholism. 1994.
95. Kraemer R, Lorenzen J, Kabbani M, Herold C, Busche M, Vogt PM, et al. Acute effects of remote ischemic preconditioning on cutaneous microcirculation--a controlled prospective cohort study. BMC Surg. 2011;11:32.
96. Ren ZH, Wu HJ, Wang K, Zhang S, Tan HY, Gong ZJ. Anterolateral thigh myocutaneous flaps as the preferred flaps for reconstruction of oral and maxillofacial defects. J Craniomaxillofac Surg. 2014;42(8):1583-9.
97. Roustit M, Cracowski JL. Assessment of endothelial and neurovascular function in human skin microcirculation. Trends Pharmacol Sci. 2013;34(7):373-84.
98. Frayn KN, Humphreys SM. Metabolic characteristics of human subcutaneous abdominal adipose tissue after overnight fast. Am J Physiol Endocrinol Metab. 2012;302(4):E468-75.
99. WorldData.info. Average height and weight by country [Available from: https://www.worlddata.info/average-bodyheight.php#by-population.
100. Lenasi H, Strucl M. Effect of regular physical training on cutaneous microvascular reactivity. Med Sci Sports Exerc. 2004;36(4):606-12.

101. Heylen E, Mansourati J, Thioub S, Saiag B, Guerrero F. Effect of exercise training on cutaneous microcirculation in rats. J Sports Med Phys Fitness. 2008;48(2):246-51.
102. Brunt VE, Miner JA, Meendering JR, Kaplan PF, Minson CT. 17beta-estradiol and progesterone independently augment cutaneous thermal hyperemia but not reactive hyperemia. Microcirculation. 2011;18(5):347-55.
103. Monfrecola G, Riccio G, Savarese C, Posteraro G, Procaccini EM. The acute effect of smoking on cutaneous microcirculation blood flow in habitual smokers and nonsmokers. Dermatology. 1998;197(2):115-8.
104. Melik Z, Princi T, Grill V, Cankar K. The effect of caffeine on cutaneous postocclusive reactive hyperaemia. PLoS One. 2019;14(4):e0214919.
105. Tesselaar E, Nezirevic Dernroth D, Farnebo S. Acute effects of coffee on skin blood flow and microvascular function. Microvasc Res. 2017;114:58-64.
106. Beier JP, Horch RE, Arkudas A, Dragu A, Schmitz M, Kneser U. Decision-making in DIEP and ms-TRAM flaps: the potential role for a combined laser Doppler spectrophotometry system. J Plast Reconstr Aesthet Surg. 2013;66(1):73-9.
107. Jones WK, Fan GC, Liao S, Zhang JM, Wang Y, Weintraub NL, et al. Peripheral nociception associated with surgical incision elicits remote nonischemic cardioprotection via neurogenic activation of protein kinase C signaling. Circulation. 2009;120(11 Suppl):S1-9.
108. Cho YJ, Nam K, Kim TK, Choi SW, Kim SJ, Hausenloy DJ, et al. Sevoflurane, Propofol and Carvedilol Block Myocardial Protection by Limb Remote Ischemic Preconditioning. Int J Mol Sci. 2019;20(2).
109. Bekara F, Herlin C, Mojallal A, Sinna R, Ayestaray B, Letois F, et al. A Systematic Review and Meta-Analysis of Perforator-Pedicled Propeller Flaps in Lower Extremity Defects: Identification of Risk Factors for Complications. Plast Reconstr Surg. 2016;137(1):314-31.
110. Yoon CS, Kim SI, Kim H, Kim KN. Keystone-Designed Perforator Island Flaps for the Coverage of Traumatic Pretibial Defects in Patients With Comorbidities. Int J Low Extrem Wounds. 2017;16(4):302-9.
111. Engbersen R, Riksen NP, Mol MJ, Bravenboer B, Boerman OC, Meijer P, et al. Improved resistance to ischemia and reperfusion, but impaired protection by ischemic preconditioning in patients with type 1 diabetes mellitus: a pilot study. Cardiovasc Diabetol. 2012;11:124.
112. McCafferty K, Forbes S, Thiemermann C, Yaqoob MM. The challenge of translating ischemic conditioning from animal models to humans: the role of comorbidities. Dis Model Mech. 2014;7(12):1321-33.

8. Declaration of own contribution

The dissertation was carried out in the BG Klinik Tuebingen under the supervision of Prof. Dr. med. Jonas Kolbenschlag, MHBA.

The study was designed by Dr. med. Louisa Thiel (supervisor), Dr. med. Ruth Schäfer, FEBHS (supervisor), Prof. Dr. med. Jonas Kolbenschlag, MHBA (doctoral supervisor), Lukas Dirschedl and myself.

The experiments were performed independently by me after instruction by Dr. med. Louisa Thiel and Lukas Dirschedl.

The statistical analysis was performed by me after consultation with the institute of clinical epidemiology and applied biometrics.

I certify that I have written the manuscript independently and that I have not used any sources other than those indicated by me.

9. Acknowledgments

I want to thank Prof. Dr. med. Jonas Kolbenschlag, MHBA, for granting me this topic for my dissertation. Furthermore, I thank him for his guidance and feedback while allowing and demanding great independence during the different stages of this project. This balance has enabled me to learn valuable skills I will need and use in my future career.

I want to thank Dr. med. Ruth Schäfer, FEBHS, for her incredible dedication to any questions I had and for actively reaching out in times of radio silence and making sure all was in order. I greatly appreciate her detailed and time-consuming feedback on my previous versions of this dissertation. Additionally, I am thankful for her honest mentorship and guidance regarding my future career.

I want to thank Dr. med. Louisa Thiel for her initial help getting me started with the clinical measurements.

I thank Dr. med. scient. Cosima Prahm, MSc BA, for introducing me to the equipment and reviewing preliminary results.

I want to thank Lukas Dirschedl for demonstrating a measurement in the very beginning. As a fellow doctoral candidate, it was great to have a fellow student to discuss ideas.

I want to thank Sabrina from the front desk, who always let me know the examination room's schedule so I could efficiently plan my measurements and complete these in a timely fashion.

I owe plenty of thanks to Lina Maria Serna-Higuita from the institute of clinical epidemiology and applied biometrics. She showed me how to tame the vast amount of data points into a manageable format relieving me of much panic. In addition, her advice on statistical methods was priceless.