

Impact of Inorganic Nitrate in Cardiovascular Ischemic Disease

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Für Papa und Opa!

Abstract

Nitric oxide (NO) is one of the most important and multifaceted messengers of the mammalian organism. To a large part it is synthesized by different isoforms of the NO-synthase (NOS). Under normoxia the enzyme metabolizes L-arginine to citrulline and NO with the assistance of oxygen (O_2). The produced NO acts as messenger mediating neurological signals and supporting a host defense. In the cardiovascular system, NO has a great impact on vasodilation and vascular homeostasis e. g. by inhibiting platelet activation. Under hypoxia and ischemia this sensitive system is impaired, as the needed cofactor O_2 is deficient. A back-up system partly compensates the emerging lack of endogenous NO, by way of NO synthesis. Gaseous NO has a short half-life and is fast oxidized to nitrite (NO_2^-) and further to nitrate (NO_3^-). These circulating metabolites could be reduced back to NO by e. g. myoglobin, hemoglobin, and cytoglobin whereby, on the whole NO_3^- is reduced to NO_2^- by commensal oral bacteria. Without O_2 , these enzymes switch from e. g. NO-scavengers to NO producing molecules absorbing reduced endogenous NO synthesis. However, NO_2^- and NO_3^- are not only endogenously produced. Both are ingredients of several foods. These ingested inorganic NO_2^- and NO_3^- also fill up the pool of reducible NO metabolites. Hence, recent works could show that an increase in dietary NO_3^- (and NO_2^-) not only increases the endogenous NO_3^- (NO_2^-) pool, but could also improve regeneration after ischemia. These works, in combination with novel considerations referring the toxicity of NO_2^- and NO_3^- resulted in a new perception of these nutritional ingredients with relativization of hazardous effects and more emphasis on its beneficial potential.

The current study hypothesized that supplementation with dietary NO_3^- improves regeneration in the murine hind limb model, an animal model of peripheral ischemia. It is supposed that regeneration is supported by revascularization, increased mobilization of Endothelial-regenerating cells and increased muscle tissue protection. Finally, it is proposed that the decrease in NO production and the subsequent decreased endothelial-regenerating cell level is associated with a severe cardiovascular disease, such as the myocardial infarction.

Accordingly, present work demonstrates that the increase of bioavailable NO by dietary NO_3^- increases perfusion of affected tissue elucidating its regenerative potential. It could be shown that mobilization and migration of Endothelial-regenerating cells is significantly improved. Moreover, it shows that not only revascularization but also tissue protection is improved. It is proposed that this cytoprotection is mediated by Endothelial-regenerating cells with the Fas/FasL pathway, as candidate for paracrine reactions. Indeed, the work in hand shows that Endothelial-regenerating cells express FasL modulated by NO_3^- intake. Presented clinical studies corroborate the hypothesis of decreased NO bioavailability in myocardial infarction with associative relation to its severity.

In conclusion, it could be demonstrated that dietary NO_3^- acts cytoprotective in the ischemic hind limb model, mediating mobilization and migration of endothelial-regenerating cells. Description of decreased NO bioavailability in myocardial infarction underscores the importance of increasing NO level in (cardio)vascular disease. The relevance of the beneficial potential of the NO_3^- - NO_2^- -NO pathway is supported, highlighting the impact of dietary NO_3^- in ischemia.

Zusammenfassung

Stickstoffmonoxid (NO) ist einer der wichtigsten, vielseitigsten Botenstoffe des Säugerorganismus. Größtenteils wird es durch verschiedene Isoformen der NO-Synthase (NOS) synthetisiert. Unter Normoxie metabolisiert das Enzym unter Zuhilfenahme von Sauerstoff (O_2) L-Arginin zu Citrullin und NO. Das produzierte NO agiert als neurologischer und immun-protectiver Botenstoff. Im kardiovaskulären System spielt NO eine wichtige Rolle bei der Vasodilatation und vaskulären Homeostase, bspw. durch die Hemmung der Plättchen-Aktivierung. Bei Hypoxie und Ischämie ist dieses System geschwächt, weil der benötigte Kofaktor O_2 fehlt. Ein Back-Up System kompensiert teilweise den Mangel endogenen NOs durch einen alternativen Weg der NO Synthese. Gasförmiges NO hat eine kurze Halbwertszeit und wird zügig zu Nitrit (NO_2^-) und weiter zu Nitrat (NO_3^-) oxidiert. Diese Metabolite können wiederum zu NO reduziert werden z. B. durch Myoglobin, Hämoglobin und Cytoglobin, wobei NO_3^- größtenteils durch kommensale, orale Bakterien zu NO_2^- reduziert wird. Ohne O_2 werden diese NO-Fänger (z. B.) zu NO produzierenden Enzymen, die den Abfall der endogenen NO Synthese auffangen. NO_2^- und NO_3^- werden jedoch nicht nur endogen produziert. Beide sind Bestandteil zahlreicher Nahrungsmittel. Das so aufgenommene inorganische NO_2^- und NO_3^- füllt zusätzlich den Pool reduzierbarer Metabolite. Daraus folgend, konnten neuere Arbeiten zeigen, dass ein Anstieg dietätischen NO_3^- (und NO_2^-) den endogenen NO_3^- (NO_2^-) Pool erweitert und so die Regeneration nach einer Ischämie verbessert. Diese Arbeiten, kombiniert mit einer aktualisierten Einschätzung der Toxizität von NO_2^- und NO_3^- , resultierten in einer neuen Wahrnehmung dieser Nahrungsbestandteile, mit Relativierung ihres Gefährdungspotentials und größerer Betonung ihres Nutzens.

Die vorliegende Studie hat hypothetisiert, dass eine Ergänzung der Nahrung mit NO_3^- die Regeneration in dem murinen „*hind limb model*“, einem Ischämie-Modell, verbessert. Es wurde vorgeschlagen, dass die Regeneration durch Revaskularisation, gesteigerte Mobilisierung Endothel-regenerierender Zellen und gesteigerten Schutz des Muskelgewebes unterstützt wird. Schließlich wurde untersucht, ob die Reduktion der NO Produktion assoziiert ist mit schweren kardiovaskulären Erkrankungen wie dem Myokardinfarkt.

Die Ergebnisse der präsentierten Arbeit zeigen, dass der Anstieg bioverfügbaren NOs durch dietätisches NO_3^- die Durchblutung in dem betroffenen Gewebes verbessert und verdeutlicht so sein regeneratives Potential. Weiterhin sind die Mobilisierung und die Migration Endothel-regenerierender Zellen signifikant verbessert. Mehr noch, auch die Geweberegeneration ist angestiegen. Es wird vorgeschlagen, dass die Zytoprotektion parakrin durch Endothel-regenerierende Zellen vermittelt wird, möglicherweise über den Fas/FasL Signalweg. Tatsächlich exprimieren Endothel-regenerierende Zellen FasL NO_3^- Aufnahme-abhängig. Die präsentierten klinischen Studien schließlich unterstützen die Hypothese einer gesunkenen NO Bioverfügbarkeit beim Myokardinfarkt.

Zusammenfassend konnte demonstriert werden, dass dietätisches NO_3^- im ischämischen „*hind limb model*“ zytoprotektiv wirkt und Endothel-regenerierende Zellen mobilisierend. Die Beschreibung einer gesunkenen NO Bioverfügbarkeit im Myokardinfarkt unterstreicht die Bedeutung zu steigernder NO Spiegel in (kardio)vaskulären Erkrankungen. Die Relevanz des nützlichen Potentials des NO_3^- - NO_2^- -NO Signalweges wird mit Betonung auf die Rolle dietätischen NO_3^- in der Ischämie unterstützt.

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Da steh ich nun ich armer Thor und binn so klug als wie zuvor!
(J.W. Goethe)

Abbreviations

AP	allopurinol
APC	allophycocyanine
BM	bone marrow
U/SA	(un)stable angina
CAC	circulating angiogenic cells
CFU	colony forming unit
Conj.	conjugated
CMR	cardiovascular magnetic resonance
cPTIO	2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide, monopotassium salt
ECM	extra-cellular matrix
EC	endothelial cells
EDTA	ethylenediaminetetraacetic acid
Eph	ephrin receptor
eGFP	enhanced green fluorescent protein
eNOS	endothelial NO synthase
FACS	flowcytometric analysis
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
FSC	forward scatter
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
G-CSF	granulocyte colony-stimulating factor
sGC	Soluble guanylyl cyclase
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
HPLC	high pressure liquid chromatography
HIF	hypoxia inducible factor
IHC	immunohistochemistry
isc/non-isc	ischemic/non-ischemic
LDPI	Laser-Doppler-Perfusion-Imaging
MCP-1	monocyte chemotactic protein-1
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NAP-2	neutrophil-attracting peptide-2
NEM	N-ethylmaleimide
NO₂⁻	nitrite
NO₃⁻	nitrate
NP-40	Nonidet P-40 (nonyl phenoxy polyethoxy ethanol)
PCI	percutaneous coronary intervention
PE	phycoerythrin
PECAM	platelet endothelial cell adhesion molecules
PDGF	platelet-derived growth factor
PMT	photomultiplier tube
PpP	platelet poor plasma

Introduction

1. Nitric Oxide

1.1 Nitric Oxide

In 1980 Furchgott et al. discovered the EDRF (endothelium dependent relaxation factor) and seven years later it was proved that EDRF was synonymous to NO^1 . Indeed, no one could believe that a pollutant could have such beneficial effects delaying evaluation². Now we know that NO is one of the most important transmitters of the cardiovascular system. It is responsible for vasodilation and the maintaining of the vascular homeostasis by inhibiting platelet activation (e. g.)². Furthermore, it has a great impact on immune responses to microorganisms and inflammation. In neurology it plays a role as a neurotransmitter and mediates neuronal plasticity².

In principle two different NO sources exist. It can be taken in either exogenously or generated endogenously. Exogenously it is inhaled or ingested with food and water, in most cases comprised in metabolites like nitrite (NO_2^-) or nitrate (NO_3^-). Endogenously it is produced via the “classical way” by NO synthases (NOS) or it is “recycled” from its metabolites NO_2^- and NO_3^- via an alternative way. Pivotal elements of the “classical way” of NO synthesis under normoxia are the NOS. The chemical reaction mediated by the enzymes is presented in figure 1, explaining the complexity of the reaction with needed O_2 and multiple cofactors. Four different NOS are responsible for NO generation: neuronal NOS (nNOS), inducible NOS (iNOS), erythrocyte NOS (which is possibly an eNOS isoform. This issue is a matter of debate), and endothelial NOS (eNOS)²⁻⁶. nNOS produced NO as neurotransmitter in the cerebellum, especially³. iNOS is not constitutive expressed, but induced in inflammatory processes⁴. Erythrocyte NOS was discovered in 2006, but is actually suggested to be an isoform of the eNOS, with important regulatory functions for erythrocytes⁵. Thus, concerning the cardiovascular system the constitutive expressed eNOS, located in the vascular wall is the most important NOS. Furthermore, eNOS derived NO mediated vessel relaxation was the first to be discovered.

From the producing endothelial cell (EC) the molecule diffuses into neighboring smooth muscle cells (SMC). Once it has arrived there, it activates the soluble guanylyl cyclase (sGC), which subsequently produces cGMP. In turn cGMP activates the signalling pathways that mediate the NO functions². However, not every synthesized NO molecule reaches its target. NO is a reactive gaseous molecule with a very short half-life of 1-2 ms and thus, a great part of the endogenously produced NO is autooxidized to NO_2^- . This NO_2^- is only a little more stable (half life of 1-5 min) and further oxidized to NO_3^- with an EC_{50} of 5-8 h⁷. Additionally, NO could react with free thiol groups of proteins. These nitrosothiols are also suggested to mediate important signals in physiology and pathology, e. g. inflammation or apoptosis⁸. Suggested reactions are presented in figure 2.

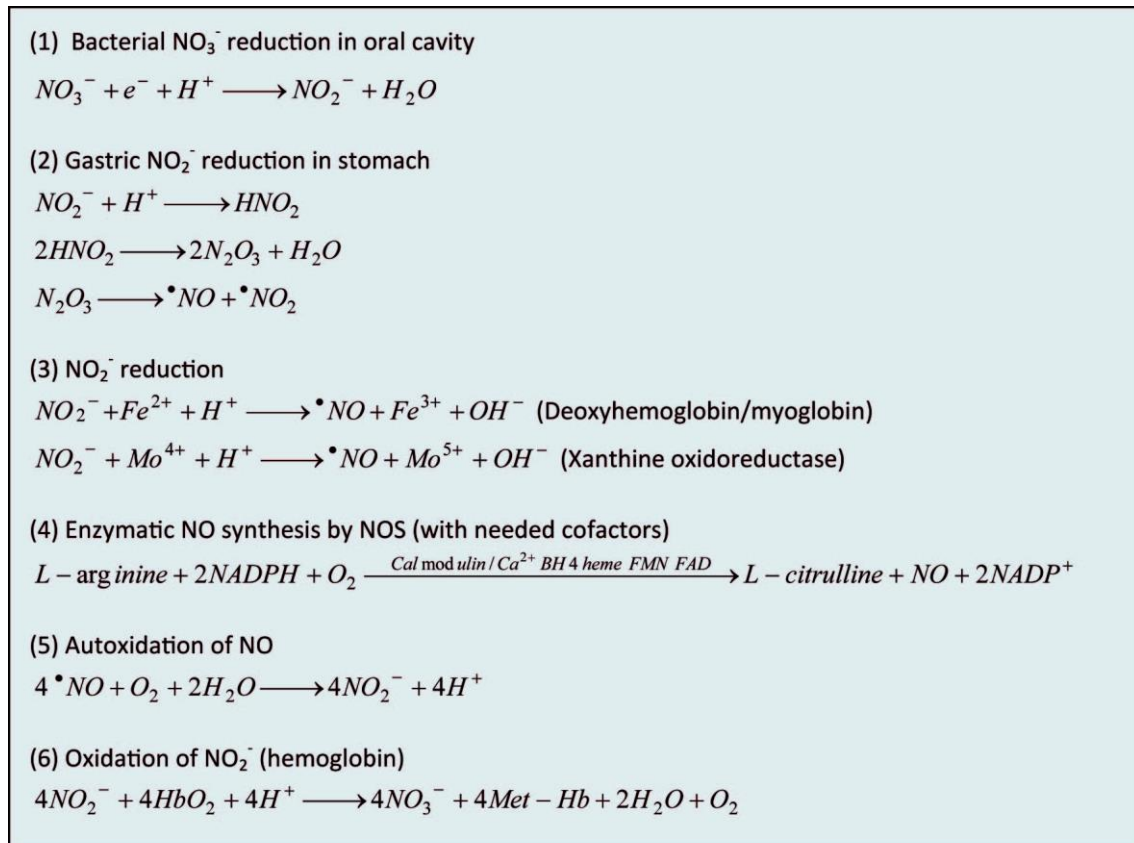


Figure 1. Formation and metabolization of NO_3^- .

Ingested dietary NO_3^- is first reduced to NO_2^- by bacterial reduction in the oral cavity (1). After passing oesophagus NO_2^- enters the stomach and is acidified to NO (2). Additionally, NO_2^- in circulation and tissue and is reduced to NO (3). At the same time, NO is enzymatically synthesized (4) and fast oxidized to NO_2^- (5). NO_2^- could be further oxidized to NO_3^- by hemoglobin (6)^{2,9}.

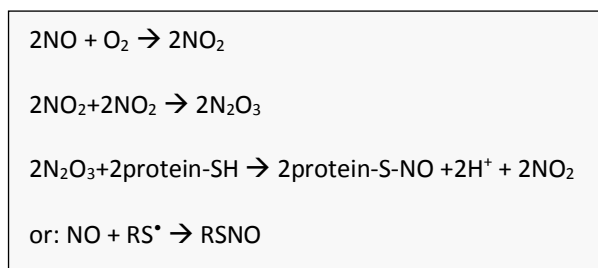


Figure 2. Generation of nitrosothiols

The reaction of NO with the $\text{O}_2^\bullet$ generates ONOO^- which could further react to NO_2^+ and $\text{NO}_2^\bullet$.

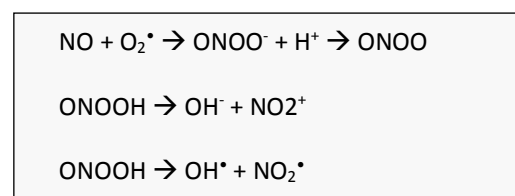


Figure 3. Peroxynitrite production and decomposition to nitrogen dioxide and hydroxyl radicals.

With oxygen, NO could react to NO_2 and further to N_2O_3 . This peroxynitrite seems to be involved in RSNO generation. Besides NO binds directly to protein building RSNO.

Finally, NO could react with reactive oxygen generating peroxynitrite. The protonated form of peroxynitrite could further react to extremely reactive, destructive radicals (figure 3).

However, amongst these reactions the autooxidation to NO_2^- and NO_3^- is probably the most common one. Moreover, it is possibly the most important one, as recent work revealed that NO_3^- presents an endogenous NO store and thus an important third NO source especially in hypoxia¹⁰. Thus,

autooxidation of NO does not produce almost inert waste products (as thought for a long time), but stores NO in a stable form until it is needed.

As diffusion is a non-directed process, endogenous NO also reaches blood stream and the above mentioned oxidative reactions produce circulating NO_3^- , which is mixed with dietary NO_3^- entering together the entero-salivary circulation by active uptake of salivary glands. This entero-salivary circulation currently provides alternatively produced NO and extends from the salivary glands (and oral cavity) to the stomach (and gut) ending in re-entering the cycle or excretion.

Experiments with dietary NO_3^- could evaluate that 25 % of ingested NO_3^- is detectable in saliva within 30-60 min¹¹. After overnight fasting basal NO_3^- concentrations in saliva were 200 μM ¹² representing highest level in human bodies except in urine. After ingestion of 10 mg/kg /100 ml NO_3^- levels increase to 800 μM . At the same time, NO_2^- concentration raises, indicating a profound NO_3^- reduction in the oral cavity. A first account of a relationship between NO_3^- content and NO_2^- concentration was done in 1976 (redox equation is shown in figure 1)^{11,13}. By testing NO_3^- reduction, after antiseptic mouthwash treatment a strong correlation between NO_3^- reduction and composition of oral microflora was proposed¹³. Different mammalian species have different compositions and numbers of commensals, but the principles are comparable, thus studies with rodents specified bacterial NO_3^- reduction to NO_2^- in mammals. From 1275 different identified strains of bacteria on rat tongues, 147 were able to reduce NO_3^- . The four main reducing strains were *Staphylococcus*, *Pasteurella*, *Streptococcus*, and *Listonella*¹⁴. In addition, bacteria were differentially distributed on the tongue. Towards the posterior end of the tongue, the general number of bacteria, and the specific number of NO_3^- reducing bacteria increased¹⁴. Acidification of NO_2^- could complete the two-step NO bioactivation from NO_3^- . Acidic milieu is supplied by (sub-)gingival microflora around the teeth and periodontal tissue and is proposed to protect against periodontal disease (written down in figure 1)¹⁵.

Two groups also independently analyzed acidic gastric NO_2^- reduction (shown in figure 1) after oral NO_3^- reduction and proposed an important function of NO for host defense against swallowed pathogens, regulation of mucosal blood flow, mucus formation, and gastric motility^{16,17}. As the lower gastrointestinal tract is also densely populated by bacteria, a similar function of acidic reduced NO_2^- was proposed for intestinal mucosa and microflora¹⁸. NO_3^- , which is not reduced to NO_2^- , arrives the gut. In the gut this NO_3^- is absorbed from the epithelium of the gut. In the next step the absorbed NO_3^- arrives the capillaries and the circulation.¹⁹

In summary, it could be estimated that 40-45 % of ingested NO_3^- was metabolized (in contrast 55 to 60 % excreted). Half of metabolized NO_3^- is processed by bacterial mechanisms and half by mammalian reactions (discussed below), as shown by Tannenbaum and Coworkers²⁰. The last sentence illustrates that, despite manifold mechanisms for the utilization of NO_3^- and NO_2^- , the majority is excreted as urinary NO_3^- . However, this urinary NO_3^- status enables an assumption about infection status, and daily ingestion^{21,22}.

Parallel to the described bacterial and acidic reduction, and the ability to reduce NO_3^- to NO_2^- and NO_2^- to NO, mammals evolved similar mechanisms. NO_3^- (and NO_2^-) crossing blood stream and tissues by diffusion and absorption could be “recycled” to NO by a variety of enzymes. In many cases these enzymes have different functions under normoxia changing to NO_2^- (or NO_3^-) reducing enzymes under hypoxia. A recent work suggested a mammalian NO_3^- reductase in tissue²³. In the study bacterial reduction was excluded by showing an effective NO_3^- reduction in germ-free mice. Instead of this, NO_3^- reduction was inhibited by treating tissue with the xanthine-oxidoreductase (XOR) inhibitor Allopurinol (AP), proposing a NO_3^- reductive function in the multipotent and ubiquitous expressed enzyme XOR²³. However, mammalian reactions metabolizing NO_2^- to NO are better known. Over the last decades several molecules were identified, which are able to reduce NO_2^- to NO, under hypoxic conditions^{24–27}. First NO_2^- reductive to be identified was hemoglobin (equation is shown in figure 1). Even in 1937, Brooks described the oxygen sensitivity of hemoglobin and a NO_2^- reductase activity, but did not discuss physiological consequences²⁸. The next groundbreaking work was done by Doyle and co-workers, who analyzed kinetics of NO_2^- reduction by deoxyhemoglobin, but were focused on the second effect of the reaction, the oxygenation of deoxyhemoglobin²⁹. Indeed, until 2003 it was thought that (concerning NO metabolism) hemoglobin physiologically only scavenges NO, lowering its bioavailability. In contrast, Cosby and colleagues identified NO_2^- -reducing deoxyhemoglobin as a physiologically important vasodilator²⁴. Efficiency increases along the vascular tree from artery to capillary, with decreasing oxygen concentration reaching its maximum at the midpoint of hemoglobin dissociation curve (oxygen saturation: 50 %). This is due to the fact that, the deoxygenated T-state of hemoglobin is necessary to bind NO_2^- , but oxygenated R-state heme is essential to increase intrinsic reactivity of the enzyme²⁴. After discovery of hemoglobin reductase activity, similar processes in myoglobin seemed natural. Indeed, our laboratory and an US group described NO_2^- reduction in the deoxymyoglobin molecule^{25,30}, written down in figure 1. Moreover, because of the lower heme reduction potential of myoglobin NO_2^- reduction occurs 32 times faster than in hemoglobin²⁵. These first studies postulated a regulatory function of NO_2^- reductase activity of deoxymyoglobin in mitochondrial respiration and cardiac energetics^{25,30}, which could be expanded to a modulating role in oxygen consumption while exercising³¹ and a protective function in I/R injury³². However, not only heme-molecules developed the ability to use NO_2^- as an electron acceptor by reducing it to NO. Several complexes of the mitochondrial electron transport chain³³, Cytochrom P450²⁶, and even NOS³⁴ shifted from molecular oxygen to NO_2^- as electron acceptor with decreasing oxygen tension. Finally, XOR provide an alternative path for NO synthesis. Under normoxic conditions, XOR reduces molecular oxygen to superoxide (O_2^-)³⁵, but with decreasing oxygen concentration catalytic domain at the molybdenum side of the enzyme (independent from the second enzymatic active FAD domain of the protein³⁶), changes from oxygen to NO_2^- reduction³⁷ (illustrated in figure 1). In the following, several studies supported the impact of non-enzymatic NO production via the NO_3^- - NO_2^- -NO-pathway under physiological and pathophysiological hypoxia^{9,38,39}. Bryan et al. even proposed a signalling function for NO_2^- itself⁴⁰. Consequently, endogenous NO_x levels (summarized in table 1) could be considered as marker for NO homeostasis, but also as a constant supported NO-store by dietary of NO_3^- (NO_2^-).

Combined, processes have evolved to ensure NO delivery for the whole range of oxygen tension, clearly illustrated in a review from 2008 as ying (normoxic enzymatic NO synthesis) and yang (hypoxic non-enzymatic NO synthesis) of NO metabolism⁹. Subsequently, the question arose as to whether an increase of NO_3^- ingestion would have a real benefit in human disease or whether risks would prevail.

Table 1. Levels of NO metabolites in health.

Listed values summarize concentrations of NO_3^- , NO_2^- and NO in biological fluids involved in the circuit of

	NO_3^- (μM)	NO_2^- (μM)	NO (nM)
Saliva	190±30	104±21	nd
Plasma	20-50	0.1-0.5	<1
Urine	250-2000	nd	nd

dietary NO_3^- metabolism^{7,12,22}.

1.2 Nitrate (NO_3^-)

Nitrogen is an essential molecule in prokaryotic and eukaryotic organisms for production of nucleic acids, amino acids, and proteins. Additionally, NO_2^- and NO_3^- are used as an energy source for various bacteria⁴¹. In ecosystems this demand is covered by the nitrogen cycle, composed of nitrification and denitrification processes. The mentioned above NO synthesis together with the entero-salivary circulation, presents a similar mammalian cycle of “nitrification” and “denitrification”. However, it is not nitrogen but NO which is the essential molecule needed. It was presented that NO is fast oxidized to NO_2^- and NO_3^- , due to its short half live^{42,43} and that NO_2^- and NO_3^- are also ingested by food and water^{9,19}. This mixture of endo- and exogenous NO_3^- circulates throughout the body and is thus present in high concentrations in the mammalian organism.

Previously it was thought that this NO_3^- was an almost inert reaction product of NO, although its “recycling” in nitrogen-cycle was known^{9,44}. It was thought that the chain ($\text{NO} \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^-$) is a one-way street, and NO_3^- is excreted unused. Indeed, it could be shown, that the reaction cascade is not a chain but a circuit. Since 1976 it has been known that circulating NO_3^- is concentrated in saliva and reduced back to NO_2^- by commensals in the oral cavity¹¹. In the 1990's, it was shown that this NO_2^- reenters circulation and is “recycled” to NO^{16,17}. Despite this physiological generation, both NO_2^- and NO_3^- were demonized to be toxic and carcinogen^{45,46}.

This attitude of hazardous NO_3^- (and NO_2^-) is somewhat astonishing considering the known importance of NO_3^- for ecosystems. Most nitrogen is stored in the atmosphere in gaseous form (N_2), but inaccessible for most plants, because there are only able to take up nitrogen as ammonium (NH_4^+) or NO_3^- . Similar counts exist for many bacteria and animals that cover their requirement by consumption of organic material⁴⁷. Other bacteria, fungi, and actinomycetes decompose dead organic material providing it for the nitrogen cycle⁴⁷. Autotrophic bacteria assimilate nitrogen by oxidization of NH_4^+ to NO_2^- . A second group oxidizes NO_2^- to NO_3^- , completing the process of nitrification⁴⁸. Subsequently, this NO_3^- is available to plants for assimilation and heterotrophic bacteria as energy source. This enzymatic respiratory reaction of denitrification closes the cycle. Obviously, NO_3^- is a key factor for life, used by a plethora of organisms and currently transformed in the nitrogen cycle. On the other hand, the first iahes

or words that spring to mind when one hears of NO_3^- are pollution, fertilization, or toxic precipitation. Increased livestock ranching and sewage waste produce large amounts of ammonia (NH_3) reaching soil and groundwater, increasing their NH_3 , NO_2^- , and NO_3^- concentrations⁴⁷. In fact, the nitrogen cycle is massively influenced by industrialization, but nevertheless it is also one of the most important nutrient cycles in terrestrial ecosystems, with NO_3^- as a key player in this biogeochemical cycle^{47,48}.

The last paragraph clarified that the dissent existing for NO_3^- (and NO_2^-), concerning mammalian physiology and health is also evident for NO_3^- in ecology. Regarding human healthcare NO_3^- and NO_2^- levels in food and water are restricted by administration to avoid risks of health (see also chapter 1.3)^{49,50}. Otherwise, NO_3^- was newly discovered as a stable but utilizable storage form of NO with a possible benefit to healthcare¹². Indeed, dietary NO derivatives were shown to have beneficial effects on cardiovascular disease, gastric ulcers, or host defence e. g. (detailed in chapter 1.5). Discovery of this non-enzymatic NO synthesis and its great potential for health care begin to change the image of NO_3^- ⁵². Obviously, the issue is complex and one does not do justice to NO_3^- by describing it either as demon or as savior. However, in recent years there has been more and more focus on dietary NO_3^- (and NO_2^-) as a beneficial supplement.

1.3 Dietary NO_3^-

As every organism needs nitrogen, but cannot assimilate and incorporate it in its molecular form, NO_3^- is a ubiquitous molecule in food and water¹⁹. Over the last decades, increased industrialization (fossil fuel), agriculture (fertilization), and livestock ranching (sewage) has meant that NO_3^- content was further amplified⁴⁷. In vegetables NO_3^- concentration is dependent on temperature, lighting conditions, and NO_3^- content in soil. High NO_3^- content in soil, in combination with lower temperature and light quantities leads to the highest NO_3^- amounts. Thus, NO_3^- content fluctuates and is higher in northern Europe than in southern Europe⁵³. Additionally, NO_3^- concentration in groundwater and accordingly in soda could be high by leaching and fertilization^{47,53}. Highest levels are found in leafy vegetables, such as spinach and root vegetables like beet root^{19,53}, summarized in table 2. Finally, NO_3^- / NO_2^- are used as a preservative for meat, fish, and cheese productions, as it inhibits late blowing⁵³.

Estimations are that the average daily intake of NO_3^- in Europe lie at between 31 - 185 mg/d and 40 - 100 mg/d NO_3^- in the US (NO_2^- ingestion varies between 0 - 20 mg/d)¹⁹. At the same time, the vegetable and fruit rich DASH diet has been recognized to lower blood pressure^{54,55}. Hord and co-workers hypothesized that this beneficial effect refers to the NO_3^- content in the diet and calculates daily ingestion for two variations of DASH diet, a NO_3^- rich one and a NO_3^- low one. The NO_3^- content varies between 174 to 1,222 mg per day, exceeding WHO recommendations by 550 % (for a 60 kg adult), but with health benefits^{12,19,49}. Apart from the question raised as to whether the WHO recommendation is in fact up-to-date (concerning daily NO_3^- uptake) the study impressively illustrates the huge range and suggestibility of dietary NO_3^- intake.

Nevertheless, only a part of this dietary NO_3^- arrives via circulation and is available for NO metabolism. Every step in the reaction cascade, results in a 3-log loss¹⁹. Only 20 % of ingested NO_3^- is

reduced to NO_2^- , which corresponds to 5-8 % of the ingested amount¹⁹. Instead of this, almost all residual NO_3^- arriving via the small intestine is absorbed¹⁹. This exogenously ingested NO_3^- is mixed with endogenously produced NO_3^- . In a healthy adult about 1 mg/kg NO_3^- is produced endogenously (e. g. 80 mg in an 80 kg adult). If both are taken into account, about two thirds of produced NO is eNOS derived and one third is delivered by diet¹⁹. This ratio elucidated that endogenous synthesis is the more important NO source for mammals and diet could only support this function. This classical way of endogenous NO synthesis via NOS is well-investigated and understood. Recent work shows that this mechanism of NO production only functions under normoxia and that under hypoxia alternative enzyme independent machinery takes over the role of NO provision, which could be well supported by diet.

Table 2. Chosen NO_3^- concentrations in food.

NO_3^- content varies from season to season and is dependent from the area of cultivation. Thus, not absolute numbers but ranges or means are listed. Additionally, indices specify the source. *Hord et al., 2009; [§]Weiß et al., 2008^{19,53}.

vegetables	NO_3^- (mg/100 g)
carrot*	92-195
lettuce*	12-268
spinach*	24-387
cabbage*	26-125
wax gourd*	36-68
cucumber*	1-14
eggplant*	25-42
beet root [§]	156-259
rucola [§]	470-480
fruits	
banana*	4.5
orange*	0.8
meats/processed meats	
bacon*	5.5
ham*	0.9
pork tenderloin*	3.3

1.4 Toxicity and Carcinogen Potential

In the 1940s a health risk was discovered, associated with the consumption of NO_3^- (and NO_2^-). In the following decades these reports about methemoglobinemia resulted in recommendations regulating NO_2^- and NO_3^- consumption. Additionally, not only methemoglobinemia but some forms of cancer were associated with excessive NO_3^- (and NO_2^-) intake. However, none of these studies could provide a real correlation and some of it showed methodical faults after retrospective analysis. Thus, reinterpretation of the results supported the actual discussion of revaluation of dietary NO_3^- (and NO_2^-).

One of the first alerting studies, published in 1948, described methemoglobinemia in infants,⁴⁶ leading to guidelines restricting NO_3^- and NO_2^- content in water. Since then, new cases have been published, but at the same time retrospective studies have also appeared impaired alerting assumptions. In methemoglobinemia NO_2^- is bound to hemoglobin suppressing oxygen binding. This alternative binding can lead to cyanosis and asphyxia, in the worst case⁵⁶. However, only after exceeding 50 % methemoglobin in blood, which corresponds to 1 - 2 g NO_2^- or 15 g NO_3^- ⁵³, could acute life-threatening poisoning occur. Physiologically, methemoglobin-reductase bind NO_2^- until the amount of methemoglobin exceeds its capacity⁵³. Although given the fact that 1 - 3 % of methemoglobin is within the physiological range⁵⁶, the likelihood of poisoning in children and adults is extremely low, with the exception of a hereditary deficit

in methemoglobin-reductase, of course. Single case studies refer to special conditions, for example in New York in 2002, when a whole family suffered from methemoglobinemia, caused by eating bad food⁵⁶. The only subpopulation, in which incidence to methemoglobinemia is increased are infants below three months of age. The three most important factors facilitating the sickening in infants are denoted below⁵⁷. They have a higher gastric pH, possibly supporting NO_3^- reducing microflora. Additionally, foetal hemoglobin is more readily oxidized by NO_2^- than adult hemoglobin, and finally methemoglobin-reductase is not as active in infants as in adults^{53,58}. Nevertheless, only infants, which are not breastfed, are vulnerable. NO_3^- ingestion of the mother does not influence NO_3^- content of breast milk and lactobacillus as the dominant commensal in the stomach and gut of breastfed children is not able to reduce NO_3^- ⁵⁸. Although the higher incidence of infants is uncontroversial, more recent studies place doubt on the fact that NO_3^- alone could be sufficient to induce methemoglobinemia^{59,60}. No NO_3^- or NO_2^- exposure study could cause the “blue baby syndrome” and bacterial contaminations of water or pre-existing gastroenteritis were suspicious to trigger methemoglobinemia in reported cases. Such infections would increase iNOS expression, possibly increasing endogenously produced NO_2^- in an acute toxic manner^{59,60}. To this end, it is not infants but adults or even elderly persons who form the demographic group thought to profit from NO_3^- supplementation. Another problem could be the blocking of the sodium-iodine symporter by NO_3^- , inhibiting uptake of iodine in the thyroid. However, epidemiological studies weakened this hypothesis by negating a correlation between goiter and NO_3^- ingestion⁶¹. At last, a carcinogen potential of NO_2^- formed from NO_3^- is discussed. NO_2^- could react with secondary amines or amides forming N-nitroso compounds (NOCs)¹¹, which were found to act as mutagen in animal models^{62,63}. Therefore, many studies analyzed the potential relation between NO_3^- and NO_2^- ingestion and risk of cancer (gastric, enteric cancer, and glioma, peculiar)^{64–66}. Nonetheless, no careful interpretation and prospective or retrospective analysis could reveal a correlation in human beings^{44,57}. For example, Michaud and colleagues combined three prospective cohort studies to analyze dietary habits of 121,700 females from the Nurses’ Health Study I (NHS I), 116,686 females from the NHS II, and 51,529 males from the Health Professionals Follow-Up Study (HPFS)^{67–69} all of whom were more than 24 years old. They screened questionnaires for meat intake, NO_3^- , NO_2^- , and even nitrosamine levels in nutrition and glioma, but did not find any relation⁷⁰.

Altogether, alerting studies led to guidelines referring maximal values in water and processed food and recommendations for daily ingestion of NO_3^- and NO_2^- ^{49,50}. However, opposing studies negated a strong relation between dietary NO_3^- , NO_2^- , and disease, in contrast they proposed a beneficial effect and suggested an actualization of guidelines^{9,60}.

1.5 NO and Metabolites as Beneficial Concomitant Therapy

Dietary NO_3^- as Beneficial Supplement

Nutrients represent an important regulating factor for good health. As malnutrition could lead to sickness (e. g. fat or sugar) the right dietary supplements could maintain good health⁷¹. Over the last decades many nutrients and ingredients were lauded as valuable with great benefit to healthcare.

Recently, polyphenols have been intensively analyzed for their protective potential, e. g.⁷²⁻⁷⁴. These secondary plant products show high anti-oxidant, -inflammatory, and -carcinogen effects⁷⁵. Among others, they are the ingredients of red wine, green tea, and cocoa and several studies analyzed correlations between health and ingestion in populations with particular high intake of these products^{76,77}. However, consumption of red wine is only one of the elements of a Mediterranean diet and the separate benefits diminish and harmful components have a greater impact. In this case, the alcohol content has to be considered. NO_3^- as an ingredient of a mass of nourishments (described in paragraph 1.3 Dietary NO_3^-) and a storage pool of NO (explained in passage 1.) with only minor risks (discussed in chapter 1.4 Toxicity and Carcinogen Potential), also becomes the focus of attention as a beneficial supplement. This is also the case for the cardiology dysregulation of NO metabolism, which is one of the key features leading to atherosclerosis⁷⁸. Therefore, it should be a goal to maintain and restore NO homeostasis. Moreover, recent work demonstrated NO mediated regenerative effects, beyond its known physiological functions in cardiology^{79,80}. It could be shown that controlled administration of NO_2^- increases revascularisation and regeneration in the animal model of hind limb ischemia. Testing different doses of chronic NO_2^- therapy, the lowest dose was the most effective. Even after three days of therapy, hind limb perfusion was significantly increased, which illustrates the potency and the efficiency of NO_2^- as a beneficial agent⁸¹. As NO_3^- is reduced to NO_2^- it could be also be a promising health supplement, even more so as its' potential for healthcare seems extensive and has still not been fully exploited.

However, in the beginning diets as a whole were analyzed without considering daily NO_3^- ingestion (or other special ingredients). The American Heart Association developed the DASH diet and described antihypertensive effects after consumption⁸², and an Italian group analyzed improved cardiovascular risk profile and oxidative stress status in Italians following a Mediterranean diet⁷⁶. After reevaluation of the health risks of NO_3^- the hypothesis raised the issue that seen positive effects in diets as DASH or Mediterranean diet, refer to their NO_3^- content. Suggested benefits mediated by dietary NO_3^- or NO_2^- concentrated on three major issues. NO operating as antibacterial agent supporting host defense⁸³, acting regenerative in ischemic diseases⁸⁴, and comprising anti-atherosclerotic and cardioprotective functions⁸⁵.

During the first developed studies, NO_2^- or NO_3^- was administered as a drug (inhaled or injected) and later on as a diet supplement. A second round of studies correlated daily NO_3^- ingestion with disease. In 1999 and 2004 two studies discovered a relation between high salivary NO_3^- concentrations and decreased caries^{86,87}. A key role in entero-gastric host defense and mucus homeostasis for non-enzymatic derived NO could be shown manifold^{16,83,88-90}. Gastric NO has an impact on protection against gastric ulcer and mucosal lesions⁸⁸⁻⁹⁰ and promotes mucosal flow and generation⁸³. In the whole gastrointestinal tract NO strengthens host defense by killing gastric and intestinal pathogens^{88,91}. Additionally, it could be illustrated that in mechanically ventilated patients NO-mediated gastric host defense is impaired. These patients who are not able to swallow saliva are fed non-orally, which represses bacterial NO_3^- reduction in oral cavity⁹². Furthermore, not only did NO_2^- and NO levels rise after NO_3^-

ingestion, but gastric RSNO levels also increased and inhibited platelet function after supplementation with KNO_3 ⁹³. A benefit of the antibacterial potential of NO was also proven on human skin and in urinary infections. NO is produced by dermal commensals from sweat controlling bacterial growth and inhibiting fungal proliferation⁹⁴. In urine, in combination with ascorbic acid, NO_2^- reduces the pH of urine, acting bacteriostatic and dietary NO_3^- supplementation was suggested as new treatment strategy in urinary infections⁹⁵.

At the same time, much research was done evaluating the impact of non-enzymatic derived NO in cardiology, underscoring the potential of dietary supplementation^{24,93,96–98}. Additionally, in the respiratory tract non-enzymatic NO unveiled its whole capability. With the discovery of inorganic NO_2^- mediated pulmonary vasodilation, new therapies for pulmonary thromboembolism and newborn pulmonary hypertension evolved^{99,100}. Consequently, the discovery of vasodilative capacity led to the broadened use of inorganic NO_2^- and NO_3^- in ischemic diseases in the cardiovascular system and other vital organs. Promising results could be gained by influencing regeneration in peripheral artery disease (PAD) and hind limb ischemia, respectively, which will be the animal model in the presented work, by increasing NO levels. Several studies could enhance outcome after hind limb ischemia by administration of NO-releasing drugs¹⁰¹ or medication, which mediates augmentation of NO synthesis⁷⁹. In 2008 the first try was done with the direct administration of intraperitoneally administered NO_2^- by Kumar and colleagues⁸¹. They could evaluate improved regeneration with concomitant increased angiogenesis in a model of murine hind limb ischemia. Accordingly, several animal studies quantified a reduced infarct size in myocardial I/R-injury^{27,85}. Parallel, experiments were expanded from heart to liver determining improved outcome in liver I/R-injury after NO_2^- supplementation^{84,102}. Subsequently, intravenous NO_2^- protects the kidney against I/R-injury¹⁰³ and reduces neurological damage after stroke¹⁰⁴. In addition, treatment with infused NO_2^- or inhaled NO show neuro-protective effects avoiding cerebral vasospasm in a primate model of subarachnoid hemorrhage¹⁰⁵ and reduces brain injury in premature newborns with respiratory failure¹⁰⁶.

Another series of work focused on the mechanism by which NO could mediate its cytoprotection. Clementi et al. could show that S-nitrosated proteins are able to inhibit mitochondrial complex I depressing mitochondrial respiration¹⁰⁷. In the following, molecular evidence for the proposed inhibition was supplied and a protective role of it in I/R-injury proposed¹⁰⁸. Mitochondrial experiments and *in vivo* models were combined enabling determination of the protective function of inhibition of mitochondrial respiration in hepatic and cardiac I/R-injury. A decrease in ROS production and prevention of mitochondrial permeability transition pore opening after reperfusion could be determined¹⁰⁹.

Preceding paragraphs summarize treatment and therapy by increasing NO bioavailability, in general, independently from source. Manifold examples emphasize the huge range of beneficial functions of NO in health care. Nevertheless, more and more studies focus on the potential of dietary supplementation able to gain sufficient results for all analyzed organ systems and syndromes presented. Already the study done by Benjamin et al. showed profound salivary NO_2^- and gastric NO formation after NO_3^- ingestion, in the following manifold confirmed^{16,83,87,90,93}. Animal models and observational studies

evaluated the vasodilative and blood pressure lowering potential of dietary NO_2^- and NO_3^- ^{19,98} and expanded analyses to I/R-injury⁸⁵. Shiva et al. transferred *in vitro* results concerning inhibition of mitochondrial respiration by NO to dietary NO_2^- ⁵.

What are the reasons for dietary approaches becoming more important during the last few years? Firstly, nothing is easier to influence than diet. Nutrients are easy to obtain and to deliver with no or less risks and side effects. Moreover, the dosage of nutrients can also be easily controlled. In addition, risks of anesthesia, surgery, or inflammation can be avoided. Costs are minimized and the problem of compliance is circumvented; although large effects could be evaluated. In the case of nitrate supplementation, it brings another advantage. By treating myocardial infarction first measure often is administration of organic nitrates like nitroglycerin (GTN) for vasodilation. However during the metabolization process to NO, organic components of such nitrates need enzymatic metabolization by mitochondrial aldehyde dehydrogenase (e. g.) inducing tolerance⁹. During the chronic administration, these enzymes are partly irreversibly modified at their active sites, lowering the efficiency of treatment¹¹⁰. This phenomenon does not occur with inorganic NO_3^- , because the critical enzymatic step is redundant. Thus, augmented daily intake of NO_3^- is assumed to significantly improve the state of health of (cardio-)vascular diseased patients.

2. Cardiovascular Ischemia

Oxygen, water and nutrition belong to the three essentials needed for life. Water is essential for organic life as it is known and every cell consists to 70-90 % of water. For example, it is a universal solvent ensuring distribution of minerals and proteins, e. g., in cells and transport within the body¹¹¹. Nutrition delivers the bricks of life. Minerals, amino acids, fats, carbohydrates, and many more are all essential to build up cells and organs and to ensure formation, proliferation, and adaptation. However, none of these key elements function without energy, which is supplied by oxygen and subsequent synthesized ATP. Consequently, deficits in oxygen delivery cause severe disease. Hypoxia is defined by a single deficit of oxygen, but in ischemia blood flow is diminished causing a deficit in oxygen and nutrients. Thus, the narrowing and occlusion of arteries lead to ischemia. One of the most prominent diseases caused by ischemia is the endothelial dysfunction, leading to atherosclerosis, peripheral artery disease, and myocardial infarction.

Severe acute ischemic events like myocardial infarction or stroke do not occur spontaneously in 50 % of cases. A long history of chronic ischemia proceeds, for which there are not necessarily any evident symptoms. The first step in the progression of disease is endothelial dysfunction provoking atherosclerosis¹¹²⁻¹¹⁴. Apart from age and hereditary risk factors like a family history of atherosclerosis, the increased prevalence of African Americans and an eNOS gene polymorphism, it is mainly life-style which is responsible for the disease¹¹⁵. Risk factors are adipositas, hyperlipidemia, smoking, and hypertension. However, diabetes mellitus type 2, chronic kidney disease, hypercoagulability (elevated levels of d-dimer, homocystein and lipoprotein A), and increased concentrations of inflammatory markers (CRP, fibrinogen, leukocytes, IL-6) are also hazard factors¹¹⁶.

Atherogenic lipids (oxLDL) induce monocyte adhesion and promote procoagulant properties of the vessel wall. Subsequently, leukocytes, smooth muscle cells (SMC) and platelets intake lipids and differentiate into foam cells forming the fatty streak. They adhere to the activated EC, incorporate into the intima of the artery and an inflammatory lesion develops (see figure 4), presenting the beginning of atherosclerosis^{117,118}. NO bioavailability is decreased, because eNOS substrate (L-arginine) is reduced and arginase II is activated (which destroys L-arginine). Moreover, eNOS is uncoupled unable to produce NO. However, it is a matter of debate whether decreased NO bioavailability is the cause or result of endothelial dysfunction. Altered enzymatic activities lead to increased synthesis of reactive oxygen species, damaging cells^{119,120}.

Next, the lesion expands representing an advanced lesion or atheroma (plaque). This vigorous cellular structure consists of EC, SMC, leukocytes, and cartilage and is covered by a fibrous cap. These atheromas accelerate in size, so that arteries are forced to compensate lowered lumen by remodeling. This includes an expansion of the media by SMC. However, atherosclerosis progresses until remodeling would be insufficient and perfusion is limited. Stenosis (narrowing of a vessel) and chronic ischemia are the consequences. Additionally, arteries lose their flexibility and become rigid. Finally, a complete occlusion elicits an acute event. Either the plaque closes up the lumen entirely or the stable plaque becomes unstable and breaks up. In the latter case the fibrous cap bursts and circulates as prothrombus. While circulating, a thrombus evolves until it obstructs an artery, e. g. in the coronary or the cerebral circulation. Indeed, aggravating atherosclerosis over decades could precede such an event^{116,118}.

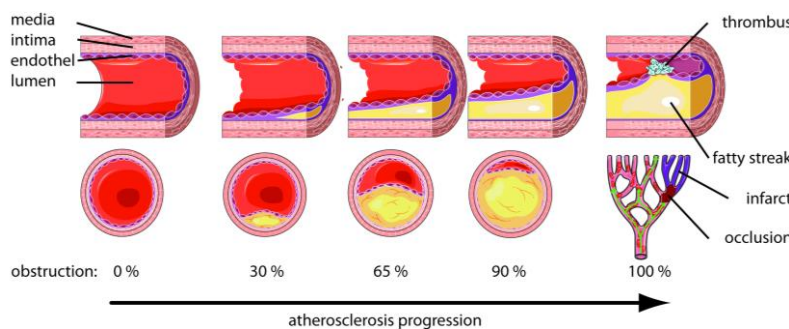


Figure 4. Atherosclerosis Progression.

The left arterial section shows a healthy artery with vast lumen. From the second to the last section, lumen is increasingly narrowed until total occlusion.

Chronic ischemia developing by progressing atherosclerosis, belongs to the group of cardiovascular diseases (CVD), could lead to CAD and PAD, dependent from the affected tissues and arteries. If a coronary circuit is involved, the patient develops a coronary artery disease (CAD), but if peripheral arteries are mainly narrowed the patient suffers from a peripheral artery disease (PAD). CVD is the most common cause of death and a third of all deaths (17.5 million) refer to it, as estimated by the WHO in 2005. In Europe even every second death is attributable to CVD¹²¹. Although neither CAD nor PAD directly cause mortality, the progress of disease leads to life-threatening acute events like myocardial infarction or stroke¹¹⁶. CAD includes patients affected with a stable angina (SA) caused by flow-limiting stenoses. In contrast to that, in unstable angina (UA) plaques disrupt and occlude arteries transiently¹²². PAD also leads to clinical symptoms by stenoses and occlusions, but is concentrated mainly on lower extremities. The animal model of hind limb ischemia (explained in detail in Methods and Materials, chapter 2.3) does not imitate these conditions, but could be observed as an artificial, general approach

enabling its analysis. In the beginning many patients are symptom-free, but in advanced stages they even experience pain while resting and they have limited mobility. The first approach for treatment of PAD is a change in life-style to reduce risk factors. Additionally, statins should lower cholesterol level and improve endothelial function. Besides, moderate exercise training had been proven to attain good results. With the progression of disease ulcerations (non-healing wounds), necrosis could emerge. At this stage interventional therapy has to be preferred to avoid amputations¹²³.

If the fibrous cap of a stable plaque of a CVD patient disrupts and a prothrombus evolves, it becomes an acute event. Subsequently, the structure circulates until it gets stuck in a small or narrowed artery which occludes it. Relative to the affected vascular network, the patient suffered a liver or a kidney infarction, a stroke, or a heart attack. Differential diagnostics describe an instable angina pectoris, a NSTEMI (non-ST-elevation myocardial infarction), and a STEMI (ST-elevation myocardial infarction). The most severe infarction, the STEMI, is diagnosed by a characterizing electrocardiogram, elevated CK (creatin-kinase), and CK-MB (creatin kinase-muscle brain) values, elucidating tissue damage. The first emergency measurements are treatment with organic nitrates. On arrival at the hospital, an early balloon catheter-assisted revascularization (PCI) would be advised, which would decrease the risk of death and increase sufficient reperfusion¹²⁴. The occluded artery would then be reopened by inflating the balloon and kept open by implanting a bare metal or drug eluting stent. Although early revascularization could be life-saving it also initiates processes that further damage the myocardium. In proportion, without reperfusion infarct size accounts for 70 % of myocardium, this is reduced to 30 % by reperfusion. Nevertheless, remaining 30 % refer to the ischemia-reperfusion injury (I/R injury) initiated by necessary revascularization¹²⁵. In order to reduce I/R injury, Encouraging results to reduce I/R injury could, up until now, only be accomplished in animal models, decreasing infarct size after reperfusion significantly^{126–128}. Indeed, to date none of the approaches could be sufficiently translated to the clinical setting¹²⁹. On the other hand, understanding of the paradox phenomenon of I/R injury has become much clearer in recent years^{125,130}.

During the so called oxygen paradox, cells come under oxidative stress attributable to reactive oxygen species (ROS) after sudden re-energization of the cells with O₂. Destructive ROS are mainly produced by the electron transport chain in mitochondria, the endothelial XOR, and the neutrophilic NADPH oxidase. In the following, ROS induces dysfunction of the sarcoplasmic reticulum, injures the cell membrane by lipid peroxidation, acts as chemoattractant for additional neutrophils, and is involved in the opening of the mitochondrial permeability transition pore (PTP). Additionally, ROS reduces NO bioavailability, repressing its cardioprotective effects^{125,128,131}. At the same time, damaged sarcolemmal membrane and dysfunctional sarcoplasmic reticulum lead to a rapid Ca²⁺ overload of the cardiac cell. The process is supported by the reversed Na⁺Ca²⁺ exchanger. In summary, the overload causes a hypercontracture and promotes opening of the mitochondrial PTP leading to cell death^{125,130}. Similarly, fast pH correction causes a paradox damaging effect. Whereas reperfusion lactic acid is washed out and supported by the Na⁺-H⁺ exchanger and the Na⁺-HCO₃ symporter pH increases to physiological values. Nonetheless, this process further facilitates hypercontracture of the cell and mitochondrial PTP opening damns the cell to

death^{125,132}. Previously, ischemic myocardial cell death induced inflammatory processes, which are further strengthened by ROS for I/R injury. A complement system is activated, and neutrophils are attracted to the cardiac tissue. Inflammatory cytokines and increased expression of adhesion molecules trapping leukocytes additionally enhance inflammation^{125,133}. Combined, all processes contribute to the opening of the mitochondrial PTP. Open pore uncouples oxidative phosphorylation and perforates mitochondrial membrane leading to mitochondrial swelling, ATP depletion and cell death^{125,134}. In conclusion, despite enhanced therapy of myocardial ischemia to date, damage induced by I/R injury still counts for 10 % of deaths^{125,135}. Thus, protection of affected tissue is still a priority goal, including myocardium and skeletal muscle tissue, as well as increased revascularization.

3. Endothelial-Regenerating Cells

On the one hand, the presented potential benefit of increased NO bioavailability by dietary NO_3^- is impressive. It was assumed that NO_3^- is reduced to NO and NO decelerate the progress of disease. On the other hand, the described mechanism of action in the cardiovascular system, namely vasodilation can only be sufficient to widen blood flow of stiffed, narrowed arteries in the initial stages of atherosclerosis. In the progressed stages of disease, occlusion reaches a level which can not be compensated by vasodilation and remodeling¹¹⁸. Accordingly, additional beneficial pathways have to be activated by NO. A first hint was given from our laboratory. A positive regulation of endothelial progenitor cells (EPC) proliferation and migration by NO was determined *in vitro*^{136,137}. Moreover, in 2010 the chemokinetic impact of NO on CAC in contrast to the chemotactic impact of chemokines and its orchestrated collaboration could be evaluated⁸⁰. Both, EPC and CAC (in the current work renamed to Endothelial-regenerating cells) are cells pertaining to the group of BM-derived progenitor cells with endothelial regenerating and angiogenic potential.

3.1 Definition

EPC are progenitor cells, which were first isolated and characterized in 1997 by Asahara and colleagues. They isolated a $\text{CD34}^+\text{KDR}^+$ subpopulation of hematopoietic precursor cells able to differentiate into CD31^+ endothelial cells (EC)¹³⁸. The markers CD34 and KDR (kinase insert domain receptor) were chosen as they are stem cell (CD34) and endothelial (KDR) markers defining the special phenotype. Subsequent work supported the assumption that EPC are mobilized from BM, differentiated into EC and are involved in neovascuogenesis^{138–140}. Neovascuogenesis, the formation of blood vessels independently from preexisting capillaries, was thought to be exclusively limited to embryonic vascularization¹⁴¹. In the adult organism only angiogenesis could be evidenced¹⁴², which is characterized by the sprouting of preexisting vessels by mature EC build up new capillaries¹⁴³. The identification of EPC derails this paradigm, revealing neovascuogenesis in adults^{138,143}. In the following, EPC were intensively analyzed, concerning mobilizing factors, migration behavior, functionality in vascularization, and identity. It became apparent that the isolated population of angiogenic precursor cells, circulating in peripheral blood is heterogeneous^{144,145}. Nevertheless, some principles are valid and accepted for the whole group. EPC reside immaturely in the BM and held there by kitL and a positive chemotactic gradient¹⁴⁶. Trigger

factors, mainly hypoxia, initiating signal cascades at which end cytokines were produced and which loosen extracellular matrix (ECM) by matrix-metalloproteinases (e. g. MMP-9)¹⁴⁷ cleave kitL to soluble kitL (skitL)¹⁴⁸ and reverse the chemotactic gradient. Now, chemokines attract EPC from BM into circulation, directing them to the sites of demand. The most important attracting factors were the granulocyte macrophage colony-stimulating factor (GM-CSF), the granulocyte colony-stimulating factor (G-CSF), the vascular endothelial growth factor (VEGF), and the stromal cell-derived factor-1 α (SDF-1 α)^{149,150}. Furthermore, nicotine, estradiol, α 4-integrin, and β 2-integrin were discovered as molecules promoting mobilization and migration of EPC^{151–154}. However, the “right” definition and the exact function of BM-derived progenitor cells are still under debate.

Determination of the “right” definition of BM-derived progenitor cells and comparison of different studies was difficult in the last decade, because of the mass of publications which all had slightly different isolation, culture, and staining methods. Hence, it is a methodological problem and a problem of heterogeneous cell population. Based on reviews and original works, the most important trends are presented leading to the definition used in the current work^{80,144,145,155–163}. Asahara et al. and Hill and coworkers isolated so called EPC from peripheral blood by selection against CD34 or KDR. Non-adherent cells were cultured for five days and the intake of acetylated low-density lipoprotein (acLDL) was confirmed. Cells were spindle-shaped forming clusters^{138,160}. Due to colonization, the cells were also called colony-forming unit-EC (CFU-EC). Another method described the culture of adherent cells for up to seven days^{158,159}, defined after one week by the binding of Ulex Europaeus Agglutinin-1 (UEA-1) and intake of acLDL. Based on surface marker expression and *in vitro* function, these cells appear similar to CFU-EC isolated from Asahara and Hill and were often grouped together as EPC. However, culturing is slightly different, and no clustering is observed. Promotion of neovasculogenesis was reported equally, because the cells were termed circulating angiogenic cells (CAC). These cells are suggested to promote neovasculogenesis mainly by secreting angiogenic factors¹⁵⁶. Thirdly, culturing of CEC (circulating endothelial cells) was extended to about 10 – 21 d¹⁶¹. These cells exhibit cobblestone morphology and are phenotypical similar to EC. Thus, this subpopulation was addressed as endothelial colony-forming cells (ECFC) supporting neovasculogenesis by differentiation into EC. In contrast to EPC, secretion of angiogenic factors is reduced¹⁵⁶. Referring to this varying paracrine potential and duration of culture, EPC were subdivided in “early outgrowth EPC” and “late outgrowth EPC” (similar to ECFC). Finally, characterization by flowcytometry (FACS) has to be linked with definition after culture. This method enables quantification and direct characterization, reflecting *in vivo* status. On the other hand, characterization by culture is more sensible, because morphology and growth behavior could be taken into account. Modern instruments lend the opportunity to multicolor FACS, compensating for that disadvantage partially by detailed surface marker characterization, but every additional marker decreases an already low number of gated cells, limiting the validity of experiment. With this limitation in mind some authors termed CD34⁺KDR⁺ (in mice: CD34⁺Flk-1⁺) cells determined by FACS “putative EPC”¹⁵⁷. A variety of markers is circulating in literature¹⁶⁴, but coming back to the roots to define a progenitor cell with endothelial characteristics, a combination of the mostly used markers CD34 and KDR should describe an “immature cell with endothelial priming”, enabling concurrent count of a valid

amount of cells¹⁵⁷. This method is confirmed by studies, which detected these cells as similar decreased in cardiovascular ill patients as shown for EPC^{165,166} and was also able to promote neovasclogenesis^{167,168}. As CD34⁺KDR⁺ cells were similarly detected to act as angiogen, circulating in peripheral blood, and acts as endothelial-regenerating, the term Endothelial-regenerating cells seems useful. Furthermore, it has to be considered that all subpopulations derived from the same origin and that, in *in vivo* all subpopulations have, possibly, parallel angiogenic eligibilities and functions. Thus, to determine the angiogenic potential of progenitor cells in ischemia, and capture the entirety of “endothelial-regenerating cells” seems favorable. It could be assumed that, *in vivo* the early outgrowth cell (CAC) secretes angiogenic factors while migrating to its target side, supporting vascularization from the time leaving BM niche until reaching ischemic tissue. Once arrived at the place of demand, the cell continuously changed from the early to the late type (late outgrowth cell or ECFC), decreasing secretion and maturing into an EC.

In conclusion, in the current work the term Endothelial-regenerating cell describes BM derived cells with angiogenic potential, circulating in peripheral blood and migrating into ischemic tissue. Recent literature confirmed an angiogenic potential for BM derived cells, but even today after 15 years of research, a consistent definition of involved subpopulations is lacking¹⁶².

3.2 Function

Mentioned above, Endothelial-regenerating cells were also defined and differentiated by their function. However, even this “parameter” could not deliver unambiguously defined populations, because it is also a matter of debate. Asahara and coworkers have already tried to reveal EPC function¹³⁹. In an extended *in vitro* series they observed a progressing EC-like phenotype, with duration of culture and the ability to form tube-like structures. Following, *in vivo* experiments were done and i. v. injected EPC were found exclusively in newly-built capillaries. They concluded that, EPC are BM-derived progenitor cells with the potential to differentiate into EC and to form new capillaries by vasculogenesis, a mechanism which was thought to be limited to embryogenesis. Further work confirmed incorporation into capillaries formed by vasculogenesis and the paradigm of embryonic vasculogenesis was replaced^{160,169–172}. Subsequently, mechanisms responsible for EPC mobilization were investigated^{148,149,172–174} and quantification in physiology and pathology followed¹⁷⁵. Quantification elucidates that Endothelial-regenerating cells are detectable in healthy volunteers but impressively increased under certain conditions, or radically repressed under distinct pathological circumstances (3.3 Endothelial-Regenerating Cells in Pathology). However, in general, the number of Endothelial-regenerating cells in peripheral blood deemed to be extremely low¹⁴⁴. Low number of cells in contrast to copious new-formed vascular networks and heterogeneous subpopulation cast the first doubt on the hypothesis drafted by Asahara et al. and others. Purhonen and coworkers contested the impact of Endothelial-regenerating cells in vasculogenesis and angiogenesis¹⁷⁶. Through detailed immunofluorescent analyses, they stated that BM derived cells are detectable perivascular to the endothelium, but not incorporated. However, total negation of an impact by Purhonen and others were focused on vascularization in tumor growth¹⁷⁶ in contrast to cardiovascular models^{158,159}. Possibly, trigger factors and mechanisms initiating

vascularization are different in these unequal models. Nevertheless, such studies brought attention to a discrepancy in the hypothesis (high number of new vessels and low number of progenitors). Moreover, advanced characterization of cells determined subpopulations unable to form tube-like structures¹⁴⁴. The *in vivo* series and clinical studies described large benefits of Endothelial-regenerating cells treatment and strong correlations between pathology and Endothelial-regenerating cells number and/or function, confirming the vast impact of Endothelial-regenerating cells^{177,178}.

It was assumed that Endothelial-regenerating cells support vascularisation by another process than incorporation into vessel wall. The group of Kalka focused on angiogenic chemokines and growth factors and analyzed Endothelial-regenerating cells concerning their paracrine potential¹⁷⁹. It became obvious that Endothelial-regenerating cells secrete angiogenic factors (VEGF, HGF, or Tie-2, e. g.) promoting vascularization, but also interleukins with anti-inflammatory functions (IL-8, IL-1Ra, or IL-10, e. g.). Moreover, conditioned medium stem-cell mobilizing factors were found in Endothelial-regenerating cells, suggesting a positive mobilizing feedback-loop (MMP-9, GM-CSF, or SCF, e. g.) together with several factors acting immunomodulatory protecting the cell or strengthen inflammatory response (MIF or NAP-2, e. g.)^{180,181}. Indeed, studies comparing the beneficial effects of Endothelial-regenerating cells conditioned medium to cell-based therapy found similar effects^{171,182}. Additionally, it could be determined that the different Endothelial-regenerating cells subpopulations show different secretion patterns. The more the cell equals an EC the less of factors are secreted¹⁴⁴. Thus, recent knowledge leads to different camps referring Endothelial-regenerating cells function. The first group is still persuaded of vasculogenetic function of Endothelial-regenerating cells by incorporation into the vessel wall¹⁸³. The second faction now believes in the great impact in cytoprotection and vascularisation by secretion of paracrine mediators¹⁴⁵. A third group regards Endothelial-regenerating cells as an artificial, not really existing cell population, whose functions are indeed fulfilled by hematopoietic and myeloid stem cells¹⁸⁴. It could be assumed that such sharp borders do not exist. Probably, other hematopoietic and myeloid stem cells are additionally mobilized from BM niche¹⁷⁵, mixed with Endothelial-regenerating cells. Evidence for paracrine effector mechanisms could be considered as proven¹⁷⁹, but endothelial priming also suggests differentiation¹⁴⁴. In conclusion, mammalian organism evolved a strong angiogenic system maintained by Endothelial-regenerating cells, but supported by additional cell groups, initiating vascularization by two functional pillars (paracrine action and build-up capillaries)¹⁸⁵.

3.3 Endothelial-Regenerating Cells in Pathology

Because hypoxia is the main trigger for Endothelial-regenerating cells mobilization, two clinical fields are mostly interested in the function and regulation of Endothelial-regenerating cells. In oncology, tumor vascularization seems to be partly mediated by similar processes as physiological vascularization and understanding these mechanisms could help to treat cancer by inhibiting vascularization, starving the tumor^{186,187}. In contrast, in cardiology, in which a multitude of diseases are caused, ischemia promotion of vascularization is desirable¹⁸⁸. Beside these mainstream fields, the impact of Endothelial-regenerating cells in rheumatid arthritis¹⁸⁹, burn injury¹⁹⁰, renal failure¹⁹¹, and many others was investigated. The

following chapter will concentrate on the impact and potential of Endothelial-regenerating cells in cardiovascular disease.

A review from 2008 stated that Endothelial-regenerating cells could be used as surrogate marker for vascular homeostasis and endothelial regenerative capacity¹⁴⁶. Indeed, Hill and coworkers could correlate decreased Endothelial-regenerating cells level with increased Framingham risk score (an artificial score describing vulnerability to atherosclerosis by risk factors)¹⁶⁰, which could be confirmed for hypercholesterolemia¹⁹² and hyperlipidemia¹⁹³, in detail. The same applies to overweight men¹⁹⁴ and passive smokers¹⁹⁵. All risk factors are associated with decreased Endothelial-regenerating cells concentration and function. Despite these clinical findings, animal studies illustrated an increased plaque size after Endothelial-regenerating cells transplantation¹⁹⁶. Progressed atherosclerosis gives rise to coronary artery disease (CAD or stable angina) and inverse correlation between Endothelial-regenerating cells level and function and CAD could be rewritten^{197,198}. In contrast, levels of unstable angina in patients seemed to increase^{199,200}. Furthermore, Leone and coworkers correlated Endothelial-regenerating cells mobilizing factors with Endothelial-regenerating cells concentrations in healthy volunteers, CAD and acute myocardial infarction (AMI) patients. A slight correlation between G-CSF and SCF (stem cell factor) and Endothelial-regenerating cells in AMI could be shown²⁰¹. Subsequently, it was hypothesized that Endothelial-regenerating cells number predict progression of atherosclerosis, prevalence of cardiovascular events and death from cardiovascular causes^{202,203}. Following, two studies could illustrate therapeutic approaches to compensate for the deficit in Endothelial-regenerating cells with flavanols⁸⁰ and moderate exercise¹⁸⁸. Parallel, the relation between Endothelial-regenerating cells and peripheral artery disease (PAD) was analyzed, determining similar decreased levels. This low concentration increased following revascularization²⁰⁴, but decreased further after vascular injury (endarterectomy)²⁰⁵. Yoon and coworkers examined the regulation of Endothelial-regenerating cells migration in the murine model of PAD (hind limb model), elucidating an impact of intracellular adhesion molecule (ICAM)²⁰⁶. Additionally, measurements in patients suffering from arterial hypertension (AH) substantiated relation between endothelial dysfunction and Endothelial-regenerating cells. In contrast to controlled AH Endothelial-regenerating cells, levels were decreased in resistant, incurable AH patients, which is supposed to be associated with chronic inflammation and progressed endothelial dysfunction^{207,208}. In chronic heart failure (CHF) a difference between the different stages of disease became apparent. From asymptomatic CHF (NYHA class I) to complaints in rest (NYHA class IV), initial elevated Endothelial-regenerating cells concentrations decrease beneath control levels²⁰⁹. This deficit could be partly improved by one acute exercise unit (increase of migratory capability of Endothelial-regenerating cells), but increase of absolute number of Endothelial-regenerating cells needed continuous training^{210,211}. Mechanistically, it could be evaluated that in diabetes, decreased Endothelial-regenerating cells number refer to dysfunctional mobilization^{150,212,213}.

In 2012, a review critically reevaluated EPC phenotypes from the perspective that EPC are proposed as biomarkers for chronic ischemic disease (pAD) and acute cardiovascular events (e. g. acute myocardial infarction (AMI))¹⁶². This review is supported by an actual study examining the metabolic activity and

composition of BM derived cells after AMI, confirming an increase of EPC¹⁶³. Shintani and colleagues could show this effect first²¹⁴, but levels were not further elevated after percutaneous coronary intervention (PCI) excluding an impact of vascular injury on Endothelial-regenerating cells mobilization^{215,216}. Numaguchi and coworkers expanded the results by demonstration of a positive relation of capability of Endothelial-regenerating cells to differentiate with an improved outcome after AMI²¹⁷. At the same time, the enlargement of G-CSF and SDF-1 α levels in relation to increased Endothelial-regenerating cells concentrations were detected^{201,218}. Moreover, G-CSF treatment showed similar beneficial effects²¹⁹ as transplantation of BM progenitor cells do²²⁰.

Accordingly, the therapeutic potential of Endothelial-regenerating cells in cardiovascular disease was analyzed. In particular, approaches were done using the rodent hind limb model with great therapeutic potential^{149,159}. Moreover, Madeddu and coworkers could show the advantage of CD34⁺KDR⁺ Endothelial-regenerating cells by improving vascularisation, in contrast to CD34⁺KDR⁻ hematopoietic stem cells²²¹. As SDF-1 α was known as potent mobilizing factor, Walter and coworkers could show that CXCR-4 signaling (SDF-1 α receptor) is impaired in CAD patients contributing to the decreased Endothelial-regenerating cells mobilization. Consequently, injection of Endothelial-regenerating cells, pre-treated with a CXCR-4 antibody in murine hind limbs, depressed outcome²²². Other studies could improve therapeutic benefit of transplanted Endothelial-regenerating cells by pre-treatment with an Ang-1/VEGF coding vector or activation of the EphB4 system^{223,224}. Additionally, sufficient clinical trials with nutritional supplementation were performed, administering e. g red wine.²²⁵

Regarding the therapeutic and diagnostic potential of EPC in coronary and peripheral artery disease, reviewed by Fadini and Dimmeler in 2012¹⁶², and the correlation between cardiovascular disease and NO, the assumption of a functional association between NO and EPC comes to the foreground. Thus, a group of studies focused on NO metabolism in ischemia and vascularization in relation to EPC. As early as 1998, Murohara et al. could show that eNOS^{-/-} mice have impaired angiogenesis²²⁶. One year later, Lee and colleagues proved that angiogenesis is a NOS-dependent process²²⁷. In 2003, Aicher et al. confirmed that impaired angiogenesis in eNOS^{-/-} mice is due to decreased Endothelial-regenerating cells mobilization¹⁷⁷. This knowledge could be expanded by the discovery that eNOS is inhibited by oxLDL, leading to Endothelial-regenerating cells dysfunction²²⁸. It could be analyzed, simultaneously, that Endothelial-regenerating cells number and function are depressed in diabetes mellitus in a NO dependent manner²²⁹. Statins were used for a long time in AMI and recent results could elucidate an underlying mechanism. Thus, the drugs mediated their beneficial effect by increasing NO bioavailability, followed by amplification of Endothelial-regenerating cells number^{230,231}. Moreover, Heiss et al. could show a direct increase in Endothelial-regenerating cells after NO₃⁻ intake²³². In summary, studies clarified that decreased NO bioavailability is jointly responsible for diminished Endothelial-regenerating cells number and function and ensuing impaired angiogenesis in endothelial dysfunction and subsequent diseases. Subsequently, studies were planned, which tried to amplify neovascularization by supporting the Endothelial-regenerating cells -NO axis. Irradiation with infrared light improved outcome after hind limb ischemia, as well as medication with organic nitrates and Benfotiamine (vitamin B3

derivate)^{79,233,234}. Thus, comprising the previous chapter (1.5 NO and Metabolites as Beneficial Concomitant Therapy), the increase of NO bioavailability to augment Endothelial-regenerating cells number and regenerative capacity seems to be a promising approach in positively influencing regeneration in cardiovascular disease. However, to construct hypothesis and make a decision which would affect chronic CAD and PAD or to intervene during an acute event like AMI, pathophysiology has to be taken into consideration.

4. Tissue Injury and regeneration

Every tissue has special features including composition, origin, resilience, and reparative mechanisms. For this reason, therapeutic approaches should be adopted to the affected organ, although a key element in treating ischemia remains restoring O₂ delivery.

In chronic ischemia, compensatory mechanisms are of paramount importance. Besides the remodeling of existing arteries, new vessels are formed from pre-existing arterioles or de novo capillaries were formed. After an acute event, regenerative processes obtain priority. Irreversible damaged cells have to be removed and the gap has to be filled. For example, skeletal muscle fibers could be renewed to a certain degree, but cardiomyocytes do not have this ability. In the heart necrotic, tissue is replaced by fibrous scar tissue.

4.1 Vascularization

Skeletal muscle of human legs is traversed by a ramified vessel network originated from three main arteries being descendent from the iliac arteries. At the groin *A. iliaca externa* changed into *A. femoralis communis*, which is subdivided into *A. poplitea* and *A. saphena* at the knee, serving the calf. Starting from these vessels, arteries ramified into surrounding tissue. These arteries changed into collaterals and arterioles, which finally end in capillaries. In this way, every station has characteristic possibilities for growth and reaction to ischemia (shown in figure 4). Conductive arteries are competent to perform remodeling. Media gradually dilates, widening lumen. However, this process is limited and only capable of compensating for lumen narrowing plaques and not for stenoses and occlusions²³⁵. On the other hand, stenoses occurring in conductive arteries were threatening and thus it was necessary to treat them. In these cases a bypass surgery could be preferred²³⁶. To a certain degree, arterioles could grow out to arteries forming natural bypasses. This process is termed arteriogenesis¹⁴³.

Initial mechanical trigger of arteriogenesis is increased to shear stress caused by elevated blood flow. In principle, two signal cascades are started. The first pathway is responsible for recruitment of BM-derived cells, supporting arteriogenesis, and the second one enables EC and SMC to re-enter the cell-cycle. Involved are MAP kinases, Rho mediated pathways, and NO dependent signaling. Nonetheless, impact of NO remains enigmatic. There are studies demonstrating impaired arteriogenesis in eNOS^{-/-} mice, which is activated by shear stress¹⁷⁷, but conflicting results were equally presented²³⁷. The peripheral nervous system is only regulatory when involved in arteriogenetic processes. As re-entry in the cell-cycle and mitosis are key features of arteriogenesis, several mitogens and growth factors were

thought to have important functions in the mechanism. However, to date none of the most abundant factors (VEGF, FGF, and PDGF) could be identified as “master factor”. Every one of them is capable of augmenting collateralization, but none reaches levels obtained by shear stress. To support local alterations, BM-derived cells are mobilized and migrated to the proliferative sites of the vessel. It is mainly angiogenic monocytes, which promote arteriogenesis by secreting paracrine factors and accumulate, but T-cells, NK cells, and BM-derived stem cells are also present²³⁸. The impact of lymphocytes becomes obvious when comparing regeneration after ischemia in different mouse strains. C57Bl/J6 mice show higher lymphocyte levels than BalbC mice, which is reflected in better regeneration (additionally, different vessel anatomy support regeneration in C57Bl/J6 mice, presented in discussion chapter 3.1). The impact of stem cells is still a matter of debate, but directs into no direct incorporation of new-formed vessels, but facilitation by paracrine mechanisms²³⁹.

Triggered by mentioned factors and pathways, all cell layers of the artery are activated, intima (consisting of EC), and media (composed of SMC), as well as adventitia (mainly fibroblasts). Activated fibroblasts in adventitia undergo inflammatory changes and attract macrophages. Fibrous and collagenous structures were dispersed by increased expressed proteolytic factors (e. g. MMPs) enabling the outgrowth of the vessel and EC activation includes proliferation and morphological changes. The cells appear swollen and surrender their orientation towards blood flow. Following, orientation seems randomized. As a consequence, in addition to a change in open-probability of Ca^{2+} -dependent Cl^- channels and –inhibitors and the proliferative status of the cells, permeability of the endothelium is elevated, facilitating both migration and outgrowth. Finally, the SMC undergoes drastic changes and accomplishes most work in arteriogenesis. Their phenotype changed from contractile to synthetic, visible by abolishment of characteristic contractile actins (mainly desmin). Yet, two zones of collateral growth could be distinguished. The inner neointima consisting of highly active, synthetic SMC and the outer media composed of less active SMC. Nevertheless, increase of tissue mass is impressive. Murine tissue could be amplified to the 3-fold and human tissue to even more than the 20-fold²⁴⁰.

Instead of the myocardium, in which arteriogenic and angiogenic processes are initiated in parallel, periphery arteriogenesis dominates only the thigh of a leg. In the calf, angiogenic mechanisms prevail. These capillary-forming mechanisms are distinguished in angiogenesis and neovasculogenesis. These processes differ in the construction of new vessels, but are initiated and realized by equal factors and pathways. Indeed, angiogenesis EC sprouts from pre-existing vessels, expanding present capillary-networks. Otherwise, neovasculogenesis capillaries are generated de-novo without an adjacent vascular tree. As described, in detail, in chapter 3.2 “Function” progenitor cells, stem cells, and circulating EC migrate into ischemic tissue. Furthermore, the cells form blood islands, differentiate into early EC, and generate a primary plexus. This primary plexus matures into a secondary plexus, presenting the new capillary network (see in figure 5)^{143,241}.

The impetus of both mechanisms is a lack of oxygen triggering expression and DNA binding of the transcription factor hypoxia inducible factor (HIF). HIF activates a multitude of pro-angiogenic genes. Expression of VEGF, the most important angiogenic mitogen, is a 30-fold increase within minutes.

Subsequently, EC derived proteolytic enzymes (MMPs, e. g.) degrade ECM and EC solve its intercellular junctions. Mediated by VEGF, capillaries are dilated and permeability is increased. Furthermore, leaked plasma proteins form a scaffold for EC migration. EC change into the activated phenotype and migrate towards a chemotactic gradient mediated by PECAM-1, VE-Cadherin and Src-kinase into tissue. Next, maturation completes the process. In a highly orchestrated manner EC fusion create anastomoses and form the lumen. The Notch-pathway mediates arterial differentiation by depressing venous signaling. At the boundaries the Eph-Ephrin system marks arterial and venous capillaries (Eph-B2 = arterial; Eph-B4 = venous). The whole process is supported by monocytes and macrophages. Finally, newly-formed vessels are stabilized by VEGF-recruited pericytes and ECM is rebuilt. In general, angiogenesis occurs more often than neovasculogenesis, but both mechanisms complement each other^{241,242}.

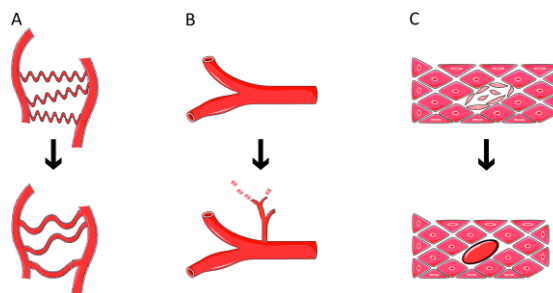


Figure 5. Arteriogenesis, angiogenesis, and neovasculogenesis in comparison.

A In arteriogenesis small collaterals grow out to arteries in response to increased shear stress. **B** Angiogenesis is triggered by hypoxia describing EC sprouting from a pre-existing network. **C** Neovasculogenesis, is able to build new capillaries de-novo without conjunction to pre-existing vessels.

4.2 Myocardium

Previously described vascular network serves myocardium and skeletal muscle tissue. Both organ systems belong to the striated muscle, but myocardium has evolved unique characteristics to accomplish its special function.

Cardiomyocytes develop from special cardiac myoblasts. This cell population does not fuse to polynuclear fibers, but remains mononuclear with intensive connections to adjacent cells. Thus, narrow connection enables fast transmission of the myogene produced potentials, inducing contraction of the muscle. Different from skeletal muscle, which is innervated by motoaxons, cardiomyocytes produce myogene potentials itself and peripheral nervous system has only modulating functions. There are three different subtypes of cardiomyocytes. First, cardiomyocytes of the sinus and atrioventricular nodes are small and only slightly contractile. They are auto-rhythmic and generate the myogene potential. Secondly, cardiomyocytes of the inner endocard are the largest and specialized to fast transmission of the myogene potential. Thirdly, the majority of cardiomyocytes is of medium size and responsible for contraction. They form the outer epicard, which is enclosed by the membranous pericard²⁴³. In contrast, myoblasts of the skeletal muscle fuse to polynuclear fibers within maturation encompassed by connective tissue, the endomysium. After differentiation nuclei are postponed to the edge of the fiber, which reach a length of many centimeters as they range from one bone or tendon to another. Next, 10 to 100 fibers are summarized to a bundle (fascicle), surrounded by the perimysium and another connective tissue layer. The epimysium forms the outer sheath of connective tissue enclosing the entire

muscle and with connection to bone and tendon. This type of striated muscle is innervated by motoaxons and is counted among the voluntary muscles²⁴³.

Under ischemic conditions, cells primarily become necrotic. Additionally, in myocardial I/R injury the opening of the mitochondrial PTP induces caspase-mediated apoptosis in cardiomyocytes. This leads to necrosis in the necrotic core zone and an apoptotic border zone within the infarct area^{125,244,245}. In the skeletal muscle, reactions are more complex because of the combination of polynucleated muscles fibers and neighboring satellite (presented in the following paragraph) cells within the tissue and their specific characteristics. Previously, it was thought that muscle fibers were not able to become apoptotic, but recently programmed cell death could be confirmed for polynucleated cells. However, it seems that apoptosis proceeds atypically and via caspase-3 independent pathways. It is assumed that single nuclei become apoptotic until the whole fiber is irreparably damaged. Otherwise, for satellite cells apoptosis is known for a long time. Thus, in summary, in ischemia and particularly I/R injury apoptosis seems to be the main cause for cell death^{246–248}; even though underlying pathways remain debatable. Controversial results were gained by referring an impact of the Fas/FasL system and possibly indicating a pleiotrophic role in myocyte apoptosis and regeneration²⁴⁹. Previously, the Fas/FasL pathway was discovered as a pro-apoptotic pathway belonging to the TNF superfamily. Despite that, increasing evidence also backs up the bidirectional role of the Fas/FasL system, which is also capable of promoting cytoprotective functions. In the current work, it is assumed that under ischemic conditions the Fas/FasL pathway could also unfold its cytoprotective, proliferative functions^{250,251}. Particularly in ischemic tissue there is a diffuse transition from unsalvageable damaged to injured, but viable cells. Hence, factors able to recognize the status of a cell and initiate appropriate pathways seem to be ideal in ischemia. The Fas/FasL system could be such a bidirectional acting pathway.

However, regeneration of the myocardium is restricted. Although, recent work could demonstrate that, in humans cardiomyocytes were replaced during life, currently no efficient therapy is known to compensate for damage and cell loss in I/R injury²⁵². To date, dead cells can not be substituted. Necrotic tissue is supplanted by fibrous scar tissue and remaining cardiomyocytes dilate (hypertrophy) to achieve needed power. Finally, the heart is pathologically enlarged and contractile muscle partly makes way for collagenous scar leading to functional depression. This process, termed remodeling could cause heart failure²⁵³.

4.3 Skeletal Muscle

In contrast to the myocardium, the skeletal muscle is a very resilient tissue, with high regeneration capacity. This restoration potential refers to the satellite cells discovered in 1961²⁵⁴. Stem cells with myogene potential placed adjacent to muscle fibers, beneath basal lamina, accounting for 2 - 5 % of nuclear mass of a muscle. The term satellite cell encompasses a heterogeneous population of myogene stem cells as different as muscle origin and types, but united in location and regenerative capacity. Moreover, it was assumed that beyond the mentioned parameters, at least two subpopulations could be distinguished. Differences are apparent based on surface marker expression suggesting a pre-mature

population ready for differentiation and an immature population, which is responsible for renewal of cell pool and proliferates before its daughter cells differentiate into muscle cells^{255,256}. In health, satellite cells rest quiescent in G₀ phase, but in response to traumata cells they were activated and re-enter cell cycle and begin to proliferate and differentiate, respectively. These activated cells present muscle precursor cells, myoblasts, recognizable in increased expression of myogenic markers like MyoD. It is assumed that the number of satellite cells is preserved constantly by self renewal and asymmetric cell division. During this mitosis, one daughter cell differentiates into a myocyte and the other remains in the stem cell status replenishing the satellite cell pool. In the elderly, muscle numbers and regenerative capacity of satellite cells is decreased²⁵⁵. Activation is initiated by infiltrating macrophages, which are responsible for eliminating cell debris after necrosis and secretion of mitotic growth factors²⁵⁶. Besides, muscular injury mobilizes several other BM-derived stem cells, which could support satellite cell driven regeneration by myogenic differentiation²⁵⁷. Finally, myoblasts differentiate into fully functional myocytes, fusing to fibers, able to replace lost muscle mass to a great extent. However, ischemia apoptosis concerns the whole muscle including satellite cells. Subsequently, not only is the adult muscle is injured, but regeneration capacity is also impaired. Consequently, approaches geared towards increasing viability of satellite cells should be a promising target for muscle preservation and regeneration. Instead of this possibility, intervention during the acute event comes too late as highly specialized organs, such as the heart muscle have a limited capacity for renewal. Moreover, the presented approach additionally supports vascularization. Thus, cause and symptoms of ischemia are affected in parallel by overcome decreased NO bioavailability. Indeed, vascularisation could be improved in an acute event, but is not sufficient if delivered tissue is irreparably injured. Thus, it is hypothesized that dietary NO₃⁻ has the potential to lower symptoms of PAD (and probably CAD) and to decelerate progression of disease preventing acute events, as presented in the “Aim of Thesis”.

5. Aim of Thesis

Decreased NO bioavailability was shown to be crucial in development of atherosclerosis. Obviously, critical incident in progression of atherosclerosis, endothelial dysfunction, is strongly related to repressed eNOS function. Furthermore, a correlation between decreased NO levels and increased risk endothelial dysfunction and atherosclerosis was determined. As atherosclerosis progresses to life-threatening diseases, such as PAD, CAD, and particularly myocardial infarction, it should be a goal to intervene, as early as possible, in this development. Recent work demonstrated that dietary NO₃⁻ could be reduced to NO increasing circulating NO pool. Moreover, evaluated beneficial effects of NO₃⁻ (e. g. blood-pressure lowering) elucidates its beneficial potential. However, mechanisms remain unknown. A first hint could be the detected NO dependent Endothelial-regenerating cells mobilization. Over the last decade intensive analyses evaluated Endothelial-regenerating cells as BM-derived progenitor cells with pro-angiogenic potential, able to increase angiogenesis and neovasculogenesis. In addition, recent results determined a high paracrine potential of these cells. Nevertheless, it remains debatable about by which key factors or pathways Endothelial-regenerating cells mediate their functions. The Fas/FasL

system was originally identified as death receptor pathway initiating apoptosis. Latest studies could extend this knowledge by showing that the Fas/FasL system could act as bidirectional initiate apoptosis and cytoprotection in parallel.

Accordingly, the aims of this study are to:

1. Analyze the beneficial potential of dietary NO_3^- in vascular ischemia. In the murine hind limb ischemia model regeneration after dietary NO_3^- supplementation will be analyzed.

- This animal model was chosen as it is no imitation of PAD, but appears to be the best approach to study regeneration after peripheral ischemia.

- Regeneration of blood flow blood is analyzed by measurement of hind limb perfusion.

2. Histological analysis should reveal potential increase of arterio- and angiogenesis.

3. Evaluate cytoprotection by determination of tissue necrosis (and apoptosis for a subgroup of experiments).

4. Determine a relation between this beneficial potential and Endothelial-regenerating cell mobilization.

- Analysis of quantity, migration, and viability should show a possible association.

5. Verify the FasL expression on Endothelial-regenerating cells, serving as first evidence of a paracrine function mediated by the Fas/FasL pathway.

- Potential relation is analyzed by quantification of FasL expression on Endothelial-regenerating cells and plasma level of FasL.

6. Deepen knowledge of NO concentration and bioavailability in cardiovascular disease (STEMI) by descriptive clinical studies.

- Determination of NO_2^- and NO_3^- level in severely ill patients and its correlation to the severity of cardiovascular events should increase knowledge about bioavailability in cardiovascular disease and strengthen the impact of the results of the animal study.

It is proposed that dietary NO_3^- increases NO bioavailability at such a rate that regeneration in the model of hind limb ischemia is significantly improved. Furthermore, NO_3^- mediated regeneration is suggested to be supported by Endothelial-regenerating cells. These BM derived cells are probably able to differentiate in EC increasing neovasclogenesis, but have also an impact on cytoprotection of surrounding tissue by paracrine functions. Regarding this, the current study assumed an involvement of the Fas/FasL system in Endothelial-regenerating cells mediated cytoprotection.

In future, increased knowledge about involved key players in regeneration after ischemia in combination with nutritional supplementation could help to improve treatment of cardiovascular disease in an early phase preventing interventional treatments.

Methods and Materials

1. Materials

If not enumerated in a table, chemicals, media, additives, assays and devices are listed in alphabetical order with the producer in parenthesis.

Antibodies

Table 3. Antibodies and isotypes used in FACS and IHC.

FACS = flowcytometry; IHC = immunohistochemistry; r.t.u. = ready to use

Name (Producer)	Description	Dilution	App.
CD11b (Miltenyi Biotec)	rat monoclonal anti-human (PE)	r.t.u.	FACS
Isotype (Miltenyi Biotec)	rat IgG2B (PE)	r.t.u.	FACS
CD31 (Santa Cruz)	goat polyclonal anti-rabbit	1:250	IHC
IgG (Abcam)	goat anti-rabbit (DyLight 594)	1:250	IHC
IgG (Invitrogen)	rabbit anti-goat (Alexa 647)	1:200	IHC
CD34 (BD)	rat anti-mouse (FITC)	r.t.u.	FACS
CD34 (Miltenyi Biotec)	mouse monoclonal anti-human (FITC)	r.t.u.	FACS
Isotype (Miltenyi Biotec)	mouse IgG2a (FITC)	r.t.u.	FACS
CD45 (Abcam)	rabbit polyclonal anti-mouse	1:100	IHC
IgG (Abcam)	goat polyclonal anti-rabbit (FITC)	1:200	IHC
IgG (Abcam)	donkey polyclonal anti-rabbit (PE)	1:200	IHC
Flk-1 (BD)	rat anti-mouse (APC)	r.t.u.	FACS
mFasL (BD)	hamster anti-mouse (PE)	r.t.u.	FACS
iNOS (Abcam)	rabbit polyclonal anti-mouse	1:100	IHC
IgG (Abcam)	goat polyclonal anti-rabbit (FITC)	1:200	IHC
Integrin- α 7 (ab-online)	mouse monoclonal anti-mouse	20 μ g/40 μ l	FACS
IgG1 (BD)	rat monoclonal anti-mouse (PE)	r.t.u.	FACS
KDR (R&D)	mouse monoclonal anti-human (PE)	r.t.u.	FACS
IgG1 (R&D)	mouse (PE)	r.t.u.	FACS
SMA (DAKO)	mouse monoclonal anti-human	1:50	IHC
IgG (Sigma)	goat anti-mouse (FITC)	1:100	IHC

Assays

cGMP Enzymeimmunoassay Biotrack (EIA) System (Amersham); In Situ Cell Death Detection Kit, Fluorescein (Roche); Mouse G-CSF Immunoassay (R&D); Mouse GM-CSF Immunoassay (R&D); Human MCP-1 Immunoassay (R&D); human NAP-2 DuoSet ELISA Development kit (R&D); PromoKine's highly sensitive nonradioactive Colorimetric Cell Viability Kit I (CCVK-I, WST-8) (Promokine); RNeasy Fibrous Tissue Mini Kit (Qiagen); Mouse/Human SDF-1 α Immunoassay (R&D); SuperScript® III First-Strand Synthesis System (Invitrogen); Mouse VEGF Immunoassay (R&D); Xanthine Oxidase Assay Kit (Cayman).

Chemicals and Narcotics

1 kb Plus DNA Ladder™ (Invitrogen); 2-propanol 99,8% (Roth); β -mercaptoethanol (Sigma); acetic acid (Roth); albumin from bovine serum, fraction V (Sigma); Annexin V (BD); Betaisodona (MundiPharma); Borgal (Virbac); Blue Juice Gel Loading Buffer (Invitrogen); CaCl₂ (Sigma); carboxy-PTIO (Cayman); citrate acid (Merck); Dako Pen (DAKO); depilatory cream (Veet); di-ethyl ether (Roth); DNase (Roche); dNTP Mix (10mM) (Invitrogen); Dulbeccos PBS (Serag Wiesner); EDTA (Sigma); Ethanol, 99%, (Roth); G6P (Roche); HCl (Merck); Heparin (Ratiopharm); Hexoral Spray® (McNeil); HgCl₂ (Fluka); Histofix (Roth); Hoechst 33342 (Sigma); iodine (Sigma); Isofluran (Forene®) (Abbott); 2-propanol (Applichem); Ketanest (Pfizer); Lysing Solution (BD); NaCl, salt (Prolabo); NaCl, solution 0.9% (Braun); N-ethylmaleimide (Calbiochem); nitrate reductase (Sigma); Nonidet P-

40 (Fluka); paraffin (Roth); PFA (Roth); Platinum® Taq DNA Polymerase (Invitrogen); potassium hexacyanoferrat (Fluka); potassium iodide (Merck); RNase Zap (Sigma); Rompun (Bayer); soda (San-Benedetto); sodium-citrate (Applichem); sodium-nitrate (Sigma); sulfanilamide (Sigma); TBE-Puffer (Bio-Rad); Temgesic (Essex Pharma); trichloroacetic acid (Roth); TRIS (Roth); TRIS-HCL (Roth); tri-sodium citrate (dihydrate) (Applichem); Triton-X (Sigma); Tween (Sigma); VECTASHIELD Mounting Medium with DAPI (VECTOR).

Buffers and Solutions

Table 4. Used buffers and solutions.

Listed are ingredients and quantities. Chemicals are solved in high purity water unless noted otherwise.

Name	Ingredients	Quantity	Final concentration
Preservation solution, 5 ml	Potassium hexacyanoferrat NEM NP40	1.32 g 0.065 g 0.5 ml	800 mM 10 mM
Stop solution	NEM EDTA NaCl 0.9 %	0.0625 g 250 µl 50 ml	10 mM 2.5 mM
Ferricyanide solution, pH = 7.5	Potassium hexacyanoferrat PBS	1.646 g	0.005 M 100 ml
Mercury solution, store dark and on ice	HgCl ₂ NaCl 0.9 %	0.1 g 5 ml	0.2 %
NO₃⁻ reductase stock, freezed aliquots (up to 3 month)	NO ₃ ⁻ reductase TRIS G6P G6PDH	2 ml 7 ml 4 ml 600 µl	
TRIS, 10 ml	TRIS	3.06 g	
G6P, 1 ml (dilute 1:20)	G6P	0.3042 g	
G6PDH, 714 µl	G6PDH (100 U/ml)	286 µg	1 mg/350 U/ml
NO ₃ ⁻ reductase, 2 ml	NO ₃ ⁻ reductase	1 vial	10 U/ml
Sulfanilamide solution, 8.7 ml (store dark, on ice)	Sulfanilamide HCl 25 %	0.5 g 1.3 g	
Triiodide solution	Potassium iodide Iod ultrapure water Acetic acid	0.81 g 0.285 g 7.5 ml 100 ml	45 mM 10 mM
FACS-binding buffer	CaCl ₂ PBS	0.022 g 100 ml	1.5 mM
20x TBS, pH = 7.5 (IHC) I	TRIS	24 g	200 mM
T-TBS/wash buffer (IHC), 1 l	20x TBS Tween 20 NaCl	20 ml 1 ml 5,8 gl	1x 0.1 % 0.1 M
Blocking buffer (IHC)	T-TBS BSA Horse serum	1 l 10 g 50 ml	1 % 5 %
Dilution buffer (IHC), 1 l	20x TBS NaCl BSA	50 ml 8.7 g 20 g	1x 0.3 M 2 %
AG-retrieval buffer (IHC), 1 l (citrate buffer, pH = 6.0)	Tri-sodium citrate (dehydrate) Tween 20	2.94 g 500 µl	10 mM 0.05 %
TUNEL fixation solution, 10 ml	PFA	0.4 g	4 %
Permeabilisation solution (TUNEL)	Triton-X Na-citrate PBS	0.01 ml 0.01 g 9.98 ml	0.1 % 0.1 %
DNase (TUNEL)	DNase Incubation buffer RNase free water	1.2 µl 12 µl 106.8 µl	
Digestion solution (satellite cells)	DMEM Pronase (10 mg/l) Hepes Pronase PBS	35 ml 4 ml 1 ml 10 mg 1 ml	25 mM
Washing buffer (FACS)	NaNO ₃ FCS PBS	0.1 g 2 ml 100 ml	

Equipment

Binocular (Leica); breathing apparatus (Penlon & Zenevar); Centrifuges (Hettich); CLD 88e (SunChrom); clean bench (Grelaire); coldlight source (Leica); ENO (Eicom); FACS Canto (BD); heating plate (Ika); Histobath (Leica); Histoembedder (Leica); incubator (Thermo Scientific); Laser-Doppler-Perfusion-Imager (Perimed); microtome (Leica); microwave (Bosch); Nanodrop (Thermo Scientific); Neubauer chamber (Brand); fluorescence microscope (Nikon); scale (Sartorius); Speed Vac (Jonan); shaker (Heidolph); suture material (Ethicon & Serag-Wiesner); vortexer (Ika); water bath (GFL).

Media and Additives for Cell Culture

Collagen (0.1%) (Sigma); DMEM (PAA); Dulbeccos PBS (PAA); FCS (Lonza); Ficoll Lympholyte Mammal (Cedarlane); fibronectin (Biochrom); goat serum (PAA); Ham's F10 with glutamine (PAA); Hepes (Sigma); horse serum (PAA); MV-2 medium (Promocell); newborn calf serum (PAA); Penicillin/Streptomycin (PAA); Pronase (Roche); Trypan-Blue (Gibco); trypsin (PAA)

Primer

Table 5. Real-Time PCR primer pairs were ordered from Eurofin.

Sequences were chosen after Yang et. al¹⁸².

Name	Sequence
XOR (forward)	GGTTGTTTCCACTTCCTCCA
XOR (reverse)	TTCCAAGGAACCTCTGTCTG
18s rRNA (forward)	CCATCCAATCGGTAGTAGCG
18s rRNA (reverse)	GTAACCCGTTGAACCCCAT

Software

Chromstar 6.3.19 (SCPA); Citavi 3.1.0 (Swiss Academic Software GmbH); DNA Baser (free test version; Heracle BioSoft S.R.L.); GraphPad Prism 5 (GraphPad Software, Inc.); LDPWin (Perimed); NCBI-Blast (www.ncbi.nlm.gov/BLAST); PowerChrom v2 (eDAQ); (<http://www.primerfox.com/toolbox.htm>) Primerfox ; Rest 2009 (M. Pfaffl, Munich & Qiagen).

2. Animal Model

2.1 Strains

All experiments were done with NMRI mice, an outbred strain from Taconic, ordered by the local animal facility. The transgene eGFP strain B6-tg(Act β -CMV-eGFP)²⁵⁸ was used for bone marrow transplantation, kindly provided by Prof. M. Okabe (Department of Experimental Genome Research, Osaka University). As a control for endothelial dysfunction eNOS knock-out mice were used, kindly provided by Prof. Dr. A. Gödecke (Institut für Herz- und Kreislaufphysiologie, Duesseldorf University Hospital)²⁵⁹.

2.2 Supplementation, Treatment, Dietary and Environmental Habit

Mice were held in groups of two to eight mice dependent on their relationship and cage size. To minimize variation in NO_x level caused by variation in tap water, the mice received soda with controlled NO_x level. Water and standard diet were given ad libitum. The NO₃⁻ group received drinking water with 1 g/L NO₃⁻ in the drinking

water. XOR mediated NO_2^- reduction should be inhibited by 0.6 g/l Allopurinol in drinking water²⁶⁰ which corresponds approximately to 100 mg/kg/day. The NO scavenger cPTIO was injected intraperitoneally, which means a slower way of application that is both less stressful and painful for the animals. They received 1 $\mu\text{l/g}$ (body weight) of a stock solution (1 mg cPTIO diluted in 1ml PBS). To control both the feeding and drinking behavior as well as the weight of the animals (in special after surgery), water bottles, food pellets and mice were weighed every day. Measurements in a subgroup of single-housed animals excluded differences in dietary habit related to supplementation or treatment. A subgroup of animals received NO_3^- once per gavage (12 mM). To inhibit bacterial NO_3^- reduction mice were treated with an antibacterial mouthspray twice a day; one pump dose administered deep into the oral cavity.

To analyze the effect of the human mouthspray on murine oral flora, an oral smear from three mice before and one hour after treatment with mouthspray was taken. The smears were partitioned and cultured on agar plates under a) aerobic and b) anaerobic conditions in the department of microbiology of the animal facility. There the bacteria were identified and after cultivation, colony forming units (CFU) were determined.

2.3 Hind limb Model

All work was conducted in asepsis. Mice were anesthetized with a Ketanest/Rompun combination-narcosis (45 and 10 mg/kg) which was given intraperitoneally. Hairs from ankle to pelves were removed by a depilatory cream. After the thigh was disinfected using Betaisodona, a 1 cm long skin incision was made on the inside of the thigh. Skin was detached, put aside, and fixed with retractors. Femoral artery was prepared free from vein and nerve. Two ligations were made at the proximal origin of the femoral and the distal end of the saphenous artery (with 0.5 mm silk thread), and artery was excised with its attached side-branches as shown in figure 6^{261,262}.

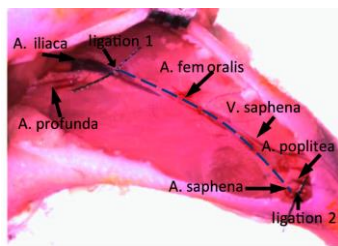


Figure 6. A murine hind limb from groin to knee.

The operative site is shown and visible arteries and veins are labeled. Because of the ligations the a. femoralis and a. saphena are not detectable and adumbrated by the blue, dashed line. The two ligations are also marked.

The skin was closed with a continuous suture done with silk. Successful induction of ischemia, defined as perfusion ratio < 20 %, was controlled by Laser-Doppler-Perfusion-Imaging (Perimed). For analgesia mice received Temgesic (0.05 mg/kg s.c.) as required.

2.4 Bone-marrow Transplantation

To trace migration of Endothelial-regenerating cells in ischemic tissue, green fluorescence protein positive (GFP⁺) bone marrow was transplanted in lethally irradiated NMRI mice. Donor mice were 8 week old males with enhanced GFP⁺ (eGFP⁺) with a C57BL/J6 background²⁵⁸.

On the first day, the eight week old male recipient NMRI mice were lethally, fractional irradiated with 6,5 Gy with 4 h in between the fractions. Irradiation was performed at the clinic for radiation of the Dusseldorf

University Hospital, Heinrich-Heine University Dusseldorf. The mice were placed into specially designed acrylic glass boxes for 10 mice. After irradiation the mice obtained water suspended with 20 µl/l of the antibiotic Borgal® (24 %). To reduce stress the mice stayed in the laboratory overnight, received the transplantation at the second day and were transported back to the animal facility. There the mice were caged for four weeks in IVC-racks for convalescence.

On the second day donor mice were anesthetized with urethane (1200 mg/kg). When they had fallen asleep and lost consciousness their fur was thoroughly cleaned with 70 % EtOH. At the back of the mouse a small skin incision was done and the abdomen and the legs were skinned. The thigh was amputated at the level of the pelvis. Muscle and fat were discarded to visualize the dark red line of bone marrow (BM). Due to the fact, that the bones must not dry they were collected in a petri dish filled with 1 ml sterile PBS. In the next step the long bones were rinsed with sterile PBS. A 20ml syringe with a small cannula (27G) and a sterile filter in between was pricked in the lumen of the bone. The bones had to be rinsed until they seemed clear. The BM was collected in a 50 ml reaction tube on ice and subsequent steps were performed under a sterile bench. Next, cells were separated by resuspending the solution with a 10 ml pipette until no red aggregations were apparent. A 40 µm strainer was laid on a new 50 ml reaction tube to filter the suspension. The cells were washed by centrifugation for 10 min at 300 g and RT. Supernatant was discarded and the pellet was resuspended in 10 ml PBS. 10 µl of this suspension was mixed with 10 µl Trypan-Blue and the cells were counted while the remaining cells were centrifuged (10 min, 300 g, RT). After removing the supernatant, cells were diluted in PBS to achieve the calculated concentration for an optimal transplantation result (2-10 Mio cells/100 µl PBS per mouse). The recipients were anesthetized with gaseous Isofluran® to allow a short but deep unconsciousness. Each 100 µl of BM cell suspension was aliquoted in syringes (1 ml, Braun) and injected via cardiac puncture. Only five syringes at a time were prepared to avoid agglutination and settling of the cells. The success of cardiac puncture should be ensured by aspiration before and after injection.

2.5 Laser-Doppler-Perfusion-Imaging (LDPI)

To control success of hind limb operation and trace regeneration of perfusion, LDPI measurements were done. In principle, a laser-beam penetrates the tissue. Moving blood cells redirect the stream of light to a photo detector. However, moving blood cells lead to Doppler-shift of the incoming light beam dependent from concentration and velocity of the cells. The detector records Doppler-shift and scattering and then calculates an artificial, color-coded perfusion value. Perfusion is defined as *concentration of moving blood cells * mean velocity of these blood cells*^{263,264}. Figure 7 illustrates the principle established⁴³⁰ and modified according to Wardell et al.²⁶³ and Perimed²⁶⁴.

First of all, the laser had to be turned on to warm up. Mice were weighed and then their legs and abdomens were depilated with depilation cream. The animals were anesthetized with the combination narcosis described in chapter 2.3. The mice were fixed on the green measurement pad lying on their back with parallel thighs and upturned soles, with a distance from the laser of 20±1 cm (from mouse surface to laser). Complete list of parameters used in LDPI measurement are summarized in table 6. Narcosis was stabilized by Isofluran, which should be administered very carefully if required.

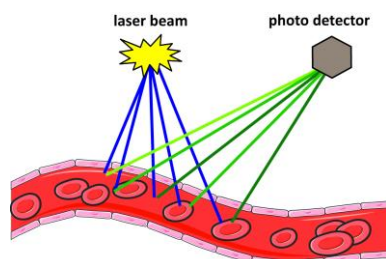


Figure 7. Concept of Laser-Doppler-Perfusion-Imaging

Emitted laser beam impinges on moving blood cells. Light is scattered and velocity changes wavelength and frequency of the streams of light (Doppler shift). The photo detector records transmitted light and connected software calculates perfusion by comparing incoming information with characteristics of emitted light²⁶⁴.

Table 6. Setting of the LDPI measurement.

Shown is the adjustment of the software.

Parameter	Setting
Width	60 mm
Height	55 mm
Auto pause	on
Number of images	3
Time delay	0
Resolution	high
Threshold	intensity 0.35
Viewing scale	5
Color scale	user defined: 400

A macroscopic necrosis score was defined to determine tissue damage (modified after²⁶⁵). The score ranges from “0” which means healthy to “6” which counts for a complete auto amputation. Figure 8 presents the whole score with illustrating photographs.

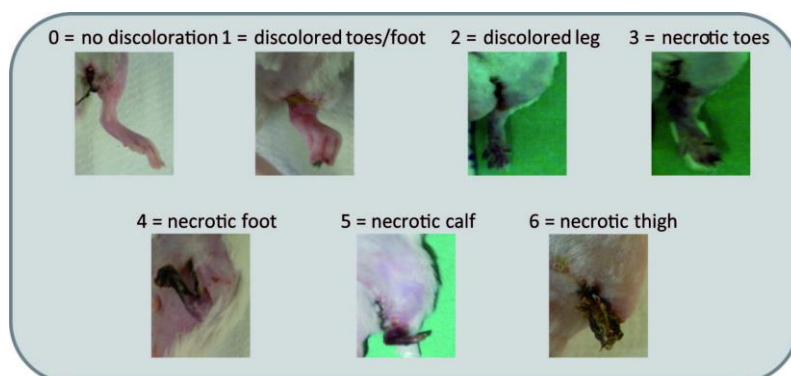


Figure 8. Murine necrosis score.

The score permits the evaluation of macroscopic tissue damage after hind limb surgery.

3. Analysis of NO and its Metabolites

Besides NO_2^- and NO_3^- RXNOs, RNNOs and NO-heme were also analyzed. Human NO_2^- and NO_3^- , and murine RXNOs, RNNOs, and NO-heme were quantified with chemiluminescence detection (CLD). Murine NO_2^- and NO_3^- were determined with the ENO (an instrument using a special HPLC technique see 3.2). All prepared samples were held at 4 °C until measurement or stored at -80 °C.

3.1 Chemiluminescence Detection (CLD)

In contrast to the ENO, that take advantage of the physical qualities of the analyzed molecules, in CLD a chemical reaction is exploited to quantify NO_x . Both are sensitive methods for analyzing NO_2^- and NO_3^- but only CLD allows quantification of more complex nitroso compounds like R_xNOs (experimental setup is shown in figure 9). In this system NO reacts with Ozone. During the course of the reaction, the electrons of nitrogen dioxide which are produced are shifted towards an excited state. While the electrons return back to their ground state they emit energy in the form of a photon in proportion to the NO content of the sample shown in the formula below.



To quantify metabolites of NO, mixtures are injected in an appropriate reaction solution which releases NO from complex metabolites. This gaseous NO is transported to the detection system with helium as carrier gas. On the way, NO passes a NaOH trap (1 M) to catch acids, higher nitric oxides and proteins.

Two basically different methods have to be distinguished in CLD, the reductive and the oxidative CLD. In the oxidative approach ferricyanide-solution oxidizes Fe_2^+ to Fe_3^+ . This conversion destabilizes NO-heme bonds releasing NO in blood or myoglobin rich tissue. In the reductive approach, valid for the detection of NO_2^- , NO_3^- , RXNOs , and RNNOs , the nitroso species are reduced to NO via the nitrosium ion (NO^+) and recorded. To quantify NO_2^- the molecule reacts direct with I_3 in the triiodid-solution. In contrast, NO_3^- , RXNOs , and RNNOs have to be pre-treated. NO_3^- is reduced to NO_2^- , as explained below. To analyze RXNOs , the sample has to be pre-treated with sulphanilamide-solution to remove NO_2^- . Finally, to determine RNNOs , the sample has to be pre-treated with mercury-solution to solve the NO^+ from RSNOs . These nitrosium ions rapidly convert to NO_2^- which is intercepted with sulphanilamide-solution. Therefore, only RNNOs react with I_3 in the reaction chamber.

For quantification a standard curve is needed. For all measurements except NO_3^- a NO_2^- calibration is prepared with the concentrations 0, 50, 100, 150, 200 nM NaNO_2 . The first four stem solutions are prepared with 0.069 g NaNO_2 in 1 ml NaCl (1 M) and three serial 1:100 dilutions (10 mM, 100 μM and 1 μM). From the 1 μM solution the calibration dilutions are prepared. Three times 100 μl sample volume was injected in the reaction solution. NO_3^- calibration was prepared with the concentrations 0, 5, 10, 15, 20 nM NaNO_3 . The first three stem solutions are prepared with 0.0849 g NaNO_3 in 1 ml NaCl (1M) and two serial 1:100 dilutions (10 mM, 100 μM). Starting from the 100 μM solution the calibration dilutions are prepared.

NO_3^- measurements with chemiluminescence detection require an additional reduction step. To get a 10 mM NADPH solution, 120 μl ultrapure water had to be added to 0.003 g NADPH (molecular weight: 833.4 g/mol). This stem solution is first diluted 1:10 and secondly 1:25 (final concentration 40 μM). One light protected aliquot nitrate reductase (680 μl) solution is diluted with 1320 ml Millipore water and mixed with NADPH in a proportion of 5:1 and stored on ice in the dark. 100 μl of this final NO_3^- reductase solution was added to 300 μl sample and incubated 1 h at RT. Calibration curve was equally treated. After incubation the enzymatic reaction was stopped by lowering the sample temperature to 4°C.

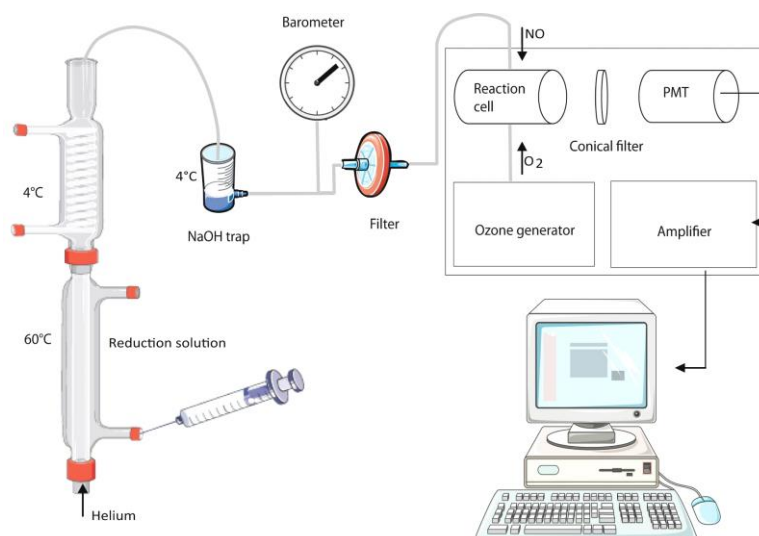


Figure 9. Experimental setup of CLD.

The carrier gas helium is conducted in the reductive reaction solution in which the sample is injected. The NO binds to the helium and flows through the NaOH trap and a sterile filter in the reaction cell. In this compound the NO reacts with the supplied ozone. The light arose is detected by the PMT, amplified and recorded by the associated computer.

After preparing the set-up, the reaction solution was applied (for reductive measurements: 40 ml 60 °C triiodid- and for oxidative analysis 20 ml 37 °C ferricyanide-solution), and calibrating a constant pressure (0 bar), analysis began with the application of the calibration curve. After successful calibration, injection of the samples started. The reaction solution should be renewed if it reached its reduction- or oxidation-capacity, respectively (shown in table 7).

Analysis was done with Chromstar 6.3.19 (human samples) or PowerChrom (murine samples). Both of the softwares save a spectrogram with peaks per sample injection. The area under the curve of every peak is in proportion to the NO concentration in the sample. First the concentrations of the calibration solutions are calculated and a linear regression was done. By calculating the formula: $c \text{ (nM)} = (\text{mean} - (\text{dilution factor} \times \text{blank})) \times \text{dilution factor}$ concentration was calculated. Regression analysis was done with GraphPad Prism 5.

Table 7. Renewing of reaction solution and list of injection volumes.

A Volumes are listed after which the reaction solution has to be changed. **B** Volumes, which are injected in the reaction chamber.

Metabolite	Kind of sample	Volume
NO ₂ ⁻	Plasma	1800 µl
NO ₃ ⁻	Plasma	1800 µl
RXNO	Plasma	660 µl
RSNO	Plasma	720 µl
NO ₂ ⁻	Whole blood	400 µl
NO ₃ ⁻	Whole blood	600 µl
RXNO	Tissue	330 µl
RSNO	Tissue	360 µl
NO-heme	Tissue	300 µl

Metabolite	Kind of sample	Volume
Calibration	-	100 µl
NO ₂ ⁻	Plasma/whole blood	100 µl
NO ₃ ⁻	Plasma	100 µl
NO ₃ ⁻	Whole blood	30 µl
RXNO	Plasma/tissue	330 µl
RSNO	Plasma/tissue	360 µl
NO-heme	Tissue	300 µl

3.2 High Pressure Liquid Chromatography combined with diazo coupling

The ENO (Eicom NO_x Analyzer, Eicom) combines the sensitive technology of high pressure liquid chromatography (HPLC) with diazo coupling accomplishing its high sensitivity. HPLC is a quantitative technology

for measuring concentrations of liquid substances by the help of standards. The questionable substance passages the stationary phase within a solvent, the mobile phase. The stationary phase consists of a separation column which retains NO_2^- and NO_3^- . This retention time is specific for the components, allowing identification and quantification by calculating the area under the peak. NO_2^- is first released by the stationary phase flowing into the reduction column followed by NO_3^- . In the reduction column the second, ENO-specific, phase of the detection reaction follows. First NO_3^- is reduced to NO_2^- with cadmium and copper. Secondly, NO_2^- reacts with the reactor solution producing a diazo compound. As diazo compounds are dyes, absorbance could be measured at 540 nm.

3.3 Sample Collection and Preparation

Sample Collection and Whole Blood/Plasma Preparation

Human blood was drawn from the sheath introducer directly before PCI or by venipuncture at the following days. For plasma samples blood was heparinized with 1 $\mu\text{l/ml}$ (5000 IU Heparin), diluted in 4 ml PBS, and centrifuged 15 min at 2170 rpm and 4°C. Plasma was drawn and stored at -80 °C until measurement. Whole blood was immediately diluted 1:4 in preservation solution to stop further reactions and heparinized with 1 $\mu\text{l/ml}$ heparin. NO_2^- in whole blood has a half-life period of 10 min, after that time NO_2^- is oxidized to NO_3^- or able to nitrosylate other proteins. The ingredients of the preservation solution inhibit these reactions. Ferricyanide reacts with hemoglobin producing methemoglobin inhibiting the modification of NO_2^- to NO_3^- . The detergent NP-40 destroys membranes of erythrocytes allowing the ferricyanide reaction. NEM alkylates proteins to mask their thiol groups, which can no longer be nitrosylated. While preparing potassium hexacyanoferrat, NEM were completely solved, before NP-40 was added. Vigorous agitation and foaming were avoided. The solution could be stored at 4°C for one week^{266,267}.

In the animal care setting, mice were anesthetized with Urethane and blood was drawn by cardiac puncture with a heparinized syringe, with the aim of obtaining up to 1.5 ml blood.

Blood was centrifuged for 1 min at 18,000 g and 4°C and the supernatant was drawn. Plasma was diluted in an equal volume of cold methanol and centrifuged for 10 min at 10,000 g and 4°C. At least 70 μl of the supernatant was given to the autoinjector.

Tissue and RXNO/RNNO Preparation

After the sacrificing and flaying of the mouse (see chapter 2.4) the complete mass of hind limb muscle was prepared free, flattened with a liquid nitrogen cooled gavel, quick-frozen in liquid nitrogen, and stored at -80 °C.

On the day of measurement samples are thawed on ice. At least 240 mg tissue was cut and weighed. Tissue was diluted 1:5 with stop solution and homogenized under ice cold conditions. Stop solution inhibits further reactions. NEM should be added to NaCl and dissolved in an ultrasonic bath. EDTA should be added last. Contamination of stop solution with NO_2^- should not be more than 50 nM because of the low level of RXNOs and was determined before sample measurement. NO-heme was directly measured in 37 °C ferricyanide reaction solution. 100 μl of the antifoam solution was added before sample injection. In the next step tissue

was diluted 1:2 with cold methanol and centrifuged (10,000 g, 4 °C, 10 min). For measuring RXNOs 30 µl sulfanilamide solution was added to a part of the sample and incubated 15 min on ice in the dark. The sample part for RSNO quantification was mixed with 30 µl mercury solution in 300 µl sample volume and incubated for 30 min in the dark on ice. After that, 30 µl sulfanilamide solution was added and incubated for an additional 15 min. Reaction chamber was filled with reducing 60 °C triiodide solution²⁶⁸⁻²⁷¹.

Urine and Feces Preparation

Fixation means stress for the animals and initiates the urinary reflex. Therefore, mice were fixed at the neck and held over a petri dish, exploiting this natural reaction. If the animals urinated less the reflex was supported with a massage of the abdomen^{272,273}. Urine was transferred into a reaction tube and diluted 1:50 with Carrier solution.

After the last treatment mice were kept in single cages without litter but with sheets of paper. After 1 h about five faeces samples were collected. Half a milligram wet weight was 1:1 diluted with sodium chloride and centrifuged for 10 min at 10,500 g at RT. Supernatant was injected into the ENO.

In general, all solvents are measured for determining blanks too. For calibration two standards were measured. All samples were measured in duplicate while 100 µl sample volume was given in the autoinjector.

3.4 EIA (cGMP)

cGMP was measured in murine plasma, skeletal muscle tissue and whole blood. Blood was drawn with heparinized syringes via cardiac puncture as described in the previous paragraph. It was immediately centrifuged for 1 min and 18,000 g at 4 °C. After the supernatant was recovered the plasma was diluted 1:10 with assay buffer. Following preparation of working solutions and EIA procedure was done in accordance with the acetylating protocol in the manufacturer's instructions (cGMP Enzymeimmunoassay Biotrak (EIA) System).

Muscle tissue was taken from hind limbs as described in chapter 3.1. On the day of measurement a piece of the sample, at least 40 mg was cropped and thawed on ice. The tissue was homogenized in cold 6 % (w/v) trichloroacetic acid to get a final concentration of 10 % and centrifuged for 15 min at 2000 g and 4 °C. The recovered supernatant was washed four times with five volumes of water saturated diethyl ether and the aqueous extract remaining dried under vacuum at 60 °C for 4 h. The dried extract was diluted in 100 µl assay buffer and preparation of working solutions and EIA procedure was done in accordance with the non-acetylation protocol in the manufacturer's instructions (cGMP Enzymeimmunoassay Biotrak (EIA) System).

After calculating the average optical density for each set of replicate wells, the percent bound for each standard and sample was calculated using the following relationship:

$$\%B/B_0 = (\text{standard or sample OD} - \text{NSB OD}) \times 100 \text{ (zero standard OD} - \text{NSB OD)}$$

Percent B/B₀ was plotted as a function of the log cGMP concentration and a two competition fit was done to calculate the unknown samples.

4. Analyses of Endothelial-Regenerating Cells

4.1 Isolation and Culture

To analyze the function of Endothelial-regenerating cells they were isolated from murine spleen. The mouse was anesthetized with the already introduced Ketanest/Rompun narcosis. A skin incision in the left, superior back of the mouse was done. Skin and muscles were thrust aside to expose spleen, which was lifted and the vessels severed. Explanted spleen was placed in a PBS-wetted Petri dish and homogenized with the stamp of a syringe (2 ml). Suspension was filled with 3 ml PBS and filtered (40 μ m) in a new tube.

Five milliliter Ficoll were carefully overlaid with the filtered suspension and centrifuged for 20 min at 400 g and RT. Important in such Ficoll-density-centrifugation is to run without brake and speed-up. The MNCs were recovered between the Ficoll- and the plasma-phase (s. figure 10), transferred into a new reaction tube and filled up to 50 ml. Cells were washed by centrifugation (350 g, 10 min, RT, brake: 9, speed-up: 9).

After removing the supernatant the pellet was resuspended in 1 ml endothelial medium. Cells were counted in a Neubauer chamber and directly analyzed or cultured. Five Mio cells were seeded on fibronectin coated 24 wells (1 ml). Coating was prepared with a fibronectin solution (10 μ g/ml); 200 μ l of the solution was dropped in every well, incubated for 1 h at 37 °C, and then excess fluid was aspirated. After 24 h 500 μ l fresh medium was added. Subsequent change of medium was done every second day including a washing step with PBS. Finally the Endothelial-regenerating cells were harvested after seven days.

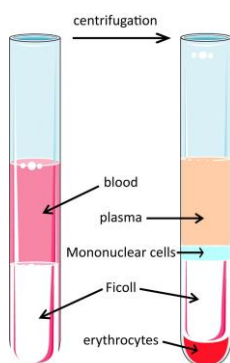


Figure 10 Ficoll density centrifugation.

Blood was carefully put over the Ficoll. After centrifugation four different phases could be seen. The mononuclear cells (MNCs) were found between the top phase consisting of plasma and the Ficoll phase. They were carefully aspirated with a small cannula. At the bottom of the centrifuge tube the erythrocytes were found.

4.2 Quantification and Characterization by Flowcytometry (FACS)

Endothelial-regenerating cells were characterized and quantified by flowcytometric analysis from murine whole blood. Endothelial-regenerating cells were defined as CD34⁺Flk⁺, a combination referring to the stem cell nature (CD34) and the endothelial character (Flk-1) of the cells. Additionally the FasL expression on the Endothelial-regenerating cells was analyzed. In a subset of samples vitality of cells was determined with Annexin V staining.

Blood was drawn by cardiac puncture with a heparinized syringe to get at least a volume of 4 x 75 μ l. After partitioning the blood was stored on ice and lysing solution was diluted 1:10 with distilled water. In case of Annexin V staining, cells were centrifuged for 5 min at 250 g and RT. After the supernatant had been discarded, the pellet was resuspended in 100 μ l binding-buffer. First 10 μ l of the antibodies against CD34 and Flk-1 were added, the sample was vortexed and incubated for 30 min in the dark on ice. Secondly 5 μ l Annexin V was added and the sample was incubated 15 min in the dark at RT. The subset of samples which were not stained

with Annexin V was mixed with 10 μ l of every antibody (CD34, Flk-1, FasL), resuspended in PBS, and incubated for 30 min in the dark on ice. Afterwards, erythrocytes were lysed with 1 ml of lysing solution. The diluted buffer was added and the samples were mixed thoroughly four times and incubated 10 min at RT in the dark. Afterwards the samples are centrifuged for 5 min at 350 g at RT. The supernatant was removed and the pellet resuspended in 500 μ l PBS. After washing by centrifuging for 5 min at 350 g and RT, the supernatant was removed another time and the sample was resuspended in 400 μ l binding buffer (after Annexin V staining) or PBS (after Endothelial-regenerating cells and FasL staining).

Flowcytometric analysis needs one control per antibody. Two types of control exist which control different artifacts. A sample stained with an isotype (immunoglobulin control) of the antibody controls for unspecific binding of the antibody. This is important in particular if you test antibodies of unknown quality or known unspecific binding. A FMO control determines autofluorescent signals of the cell. It is especially important to control that if you want to, for example characterize erythrocytes with a known high autofluorescence. Recently in “normal” not especially difficult but multicolor flowcytometric staining the FMO control has become routine. FMO is the abbreviation of “fluorescence minus one” and means that all antibodies are used minus the one this sample controls. In the described work three controls, in addition to the staining, were carried out: 1. CD34+FasL (-Flk-1); 2. Flk-1+FasL (-CD34); 3. CD34+Flk-1 (-FasL).

Samples were counted in the FACS Canto and gated in a SSC vs. FSC plot. The Endothelial-regenerating cells were detected in a gate including monocytes and lymphocytes. In the next step the FITC fluorescence (CD34) is plotted versus the APC fluorescence (Flk-1). Double positive events were defined as Endothelial-regenerating cells. PE positive Endothelial-regenerating cells were finally quantified as FasL⁺ Endothelial-regenerating cells (figure 11).

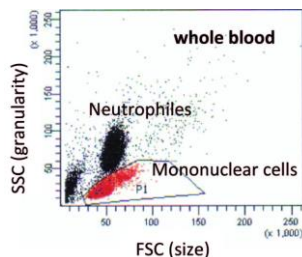


Figure 11. FACS measurement.

Whole blood was stained with CD34, Flk-1, and FasL. The scatter plot shows the different cell populations, and mononuclear cells could be gated separately from the neutrophils.

4.3 Immunofluorescent Determination of Angiogenesis and Neoangiogenesis

After injection of the lethal narcosis (1200 mg/kg Urethane, i.p.), the skin of the thorax was opened and heart and liver were prepared. To remove blood from the tissue the mouse was perfused with 20 ml PBS via the cardiac apex (using a 20 ml syringe and a 20G cannula). Success of perfusion was controlled by a grey discoloration of the liver. Subsequently, the mouse was perfused with 20 ml Histofix to support following fixation.

After skinning has been completed, the legs were amputated at the groin and placed in 10 ml Histofix (10 ml/g tissue) for 24 h at RT. On the second day, the solution was changed to a decalcifying solution with EDTA in which the tissue remained for four to five days. To dehydrate the tissue, samples passed an ascending alcohol series: washing 30 min in PBS – swivel in 70 % isopropanol overnight (4 °C) – swivel in 80 % isopropanol for 2 h (RT) – in 90 % isopropanol for 2 h (RT) – 96 % isopropanol for 2 h (RT) – 100 % isopropanol for 2 h (RT) and in

Histol again overnight. However, before starting with the ascending alcohol series the legs were separated at the knee and fringed ends were smoothed cutting the thigh and the calf in square, regular blocks. On the ninth day the tissue was penetrated with 37 °C hot paraffin for at least 4 h and thigh and calf were embedded in one paraffin block. These paraffin blocks were cut into 8 µm sections. From a whole leg 100 slides were made, 10 slides in 10 levels. Staining of the capillary system and the Endothelial-regenerating cells was done in the upper calf (level 1). For reliable staining of the capillaries (Endothelial-regenerating cells) a double (triple) staining was done to exclude CD31⁺ leukocytes. Thus, capillaries were defined as CD45⁻CD31⁺ and Endothelial-regenerating cells were defined as CD45⁻CD31⁺GFP⁺. Antibodies were diluted in TBS with 0.3 M NaCl and 2 % BSA, pH 7.5.

While the slides passed a descending alcohol series (1. Xylol/Histol: 15 min., 2. Xylol/Histol: 15 min, 1. 100 % isopropanol: 5 min, 2. 100 % isopropanol: 5 min, 96 % isopropanol: 5 min, 70 % isopropanol: 5 min, T-TBS: 5 min) buffers and solutions are prepared. A 20x TRIS, pH 7.5 solution serves as stock for the remaining solutions. The washing buffer (T-TBS) additionally contains Tween 20 and NaCl, the blocking buffer BSA and horse serum. For antigen retrieval a citrate buffer, pH 6.0 was prepared.

After hydration the antigens in the tissue were unmasked by boiling in citrate buffer (20 min, 900 W in a microwave). After 20 min cooling time and washing twice for 5 min in T-TBS the samples were blocked for 1 h and RT in blocking buffer. In the next step the samples were framed with a marking pencil and 50 µl of the first antibody was dropped onto the slide. Incubation took place overnight in a humidified chamber at 4 °C. On the following day the slides were washed three times 5 min in T-TBS and 50 µl of the secondary antibody was added. The samples were incubated for 1 h in a humidified chamber in the dark at RT. Finally the samples were washed another three times for 5 min in T-TBS and mounted in one drop Vectashield® with DAPI.

Regularly, one day after staining slides were analyzed with a fluorescence microscope. Five pictures of every slide and every dye were recorded with 20x magnification, trying to cover the whole slide. During analysis the CD45⁻CD31⁺ (CD45⁻CD31⁺GFP⁺) cells of every field of view were counted and averaged. This value was extrapolated to 1 mm².

4.4 Analysis of Viability by Tetrazolium Salt (WST-8) Assay

The spleen isolated Endothelial-regenerating cells were cultivated for 7 days as described in chapter 2.1. After that the cells were trypsinized and a suspension with 300,000 cells/300 µl was prepared. The cells were given in a 96well plate in triplicate (3 x 100 µl). In three wells the medium was filled as blank. To every well 10 µl of the reaction-solution (CCVK-I) was added and 4 h incubated in the incubator. After that time absorption was read at 450 nm. The level of absorbance directly correlates to the viability of the cells.

4.5 Quantification of sFasL by Enzyme-linked-immunosorbent Assay (ELISA)

Blood was drawn by cardiac puncture with heparinized syringes and plasma separated by centrifugation for 15 min at 2170 rpm and RT. Samples were stored at -20°C until analyzing. On the day of analysis all reagents and samples were brought to RT and 50 µl assay buffer was added to 50 µl sample filled in the wells. Every sample was analyzed in duplicate. The following procedure was done in accordance with the manufacturer's instructions and optical density was read at 450 nm and subtract readings at 540 nm. For the calculation the

duplicate readings were averaged and the blank was subtracted. A standard curve was generated and unknowns were calculated with the slope.

5. Analysis of Satellite Cells

5.1 Isolation

The mice, which were donor animals for the BM transplantation, were sacrificed and the legs amputated as described in chapter 2.4. The muscle was separated from bone, fat, and fibrous tissue under the clean bench and cut into small pieces with a scissor. With the stamp of a syringe the cells were further homogenized. After this mechanic digestion the tissue was brought into an enzymatic digestion solution and incubated for 1 h on a shaker at 37 °C.

Subsequently the homogenate was filtered first with a 100 µm cell strainer and second with a 40 µm cell strainer. The suspension was filled up to 50 ml with PBS and centrifuged (10 min, 350 g, RT). After discarding the supernatant the pellet was resuspended in 1 ml PBS and filled up to 50 ml. The cell suspension was split into five petri dishes (10 cm diameter). Because only fibroblasts are able to adhere to uncoated material in the short term, after 1 h incubation (37 °C) the cells could be separated in mainly adhesive fibroblasts and floating satellite cells. The enriched satellite cells were washed (centrifugation for 10 min at 350 g and RT), resuspended in 1 ml PBS, and counted with a Neubauer chamber.

5.2 Characterization by Culture and FACS

Culture of Satellite Cells

The cells were first characterized by culturing and qualitative morphologic observation. Isolated cells were cultivated on collagen-coated plates for 16 d. Every three days the medium was changed and cells were microscopically assessed and photographed. After 11 d satellite cells began to differentiate developing their characteristic spindle-like shape as shown in figure 12. In the following week the cells started to fuse into polynuclear myofibers which could be seen after 16 d.

For coating, collagen solution (10 ng/100 µl/cm²) was prepared and 100 µl/cm² were pipetted on the plates and incubated at 4 °C overnight. On the next day, the remaining fluid was aspirated and the plate dried under the clean bench. Subsequently 100,000 cells/cm² were seeded in 6well plates in satellite cell specific medium (Ham's 10 with glutamine, with 100 ml FCS (10 %), 50 ml horse serum (10 %), and 1 ml Penicillin/Streptomycin (0.2 %)).

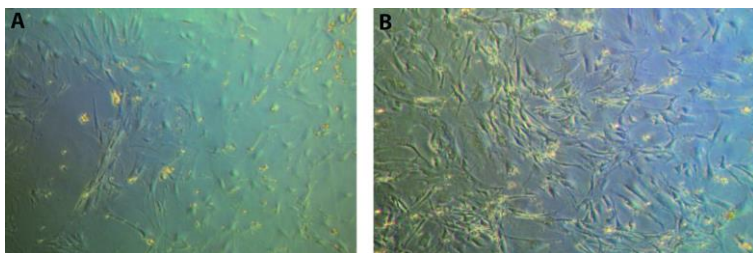


Figure 12. Morphology of primary isolated satellite cells.

Samples derived from cultures after 11 (A) and 16 (B) days of culturing. After 11 d the cells began to differentiate and the long spindle shape arose. After 16 d the differentiated cells began to fuse forming polynuclear fibers.

Flowcytometric Characterization of Satellite Cells

Secondly, satellite cells were characterized by flowcytometric measurement. The cells were stained with an Integrin- $\alpha 7$ antibody which is specific for myocyte progenitors. One million cells suspended in 500 μ l PBS were introduced in the analysis and washed twice by centrifugation in washing buffer (5 min, 350 g, RT). Next the cells were stained with 40 μ l of the primary antibody and incubated for 30 min at RT. After another washing step with 1 ml washing buffer, 30 μ l of the secondary antibody was added and incubation started (15 min, RT, in the dark). Finally the cells were washed like before and resuspended in 500 μ l PBS. An unstained sample was used as control and all cells were gated (s. chapter 4.2). Mean fluorescence intensity was verified in histograms similar to those presented for the FasL expression analysis in chapter 4.2.

5.3 Analysis of Apoptosis by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

The fresh isolated satellite cells were washed three times in 1 ml PBS (centrifugation: 350 g, RT, 5 min). Two million cells were resuspended in 100 μ l PBS; at least one sample had to be separated into thirds à 2 Mio (1 unknown, 1 positive control, 1 negative control). After adding 100 μ l fixation solution and the cells incubated for 1 h at RT on a shaker. Two more washing steps were affiliated with 200 μ l PBS (5 min, 300 g, RT).

Subsequently the cells were resuspended in permeabilisation solution and incubated for 2 min on ice. Two additional centrifugation steps in 200 μ l PBS followed (5 min, 300 g, RT). At the same time, the positive control was resuspended in 50 μ l DNase and incubated for 10 min at RT. At the end of this incubation, samples were resuspended in 50 μ l TUNEL-reaction-mix (enzyme and label solution) and negative control (only label solution without enzyme). Samples and controls were incubated for 1 h (37 °C)²⁷⁴. After completing the TUNEL reaction, cells were washed twice in PBS (5 min, 350 g, RT) before a Hoechst staining completes the assay. 300 μ l of Hoechst solution (1:5000 diluted in PBS) was added to the samples and incubated for 5 min. Two more washing steps followed as described and the resuspended cells (250 μ l PBS) were transferred into a 96-well plate. On the next day, the plate was analyzed by fluorescence microscopy. Three fields of view were counted with FITC (TUNEL positive nuclei) and UV (DAPI positive nuclei). An apoptotic ratio was calculated. The proportion of apoptotic cells from total in the ischemic and the non-ischemic leg was counted and divided.

6. Immunofluorescent Analysis of Arteriogenesis and iNOS Expression

The same tissue and procedure which was used for Endothelial-regenerating cells and endothelial cell immunohistochemical staining (s. chapter 4.3) was used for the detection of arterioles and inflammation.

Arterioles were detected by staining for SMA- α in level 1 of the thigh. Inflammation was depicted by iNOS (level 1 of the calf). Because iNOS is an intracellular protein, permeabilization of the cell membranes were needed and achieved by adding Tween 20 to the antigen retrieval buffer.

7. Biochemistry and Molecular Biology

7.1 Determination of Angiogenic Cytokines by ELISA

For all enzyme linked immunosorbent assays (ELISA) cardiac blood was drawn with heparinized syringes and centrifuged 1 min at maximum speed and 4°C. All immunosorbent assays listed were done in accordance with the manufacturer's instructions and absorbance measured at 450 nm. Differences only occurred in sample preparation, thus these steps of the analyses were detailed below.

GM-CSF

For analyzing GM-CSF plasma samples were centrifuged a second time for 5 min at 13,000 g and RT to get PpP.

G-CSF

For the determination of G-CSF the samples were treated as described for measuring GM-CSF. Plasma samples were centrifuged a second time for 5 min at 13,000 g and RT to get PpP, but were diluted 1:2 in assay diluent.

VEGF

VEGF was determined in plasma and tissue. 30-50 mg of the tissue were homogenized in PBS (1:20 dilution) and stored overnight at -20°C. After two freeze-thaw cycles (according to manufacturer's instructions) to break the cell membranes, the homogenates were centrifuged for 5 min at 5000 g and assayed immediately. In contrast heparinized plasma was only diluted 1:5 in calibrator diluent and further processed.

SDF-1

The samples for SDF-1 α detection were centrifuged a second time for 5 min at 13,000 g and RT to get PpP.

7.2 Quantification of XOR activity by an Enzymatic Assay

XOR activity was measured in murine skeletal muscle tissue with an enzymatic fluorescence assay. After sacrificing and flaying the mouse, the pelage and skin were removed as described in chapter 3.1. The complete mass of hind limb muscle was prepared and 1 g tissue homogenized in 1 ml cold Tris-HCl buffer. Homogenate was centrifuged for 15 min at 10,000 g and 4°C and the supernatant was analyzed in the assay, which was done in accordance with the manufacturer's instructions and fluorescence measured at 540 nm.

7.3 XOR Expression Analysis by Real Time PCR

In murine skeletal muscle XOR mRNA expression was analyzed. Muscle tissue was raised in the same manner as for "Analysis of NO and its Metabolites" (chapter 3.1). Primer pairs were ordered according to Yang et. al¹⁸².

RNA was isolated according to manufacturer's instructions by the "RNeasy Fibrous Tissue Kit" except for three steps. According to the large amount of tissue, it was not homogenized once but 4 times in 200 μ l RLT buffer. Hereafter, these four aliquots were pooled to two samples given on two RNeasy mini columns. While the RNA was eluted from the column with H₂O, the samples were incubated for 1 min. Concentration was determined with a Nanodrop and quality additionally with a NanoChip 6000. This chip analyzes the quality of

the 18s and 28s rRNA, which have to stand at the rate of 1:2. The software then calculates the RIN (RNA integrity number), which should be between 7 and 10.

In the next step cDNA was synthesized with “superscript III” reverse transcription kit (Invitrogen©) according to manufacturer’s instructions. In every case 1 µg RNA was introduced into the synthesis.

Thereafter, Real-Time PCR analysis of XOR with SYBR Green reagent was conducted (PCR settings are listed in table 8). The mastermix (for one reaction) contained: 2x SYBR Green reagent (12.5 µl), 3 µl forward primer (table 5), 3 µl reverse primer (table 5), 5 µl template, and 1.5 µl molecular biology grade water. The volumes guaranteed a final concentration of 2 ng/µl template and 5 µM primers. A primer pair from 18s rRNA served as reference control. Analysis was done after Pfaffl et al. with REST software²⁷⁵.

Table 8. PCR settings were set as shown in the table below.

Name (Producer)	Temperature	Time
ApliTa1Gold activation	95 °C	10 min
Denaturation	95 °C	15 s
Annealing	60 °C	1 min
Extending	60 °C	1 min
Number of cycles: 40		

8. Clinical Studies

(“New techniques in cardiac imaging and diagnostic of the ischemia/reperfusion-injury in the acute myocardial infarction” and “Serial measurements of Whole Blood Nitrite in an Intensive Care Setting”)^{276,277}

Objective of the studies was to examine NO metabolites of critically ill patients in comparison to organ function and inflammatory status. Two sets of patients were assessed. In the first setting, blood was drawn from all ICU patients each morning via an 18- or 20-gauge arterial catheter over a period of 10 days. In the second series, there was an attempt to track the hemodynamic and inflammatory status, for the period of one week, of patients suffering from a STEMI. Blood was drawn directly before PCI, 1 d later and 6 d afterwards.

8.1 Patient Population

30 patients of the cardiology ICU were included in the study (“Whole blood nitrite in an intensive care setting”¹) irrespective of their disease. The sole exclusion criterion was patients who had been administered NO-donating drugs less than 8 h before blood collection. The patients were enrolled for a periods of 10 d., which was the predefined investigation period. Patients suffering from a STEMI were included in the second study, if they were older than 18 years and when admission occurred less than 12 h after the onset of the symptoms. Only patients who had had a first myocardial infarction or if their infarct related artery had been affected for the first time were considered. In the case of chronic kidney disease with a glomerular filtration rate below 60 ml/min/1.73 m² calculated by the Modification of Diet in Renal Disease Study equation⁴¹⁹ or pregnancy and lactation, patients were excluded from the study. The study was conducted in accordance with the provisions of the Declaration of Helsinki and with the International Conference on Harmonization Good Clinical Practices, and protocol approval was obtained from the local Ethics Committee. All patients gave written informed consent.

8.2 Analysis of Nitrite and Nitrate

Analysis of nitrite and nitrate was done from arterial or venous blood with chemiluminescence detection as described in chapter III. Blood was collected in tubes with 1 µl heparin (5000 U) and 4 ml PBS per milliliter blood. Immediately after taking the blood sample the fluid was centrifuged (15 min, 2170 rpm, 4 °C) and the supernatant stored at -80 °C.

8.3 Description of Inflammatory and Angiogenic State

SDF-1, NAP-2, and MCP-1

Levels of the cytokines SDF-1, NAP-2, and MCP-1 were detected in plasma of STEMI patients. All cytokines were quantified in venous blood but the anticoagulant was heparin for SDF-1 α , citrate for NAP-2, and EDTA for MCP-1. Plasma was separated by centrifugation as described above (chapter 7.1). Moreover, the PrP was centrifuged a second time for getting PpP for quantification of NAP-2 (5 min, 13,000 g, RT). All assays were done according to manufacturer's instructions.

Quantification of Endothelial-Regenerating Cells by FACS

According to the protocol listed in chapter 4.2, whole blood of STEMI patients was stained and Endothelial-regenerating cells quantified. Variance was given in anticoagulant (citrate), volume (1 ml), controls (isotype-controls) and human antibodies. In addition to the quantification of Endothelial-regenerating cells, blood was stained for CD11b, a marker for activated neutrophils.

Clinical Chemistry

All analyses were performed in the laboratory of the Institute for Clinical Chemistry of the University Hospital Aachen. The inflammatory parameters IL-6, (hs)CRP, and TNF- α were determined by serum samples analyzed within 30 min after collection. TNF- α and IL-6 were determined by chemiluminescent immunoassay. CRP was assessed by immunoturbidimetry and nephelometry (hsCRP), respectively.

9. Statistics

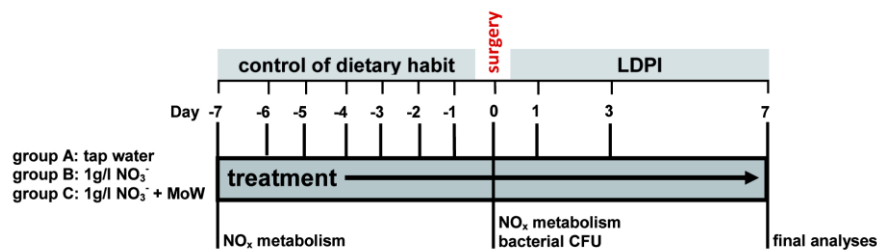
Statistics were calculated with GraphPad Prism 5. Firstly, distribution of the data was determined with a Kolmogorov-Smirnov test (with Dallal-Wilkinson-Liliefors p value) or D'Agostino and Pearson omnibus normality test. Subsequently, two samples were analyzed using a t-test (parametrical data) or a Mann-Whitney test (non-parametrical data). The paired data of the clinical study was analyzed by a Wilcoxon matched paired test. If three or more data sets had to be compared a variance analysis was done. In the case of parametrical data an ANOVA with Bonferroni post-test was calculated, but if the data showed a non-parametrical distribution a Kruskal-Wallis analysis and Dunn's post-test was chosen. Three or more data sets of the paired clinical data were analyzed by a Friedman-test, including Dunn's post-test. Outliers were detected by calculating twofold SD and fitting the data with non-linear regression and automatic outlier detection with GraphPad Prism²⁷⁸. Real-Time PCR results were calculated by REST© by a Pair Wise Fixed Reallocation Randomisation Test and S.E. estimation via a Taylor algorithm²⁷⁵.

Results

1. Animal Study

The awareness that in atherosclerosis there is a need for NO and that its' metabolites NO_2^- and NO_3^- could be recycled brought about the idea of positively influencing NO metabolism in ischemia with NO_3^- . As a result, an animal study was designed in which a severe ischemia was induced and diet was supplemented with NO_3^- .

In the following, the mice, which got soda without supplement, are termed “control”. Subsequently, the group, which drunk soda supplemented with NO_3^- , is named “ NO_3^- ”. The animals, which were supplemented with NO_3^- and treated with antiseptic mouthwash, are denoted as “MoW”. Figure 13 clarifies the study design for the main series; if other set-ups were designed to analyze special features they are explained coupled with



corresponding results.

Figure 13. Study design of *in vivo* experiments.

NO_3^- supplementation began seven days before surgery and persists a further seven days after it. Mice that were administered mouthspray twice a day in addition to a NO_3^- supplementation were used as negative control. In the week before surgery, the influence of supplementation and treatment on food and water consumption was excluded by determination of NO_3^- and NO_2^- levels. Before and four times after surgery perfusion was assessed. All other analyses were done seven days after surgery.

1.1 Dietary and Environmental Habit

As described, the mice obtained 1 g/l NO_3^- in drinking water ad libitum (and MoW twice a day). Through regular weighing, it was ensured that the supplementation and treatment did not influence water and food consumption, and the weight of the mice. As shown in figure 15, water consumption remained invariable over the period of experiments and did not differ; the mice got neither NO_3^- -water and antibacterial mouthwash nor normal soda and placebo mouthwash. *Control* mice consumed 0.28 ± 0.04 ml water/g body weight, NO_3^- mice drunk 0.28 ± 0.04 ml water/g body weight, and *MoW* animals needed 0.29 ± 0.03 ml water/g body weight. An analysis of variance did not show a significant difference ($p=0.98$). At the same time, food consumption remained constant independently from the collective (*control*: 0.18 ± 0.02 g; NO_3^- : 0.2 ± 0.01 g; *MoW*: 0.19 ± 0.01 g; normalized to body weight) as an ANOVA confirmed ($p=0.56$). Finally, the weight of the mice was determined once, in the week before surgery determining the effect of supplementation and treatment and for a second time in the week after surgery to detect any effects of surgery. Neither in the first nor in the second case could a discrepancy be established. Thus, a variance analysis failed to detect a significant difference between the weights of the groups ($p=0.42$ when supplementation and treatment started; $p=0.46$ 7 d after it). The absolute values are listed in table 9. The same applied for the loss of weight seven days after surgery,

which amounted to 2.60 ± 0.46 g for the *control* group, 2.54 ± 0.58 g for the NO_3^- animals, and 3.48 ± 1.20 g for the *MoW* mice (ANOVA and Bonferroni post-test, ns, $p=0.56$).

Table 9. Comparison of weights dependent from collective.

Shown are weights before the start of supplementation and treatment (day -7) and seven days afterwards (day 0). In addition to the comparison of different groups at a particular time-point, the groups were tested among each other. Weights remained constant among the groups (paired t-test). Data are given as mean $\pm$ SEM ($n=5$).

Group	Weight day -7 (g)	Weight day 0 (g)	p-value
<i>control</i>	43.6 ± 0.92	43.7 ± 0.99	0.83
NO_3^-	41.2 ± 1.49	41.0 ± 1.36	0.31
<i>MoW</i>	41.7 ± 1.4	41.9 ± 1.95	0.78

The success of bacterial inhibition by *MoW* was analyzed by counting CFU (see figure 14). Only one hour after treatment the oral flora quantities decreased significantly compared to basal values (Wilcoxon-matched-paired test, *, $p=0.03$). Before *MoW* 273 ± 35 CFU of aerob and anaerob bacteria were counted and one hour after treatment the values decreased to 21 ± 5 CFU. Bacteria were also qualitatively determined and *Staphylococcus xylosus*, *Staphylococcus aureus*, *Diplococcus*, *Proteus* sp., *Streptococcus viridans*, and spore-forming bacteria were found in the oral cavity before treatment, but only the two staphylococci and the spore-forming bacteria were found after treatment.

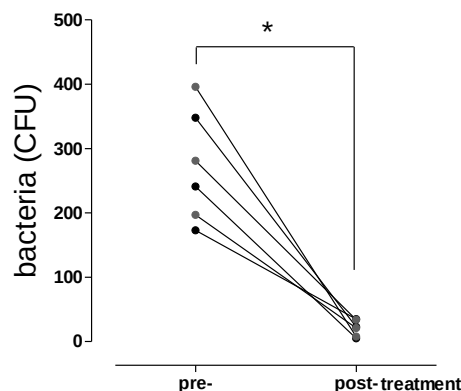


Figure 14. Quantification of oral flora.

Both aerob bacteria (black circles) and anaerob bacteria (grey circles) were cultured and CFU counted. The populations did not differ significantly and therefore shown in one graph. The decrease of oral bacteria one hour after treatment was significant (Wilcoxon-matched-paired test, *, $p=0.03$). Data are given as mean $\pm$ SEM ($n=6$).

Finally, kinetics of NO_3^- and NO_2^- processing was tested. To obtain as many samples as possible from one animal and to combine as many animals as possible on one day, sample withdrawal lasted no longer than two hours. Subsequent analysis (figure 16) showed that long intervals between last treatment and euthanasia did not influence following results. NO_3^- was given per gavage to equals uptake (12 mM, which refers to the average daily uptake); half of the mice were treated with *MoW*. Nevertheless, NO_3^- circumvented direct reduction in the oral cavity if given per gavage, it is reduced by absorption in the saliva. Six hours after the last treatment, no differences in NO_3^- levels could be measured. In the NO_3^- group levels started at 44.73 ± 4.05 μM , went to 146.10 ± 58.21 μM , and ended at 88.94 ± 18.12 μM (Friedman test and Dunn's post-test, ns, $p=0.19$). Even more stable were the levels after *MoW* with 29.33 ± 1.34 μM , 29.789 ± 3.24 μM , and 45.99 ± 4.54 μM (Friedman test and Dunn's post-test, ns, $p=0.19$). The NO_2^- measurements showed similar results. The level in

the NO_3^- group ranged from $0.57 \pm 0.13 \mu\text{M}$, to $0.3 \pm 0.04 \mu\text{M}$, and $0.33 \pm 0.15 \mu\text{M}$ (Friedman test and Dunn's post-test, ns, $p=0.19$). Although the NO_2^- level in MoW mice were statistically significant (Friedman test and Dunn's post-test, *, $p=0.03$) no substantial association was assumed, because of the high variance in the basal measurement (0 h: $0.84 \pm 0.35 \mu\text{M}$; 2 h: $0.13 \pm 0.02 \mu\text{M}$; 6 h: $0.25 \pm 0.04 \mu\text{M}$).

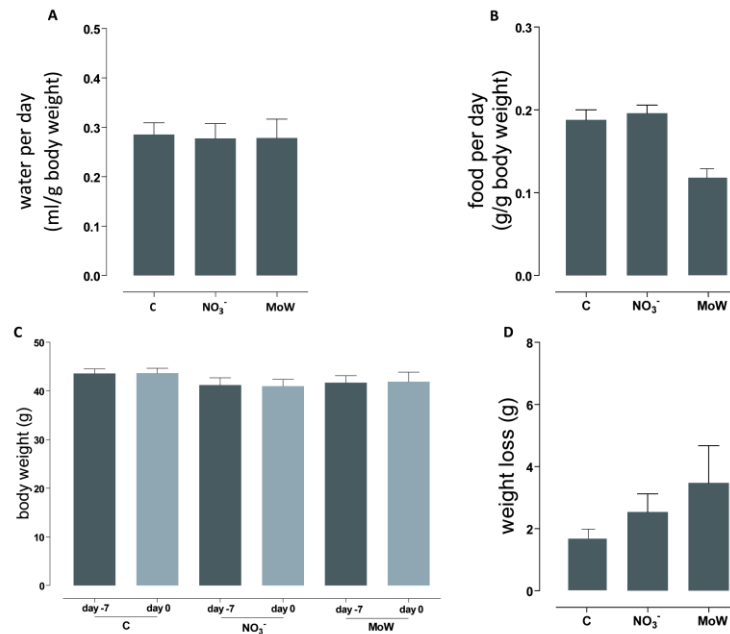


Figure 15. Exclusion of treatment effects on dietary habit.

A and **B** Water and food consumption were normalized to body weight. All three groups drank and ate the same amount of soda and pellets independently of supplementation and treatment. Shown are measurements determined in the first week of supplementation and treatment before surgery (ANOVA and Bonferroni post test, ns, $p=0.98$ and $p=0.56$). **C** and **D** All animals started the series at a similar weight, which remained equal after seven days of supplementation and treatment (ANOVA and Bonferroni post-test, ns, $p=0.42$ and $p=0.46$). Moreover, the loss of weight was constant among the groups (ANOVA and Bonferroni post-test, ns, $p=0.56$). Data are given as mean $\pm$ SEM ($n=5$).

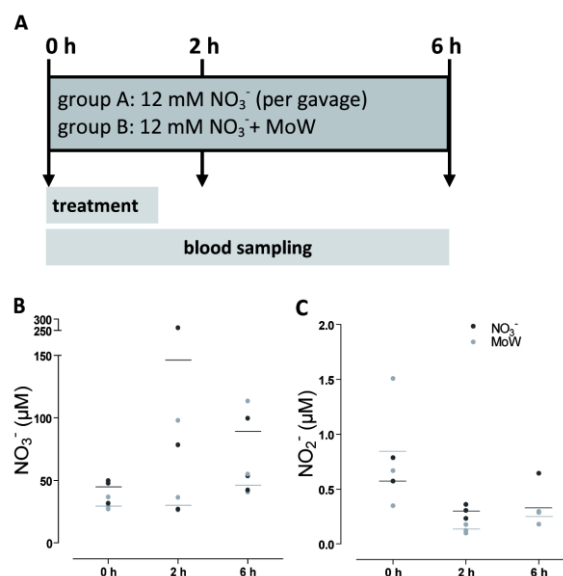


Figure 16. Influence of supplementation on plasma NO_2^- and NO_3^- level over the experimental course.

A Study set-up shows that after NO_3^- supplementation $\pm$ MoW blood samples were drawn directly, 2 h, and 6 h afterwards. **B** and **C** NO_3^- and NO_2^- levels stayed mainly constant over 6 h, simulating an experimental day (Friedman test and Dunn's post-test, ns, NO_3^- : $p=0.19$ and $p=0.19$; NO_2^- : $p=0.19$ and $p=0.03$). Data are given as mean $\pm$ SEM ($n=3$).

1.2 NO Metabolism

1.2.1 Non-ischemic Measurements

The NO_x levels in the analyzed groups were influenced by NO_3^- , MoW and ischemia. To determine the single effect of dietary NO_3^- and MoW on NO_x metabolism, diet was supplemented and mice were treated for seven days and plasma, urine and feces were collected at day 0 before surgery. Besides the known groups, a fourth group (“*basal*” without placebo MoW) was introduced to exclude the influence of spraying a fluid twice a day.

Figure 17 A presents the NO_3^- levels in plasma. Both, *basal* and *control* mice had equal low levels (*basal*: $25.8 \pm 3.5 \mu\text{M}$; *control*: $23.2 \pm 5 \mu\text{M}$). With NO_3^- supplementation the levels rose to the sevenfold ($190 \pm 20.4 \mu\text{M}$). However, after additional MoW treatment the levels decreased significantly ($59.4 \pm 32.3 \mu\text{M}$). Put together, all control groups showed similar NO_3^- levels without statistical differences. Conversely, the NO_3^- animals showed significantly higher NO_3^- levels (ANOVA and Bonferroni post-test, ***, $p < 0.0001$; ***, *basal* vs. NO_3^- ; ***, *control* vs. NO_3^- ; **, *MoW* vs. NO_3^-).

Additionally, NO_2^- levels in plasma were determined, shown in figure 17 B. The NO_2^- levels after NO_3^- supplementation rose significantly (ANOVA and Bonferroni post-test, *, $p = 0.02$; *, *control* vs. NO_3^- ; *, *MoW* vs. NO_3^-) compared to the levels in the *basal*, *control* and *MoW* group (NO_3^- : $1.6 \pm 0.35 \mu\text{M}$; *basal*: $0.90 \pm 0.13 \mu\text{M}$; *control*: $0.78 \pm 0.05 \mu\text{M}$; *MoW*: $0.73 \pm 0.13 \mu\text{M}$).

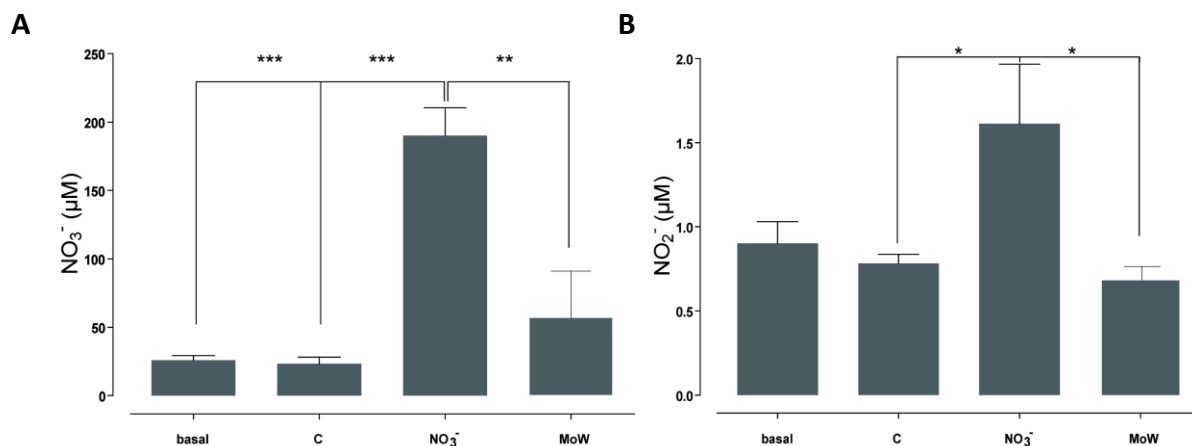


Figure 17. NO metabolites measured in plasma before surgery.

A After seven days of NO_3^- supplementation, but before induction of ischemia, differences from NO_3^- supplementation and MoW treatment are distinct. *Basal* and *control* levels are significantly lower than the increased levels in NO_3^- mice (ANOVA with Bonferroni post-test, ***, $p < 0.0001$; ***, *basal* vs. NO_3^- ; ***, *control* vs. NO_3^- ; **, *MoW* vs. NO_3^-). **B** At the same time NO_2^- levels were determined. Levels of NO_3^- animals increased twofold in comparison to *basal* and *control* levels. Consequently, after additional MoW treatment, NO_2^- decreased significantly. (ANOVA with Bonferroni post-test, ***, $p = 0.016$; ns, *basal* vs. NO_3^- ; *, *control* vs. NO_3^- ; *, *MoW* vs. NO_3^-). Data are given as mean $\pm$ SEM ($n = 4-5$).

At the same time, urinary NO_3^- and NO_2^- levels were detected. When tested, collective NO_3^- levels rested low ($1471 \pm 324 \mu\text{M}$). Conversely, the levels in the NO_3^- and *MoW* groups increased significantly (ANOVA and Bonferroni post-test, ***, $p < 0.0001$; ***, *control* vs. NO_3^- ; ***, *MoW* vs. NO_3^-) to $9184 \pm 125 \mu\text{M}$ (NO_3^-) and $9229 \pm 1622 \mu\text{M}$ (*MoW*). The analyzed NO_2^- levels tended to increase after NO_3^- supplementation and *MoW* treatment, but lacked significance (*control*: $1.13 \pm 0.27 \mu\text{M}$; NO_3^- : $2.21 \pm 0.49 \mu\text{M}$; *MoW*: $1.94 \pm 0.3 \mu\text{M}$; ANOVA

and Bonferroni post-test, ns, $p=0.14$). These results are shown in figure 18 elucidating that NO_3^- arrived circulation after MoW as shown in figure 18.

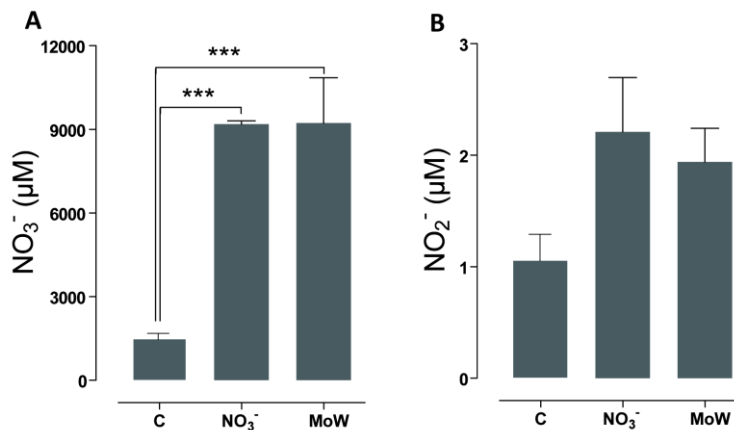


Figure 18. Urinary NO metabolites measured by diazo coupling and HPLC after supplementation.

A NO_3^- levels were, in total, very high after 7 d of supplementation. However, the levels in the NO_3^- and MoW groups were significantly elevated from *control* mice (ANOVA and Bonferroni post-test, ***, $p<0.0001$; ***, *control* vs. NO_3^- ; ***, MoW vs. NO_3^-). **B** NO_2^- levels in murine urine are low in comparison to urinary NO_3^- . The levels after 7 d of supplementation and treatment tended to increase but remained statistically indifferent to the *control* collective (ANOVA and Bonferroni post-test, ns, $p=0.14$). Data are given as mean $\pm$ SEM ($n=4-5$).

1.2.2 Ischemic Measurements

For completion of the comprehension of NO_x metabolism, NO_x levels in ischemia were determined. At the start, plasma NO_3^- (and NO_2^-) levels showed higher variance than before surgery (figure 19). In particular, data of NO_3^- and MoW mice showed a big scattering and because of that these data are shown as box plots.

The NO_3^- levels in the NO_3^- and MoW groups were significantly increased in contrast to *control* animals, but without significance among these groups (ANOVA and Bonferroni post-test, **, $p=0.006$; **, *control* vs. NO_3^- ; *, *control* vs. MoW). The levels of the *control* group lie at $28.2\pm3.4 \mu\text{M}$, the levels of the NO_3^- samples at $173.2\pm30.7 \mu\text{M}$ and these of the MoW collective at $127.8\pm32.3 \mu\text{M}$.

NO_2^- levels only showed a trend to increase after NO_3^- supplementation and MoW treatment (ANOVA with Bonferroni correction, ns, $p=0.27$). Levels were $0.3\pm0.03 \mu\text{M}$ (*control*), $0.38\pm0.03 \mu\text{M}$ (NO_3^-), $0.39\pm0.04 \mu\text{M}$ (MoW). These levels, determined at day 7, seemed lower than the levels determined at day 0 (figure 19). Statistical analysis confirmed a significant decrease of NO_2^- levels in ischemia, independent from observed group as shown in figure 19 C (t-test, ***, $p<0.0001$, *control* day 0 vs. *control* day 7; **, $p=0.001$, NO_3^- day 0 vs. NO_3^- day 7; **, $p=0.006$, MoW day 0 vs. MoW day 7). NO_2^- levels in *control* mice decrease from $0.78\pm0.05 \mu\text{M}$ to $0.3\pm0.3 \mu\text{M}$, the levels from NO_3^- animals from $1.6\pm0.35 \mu\text{M}$ to $0.38\pm0.03 \mu\text{M}$, and the levels from MoW mice from $0.73\pm0.13 \mu\text{M}$ to $0.39\pm0.04 \mu\text{M}$.

Furthermore, NO metabolites in skeletal muscle were quantified. Paraller, to NO_3^- and NO_2^- levels, the amount of NO-heme, and RXNOs were determined. All results from the ischemic and the non-ischemic hind limb muscle were shown in comparison in figure 20 A-D.

The stable NO_3^- levels of *control* mice were $278.3\pm87.8 \mu\text{M}$ (non-ischemic) and $274.6\pm100 \mu\text{M}$ (ischemic). The not significant but higher levels of NO_3^- animals were $364.9\pm148.7 \mu\text{M}$ (non-ischemic) and $465.3\pm105.1 \mu\text{M}$ (ischemic).

(ischemic). Finally, the levels of *MoW* mice were $45.6 \pm 6.5 \mu\text{M}$ (non-ischemic) and $54.6 \pm 7.8 \mu\text{M}$ (ischemic). The ischemic level of the NO_3^- and the *MoW* group differed significantly (Kruskal-Wallis test with Dunn's post test, *, $p=0.01$; *, NO_3^- isc vs. *MoW* isc).

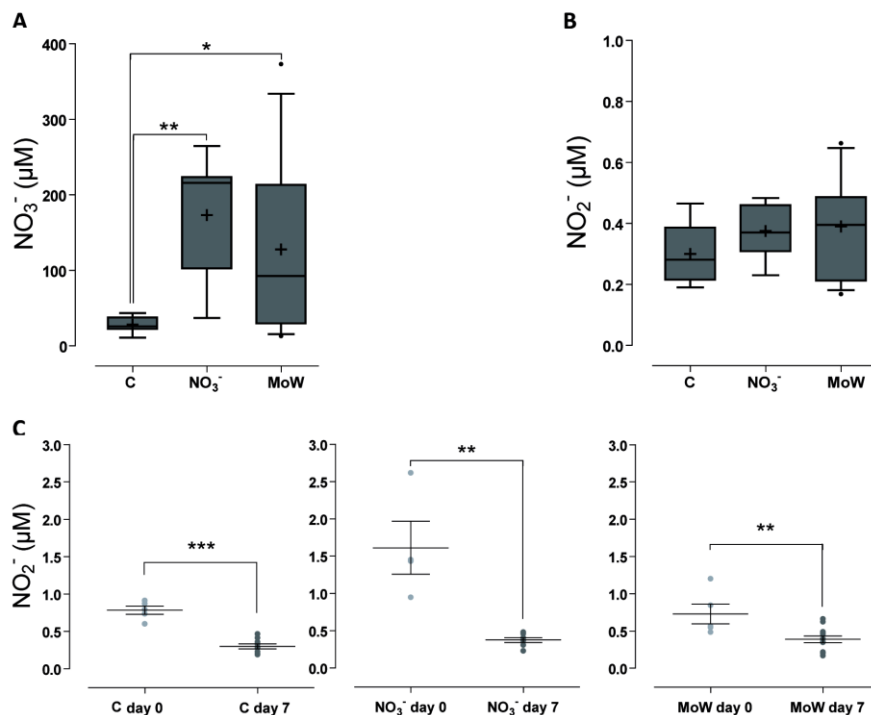


Figure 19. Plasma NO metabolites after 14d of supplementation and under ischemic conditions.

A NO_3^- levels after surgery were increased as well in NO_3^- as in *MoW* mice (ANOVA and Bonferroni post-test, **, $p=0.006$; **, *control* vs. NO_3^- ; *, *control* vs. *MoW*). **B** In general, NO_2^- levels after surgery were lower in relation to plasma levels before surgery without a difference among the groups (ANOVA and Bonferroni post-test, ns, $p=0.268$). **C** A comparison of the data sets before and after surgery elucidated a significant difference amongst the groups between day 0 and day 7 (t-test, ***, $p<0.0001$, *control* day 0 vs. *control* day 7; **, $p=0.001$, NO_3^- day 0 vs. NO_3^- day 7; **, $p=0.006$, *MoW* day 0 vs. *MoW* day 7). Data are given as mean $\pm$ SEM ($n=9-12$; day 7).

The picture of the NO_2^- levels was slightly different (figure 20 B). In all three groups a trend of increased levels in the ischemic legs in comparison to the non-ischemic legs could be detected, obtaining significance in the NO_3^- collective (Mann-Whitney test, *, $p=0.03$). In contrast to the NO_3^- levels, no significance could be detected between the groups (ischemic) (Kruskal-Wallis test with Dunn's post test, ns, 0.08). Levels were $1.6 \pm 0.3 \mu\text{M}$ (*control*), $2.7 \pm 0.1 \mu\text{M}$ (NO_3^-), and $3.8 \pm 0.7 \mu\text{M}$ (*MoW*) for the ischemic tissue and $1.1 \pm 0.09 \mu\text{M}$ (*control*), $1.2 \pm 0.07 \mu\text{M}$ (NO_3^-), and $3.4 \pm 0.5 \mu\text{M}$ (*MoW*) for the non-ischemic legs.

In figure 20 C, NO-heme levels are shown. Similarly to NO_2^- levels, the ischemic samples had higher levels of NO-heme than the non-ischemic (Mann-Whitney test, **, $p=0.008$, *control*; *, $p=0.03$, NO_3^- ; ns, $p=0.2$, *MoW*). In contrast, no significant difference could be measured between the three collectives, in spite of an obvious trend to increased levels after NO_3^- supplementation (Kruskal-Wallis test with Dunn's post-test, ns, $p=0.8$). Detected levels were $14.9 \pm 2.6 \text{ nM}$ (*control*), $25.2 \pm 15.5 \text{ nM}$ (NO_3^-), and $18.8 \pm 4.7 \text{ nM}$ (*MoW*) in the ischemic legs and $0.4 \pm 0.2 \text{ nM}$ (*control*), $0.7 \pm 0.1 \text{ nM}$ (NO_3^-), and $11.4 \pm 4 \text{ nM}$ (*MoW*) in the non-ischemic muscle samples.

RNNO and RSNO levels were shown in stacked bars (figure 20 D). These two halves result in the entirety of protein-bound NO (RXNO). Only *control* and NO_3^- animals (ischemic legs) were tested. The first results missed a clear shift by supplementation to an alteration between the RNNO-RSNO proportions, which corroborated the hypothesis. Therefore, these experiments were closed. Consequently, and because of high scattering, a statistical evaluation was resigned, but the results were shown graphically to complete this complex of analyses.

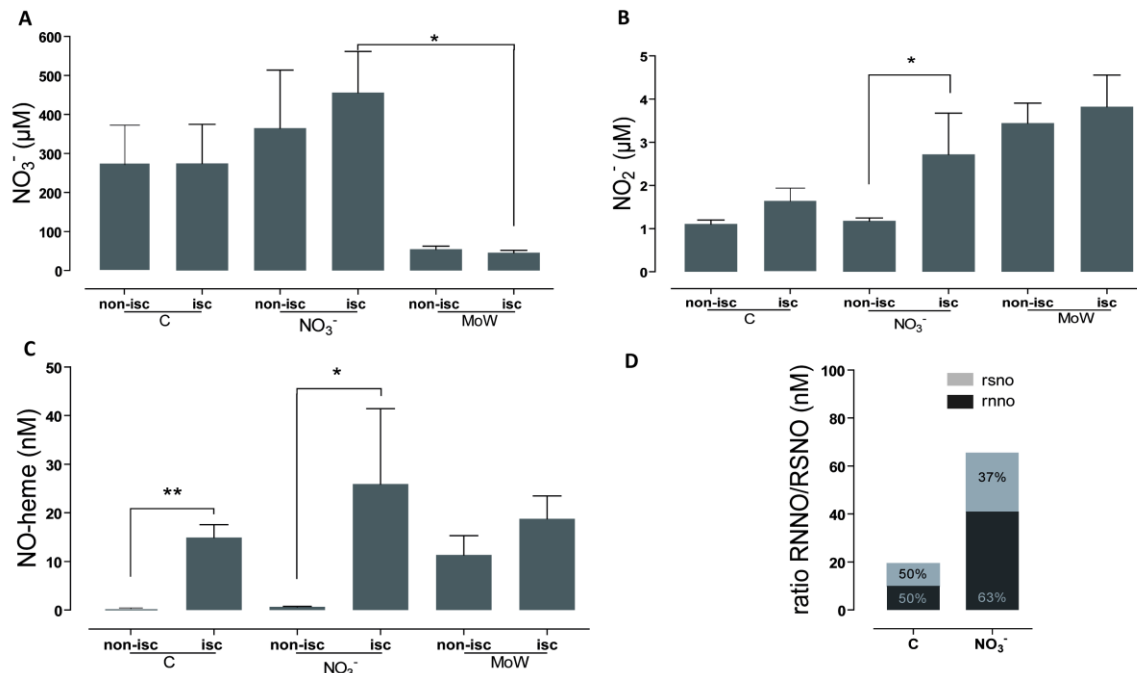


Figure 20. Juxtaposition of NO metabolites in muscle tissue of non-ischemic and ischemic legs.

A In conclusion, after NO_3^- supplementation the NO_3^- levels tended to increase, but were significantly decreased after additional MoW treatment (Kruskal-Wallis test with Dunn's post test, *, $p=0.01$; *, NO_3^- isc vs. MoW isc). **B** The ischemic NO_2^- levels were inclined to increase (in relation to non-ischemic levels), and attained significance after NO_3^- supplementation (Mann-Whitney test, $p=0.03$, NO_3^- isc vs. NO_3^- non-isc). There is a lack of significance among the groups (Kruskal-Wallis test and Dunn's post-test, ns, $p=0.08$). **C** In the ischemic legs NO-heme levels remained constant independent from analyzed collective (Kruskal-Wallis test with Dunn's post-test, ns, $p=0.8$). Instead of this, the levels in the non-ischemic legs were significantly lower (Mann-Whitney test, **, $p=0.008$, *control*; *, $p=0.03$, NO_3^- ; ns, $p=0.2$, MoW). **D** In the *control* group RNNOs and RSNOs were parted in two halves amounting 50 %. After NO_3^- supplementation this proportion lacked an obvious variation, but overall the levels appeared higher after NO_3^- supplementation. Data are given as mean $\pm$ SEM ($n=3-5$).

1.2.3 cGMP Measurement

NO acts by binding to the Guanylyl cyclase, which subsequently produces cGMP²⁸⁰. Thus, cGMP is regarded as a marker for NO level, whose direct measurement is difficult. Based on this background, cGMP levels in the ischemic tissue were analyzed. In contrast to the hypothesis, the samples lacked a difference between *control*, NO_3^- and MoW mice (ANOVA and Bonferroni post-test, ns, $p=0.26$). Moreover, the levels after NO_3^- supplementation tended to decrease (4.2 ± 2 fmol/mg tissue) in contrast to the control groups (8 ± 1.5 fmol/mg tissue, *control*; 9.3 ± 2.9 fmol/mg tissue, MoW), as shown in figure 21.

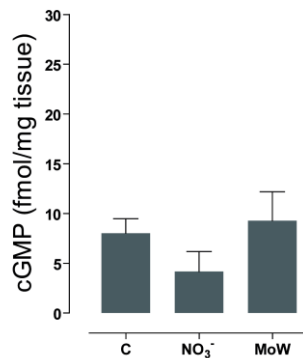


Figure 21. cGMP levels determined in ischemic legs.

Despite a drift to lower levels after NO₃⁻ supplementation no significance was obtained (ANOVA and Bonferroni post-test, ns, $p=0.23$). Data are given as mean $\pm$ SEM ($n=6$).

1.3 Outcome – Tissue Injury and Regeneration

1.3.1 Perfusion, Necrosis and Characterization of Satellite Cells

In pAOD, the undersupply caused by ischemia in the extremities could lead to severe tissue damage. Therefore, a major goal of the study was to recover perfusion in ischemic legs to ensure nourishment and prevent necrosis. LDPI measurements, supported by macroscopic evaluation of necrosis, were used to follow the regeneration process.

Perfusion was tracked over one week and the outcome defined as perfusion after seven days, is presented in figure 22. Unfortunate restoration in *control* mice ($59 \pm 4\%$) was significantly augmented by NO₃⁻ supplementation ($73 \pm 4\%$). Without bacterial NO₃⁻ reduction (*MoW*) the effect disappeared ($55 \pm 3\%$). Indeed, both changes were significant (ANOVA with Bonferroni correction, **, $p=0.003$; *, *control* vs. NO₃⁻; **, *MoW* vs. NO₃⁻). As described, parallel to perfusion, necrosis was determined and outlined in figure 23. To compare the degree of necrosis, a score was developed and every mouse evaluated at the day of the final measurements. No significant change in the rate of necrosis was detected (Chi-square test, ns, $p=0.13$). Nevertheless, the difference between the collectives was impressive as figure 23 clarifies.

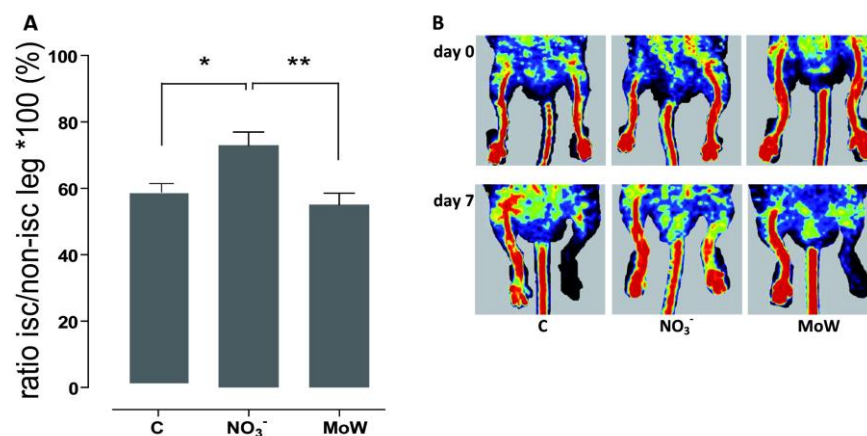


Figure 22. Regeneration of perfusion after severe ischemia by dietary NO₃⁻.

A The histogram illustrates the final perfusion measurement. Without support, perfusion only regenerated to about 60 %. This poor outcome is significantly improved by dietary NO₃⁻. After antiseptic MoW the protective effect is abolished (ANOVA and Bonferroni post-test, **, $p=0.003$; *, *control* vs. NO₃⁻; **, *MoW* vs. NO₃⁻). Data are given as mean $\pm$ SEM ($n=21-23$) **B** The collage summarizes representative pictures of the measurement of perfusion. In the upper row mice are shown before surgery, when both legs were equally perfused. Seven days after ischemia-induction (lower row) in the *control* and the *MoW* mouse perfusion remained low. In contrast, the perfusion in the NO₃⁻ mouse was considerably higher. Perfusion is calculated in arbitrary units, and shown in pseudo colors (black = low; red = high).

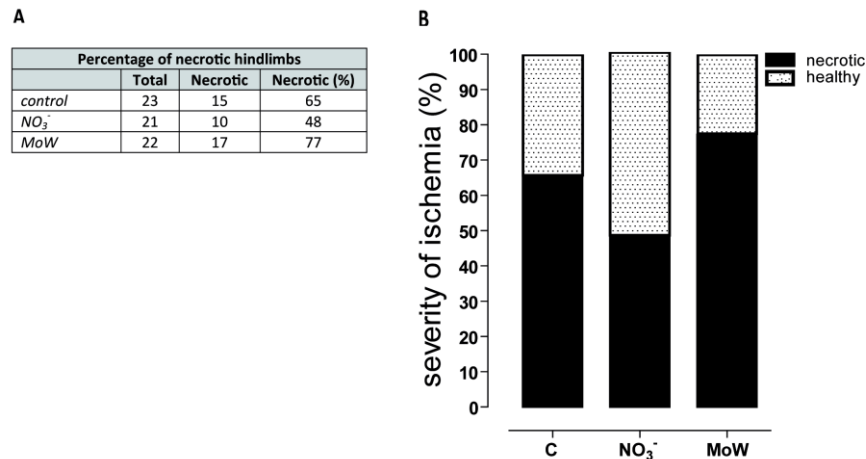


Figure 23. Necrosis determined seven days after hind limb surgery.

A Tabular proportion of healthy to necrotic legs between the groups. Whereas more than 50 % of ischemic legs in the control groups were necrotic; in the NO₃⁻ group less than 50 % of legs evolved necrosis. **B** Graphic illustration of tabular values (Chi-square test, ns, $p=0.13$; $n=21-23$). Necrosis score: 0= no visible necrosis; 1= discoloration of the toes/feet; 2= necrosis of the toes; 4= necrosis of the foot; 5= necrosis of the calf; 6= necrosis of the thigh

Dietary NO₃⁻ supplementation improved perfusion and reduced necrosis. Besides, the question of whether viability of muscle progenitor cells (satellite cells) is also positively influenced by NO₃⁻ supplementation should be answered. To answer this issue, the isolation of satellite cells was established and proved by FACS measurement after $\alpha 7$ -Integrin staining (shown in figure 24). The results demonstrated a high purity of isolated satellite cells, comparable to the result in a myoblast cell line (C2C12) (control: $2.8 \pm 0.1\%$; stained: $94.4 \pm 0.9\%$; t-test, ***, $p < 0.0001$). Subsequently, the examination of the satellite cells for apoptosis and viability was done and presented by cand. med. Andreas Schicho whose doctoral thesis was designed and done in close collaboration with the presented work.

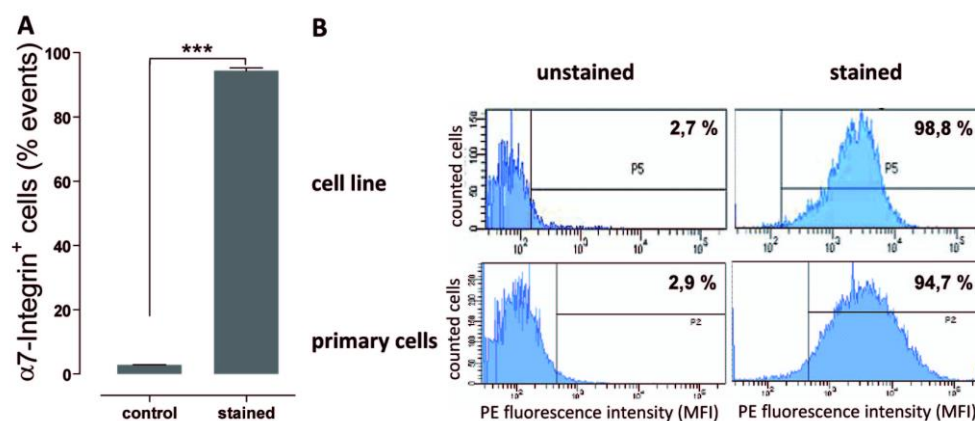


Figure 24. Characterization of satellite cells.

A About 95 % of stained cells were $\alpha 7$ -Integrin positive. Thus, it could be concluded, that isolation and enrichment was successful. **B** Representative FACS histograms with C2C12 cells as positive control in comparison with histograms of primary isolated satellite cells visualize purity of the isolation (t-test, ***, $p < 0.0001$). Data are given as mean $\pm$ SEM ($n=3$). (PE= dye; MFI=mean fluorescence intensity, arbitrary units).

1.4 Vascularization

1.4.1 Isolation, Culture, and Characterization of Endothelial-Regenerating Cells

Endothelial-regenerating cells are pro-angiogenic progenitor cells. They are mobilized from bone-marrow and are involved in angio- and neo-angiogenesis¹³⁸. Besides, recent work could show that NO acts as chemoattractor for Endothelial-regenerating cells⁸⁰. The following results should demonstrate that Endothelial-regenerating cells are also concerned in the regenerative effects mediated by dietary NO₃⁻.

Isolation of Endothelial-regenerating cells from murine spleen was established to investigate vitality of these cells after NO₃⁻ supplementation. As described in chapter 4.1 (Methods and Materials) the cells were cultured for seven days under endothelial conditions. After the seven days, the cells were characterized with a DilacLDL/UEA staining to define their endothelial phenotype. From small murine spleens only a small amount of cells could be extracted. Nevertheless, figure 25 shows a representative picture of a vital (regularly tested by Trypan blue staining during cell counting), typical spindle-shaped culture.

As described, quality of the culture was ensured by a DilacLDL/UEA lectin staining. Figure 26 exposes the single stainings and the merged picture. All stained cells, detectable by the blue nuclei staining, were also positive for the red DilacLDL and the green UEA lectin, which become visible in the forth merged picture.

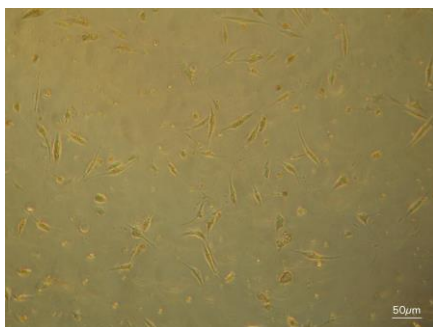


Figure 25. Representative photography of a splenic Endothelial-regenerating cell culture.

The progenitor cells were isolated via Ficoll-density centrifugation and subsequently concentrated by culturing under endothelial conditions. The characteristic spindle shape of the cells is apparent. Magnification, x 100.

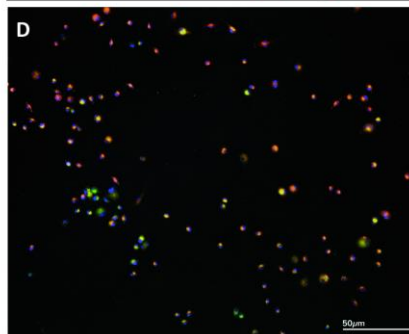
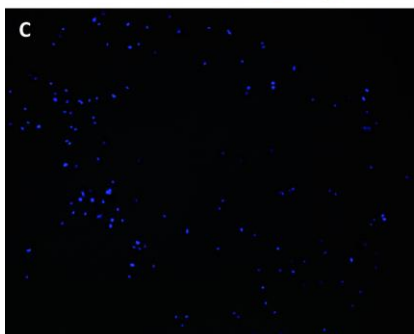
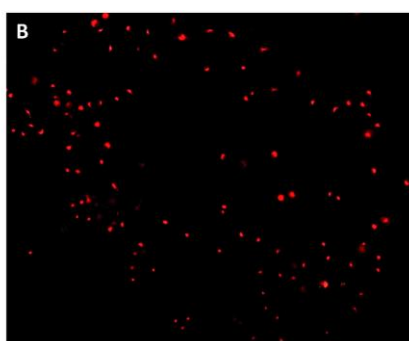
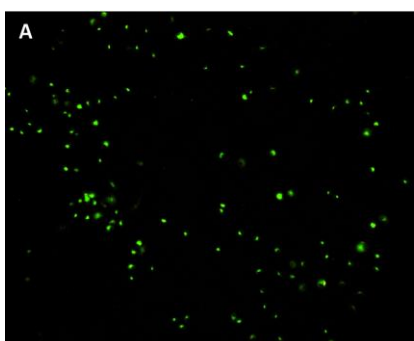


Figure 26. Representative images of DilacLDL/UEA lectin stained Endothelial-regenerating cells.

A The first picture shows the FITC conjugated UEA lectin, fluorescing green. **B** The red DilacLDL staining perfectly mirrored the picture of **A**. **C** The same counts for the third photography, which shows the Hoechst nuclei staining to define real cells. **D** Finally, the last picture presents the overlay of previous images. Magnification, x 100.

1.4.2 Endothelial-Regenerating Cell Mobilisation

In addition to the splenic Endothelial-regenerating cells the progenitors were stained in whole blood and quantified by FACS to determine the impact on mobilization by dietary NO_3^- .

Figure 27 B illustrates the procedure. According to their size and granularity the Endothelial-regenerating cells were assumed within the mononuclear cell (MNC) population, including lymphocytes and monocytes. In the next step, $\text{APC}^+\text{FITC}^+$ ($\text{Flk-1}^+/\text{CD34}^+$) double positive cells were defined as Endothelial-regenerating cells. *Control* mice had 0.1 ± 0.03 % MNC Endothelial-regenerating cells and the same pertained for the blood of *MoW* animals (0.1 ± 0.03 %MNC). In contrast, the levels are significantly increased after NO_3^- supplementation to 0.4 ± 0.09 %MNC (Kruskal-Wallis test and Dunn's post test, ***, $p = 0.0002$; **, *control* vs. NO_3^- ; **, *MoW* vs. NO_3^-). The results were summarized in figure 29 A.

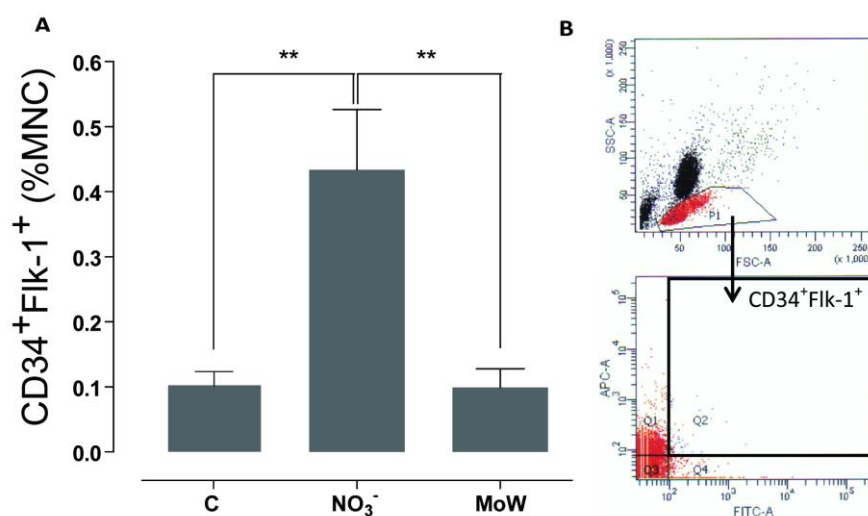


Figure 27. FACS analysis of Endothelial-regenerating cells in whole blood.

A Quantitative analysis reveals a significant increase of circulating Endothelial-regenerating cells ($\text{CD34}^+\text{Flk-1}^+$) within the MNC population after NO_3^- supplementation. This increase is abolished by *MoW* (Kruskal-Wallis test and Dunn's post test, ***, $p=0.0002$; **, *control* vs. NO_3^- ; **, *MoW* vs. NO_3^-). **B** Representative FACS charts point up the stained populations. Data are given as mean $\pm$ SEM ($n = 13 - 16$). (FITC = dye; PE = dye; APC = dye; SSC = side scatter; FSC = forward scatter)

1.4.3 Endothelial-Regenerating Cell Migration

Bone-marrow transplantation control

After migration into tissue, Endothelial-regenerating cells begin to differentiate into endothelial cells⁴²⁷. Accordingly, differentiated Endothelial-regenerating cells without stem cell character can not be retraced. Therefore, bone-marrow transplantation with labeled stem cells was conducted. The scheme in figure 28 illustrates the experimental set-up. After irradiation and transplantation the mice got four weeks to convalesce. After this time, supplementation and treatment were the same as in the previously described series. On day seven the hind limbs were explanted and prepared for immunofluorescent staining.

Success of transplantation was proved qualitatively with blood smears. Figure 29 demonstrates that despite the autofluorescence of erythrocytes the green glaring of GFP^+ cells is distinctive. In Row I and II photographs of a chimera and a transgenic eGFP-mouse are displayed. Both mice had similar amounts of GFP^+ blood cells.

However, only a part of the visible cells shine green, because erythrocytes do not express the transgene²⁵⁸. Instead, the blood smear of the control mice (row III) without GFP⁺ cells lacked positive cells.

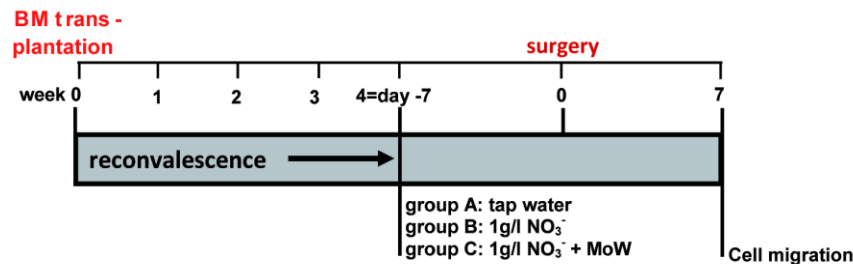


Figure 28. Scheme of experimental set-up including BM-transplantation.

Four weeks before starting the NO₃⁻ supplementation, the mice were lethally irradiated and GFP⁺ BM was transplanted 24 h later. Mice need four weeks to convalesce and become sufficient healthy to sustain further supplementation, treatment, and surgery.

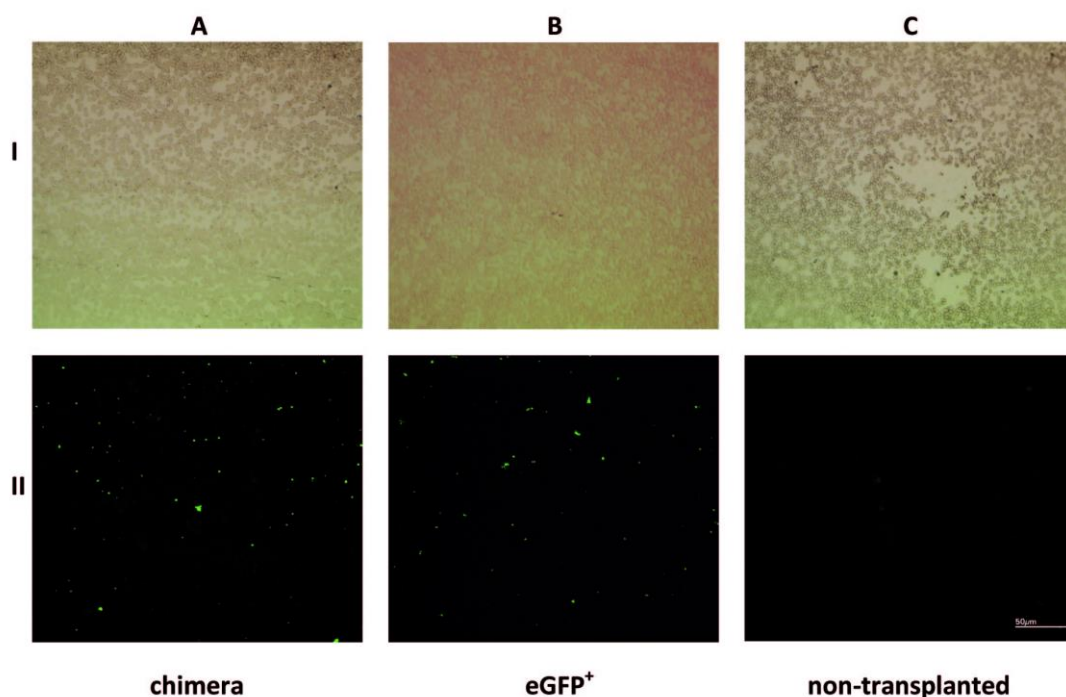


Figure 29. Quality control of BM transplantation by blood smears.

I The upper row includes the bright-field photographs of the blood smears. **II** The lower row contains the green fluorescent pictures, determining GFP-fluorescence. **A** The column demonstrates that in the chimera, six weeks after BM transplantation, GFP⁺ cells outshined the autofluorescent erythrocytes. **B** The mid-column elucidates that the amount of GFP⁺ cells in the transplanted animals is similar to the transgene eGFP⁺ animals. **C** In the right column the blood smear of a non-transplanted control mouse is displayed without distinct GFP fluorescence. Magnification, x 100.

Endothelial-Regenerating Cell Migration

Subsequently, migration was determined. Increasing amounts of Endothelial-regenerating cells were not only expected in the circulation, but also in the ischemic tissue. These differentiated Endothelial-regenerating cells could be detected as GFP⁺ after bone-marrow transplantation. In addition to this “bone-marrow marker” the cells were defined as CD45⁻CD31⁺ to exclude lymphocytes and define the endothelial character. Figure 30 and 31 summarize the results. Figure 30 demonstrates that after NO₃⁻ supplementation more Endothelial-regenerating cells migrated into the ischemic tissue in contrast to *control* mice and that this increase is

abolished by additional MoW. Moreover, in figure 33 the statistical analysis of this staining quantifies the significant rise of Endothelial-regenerating cells migration (ANOVA and Bonferroni post-test, **, $p = 0.004$; **, NO_3^- vs. *control*; **, NO_3^- vs. *MoW*). Indeed, on average 10.5 ± 2 $\text{CD31}^+\text{GFP}^+\text{CD45}^-$ cells/ mm^2 were mobilized by dietary NO_3^- . However, in the *control* mice only 3.5 ± 0.9 $\text{CD31}^+\text{GFP}^+\text{CD45}^-$ cells/ mm^2 were identified and in the *MoW* animals only 3.3 ± 1.1 $\text{CD31}^+\text{GFP}^+\text{CD45}^-$ cells/ mm^2 .

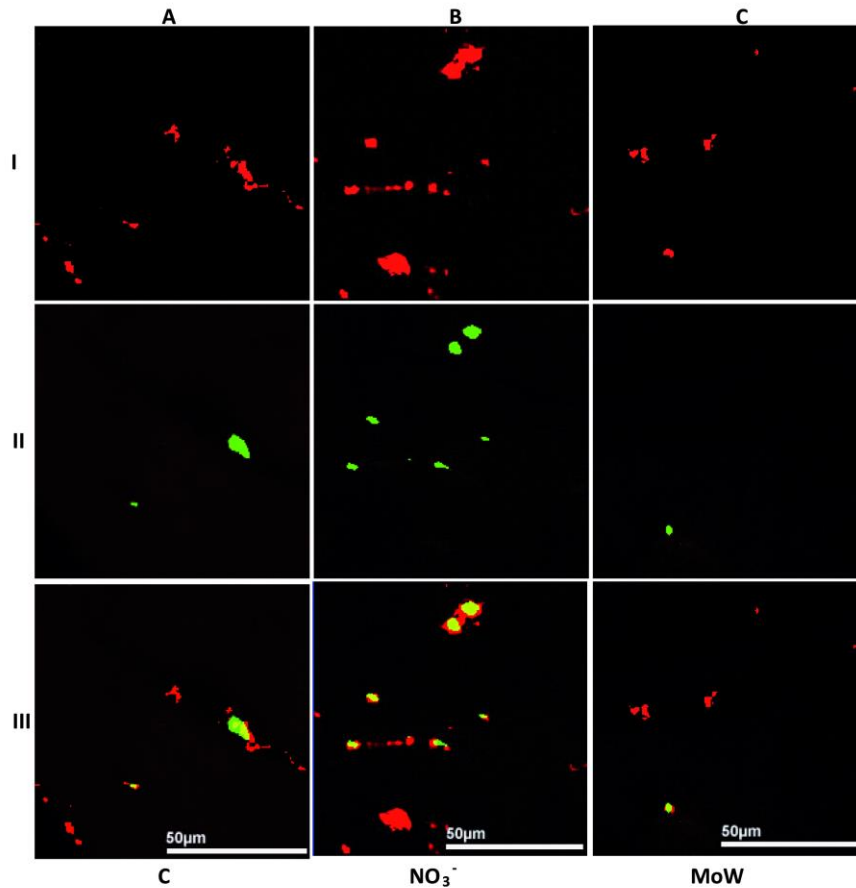


Figure 30. Migration of Endothelial-regenerating cells in the skeletal muscle.

I The upper row unifies the red CD31 staining, II The second row presents the amount of green GFP⁺ cells in the hind limbs. III The last row encloses the merged pictures. To exclude false positive staining and lymphocytes, a CD45 and a Hoechst staining were done but not shown to improve clarity. The representative photographs visualize the difference between the groups. Into the legs of NO_3^- mice were much more Endothelial-regenerating cells migrated than into the *control* limbs. Columns represent the different groups (A = *control*; B = NO_3^- ; C = *MoW*). Magnification, x 200.

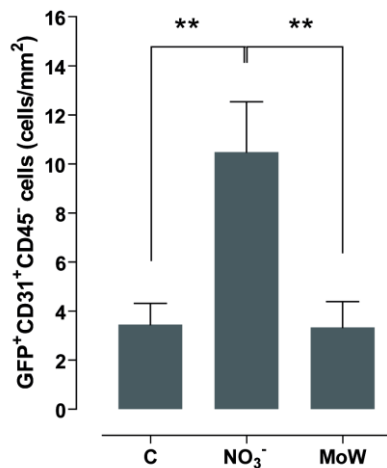


Figure 31. Migration of Endothelial-regenerating cells in murine hind limbs.

Endothelial-regenerating cells were detected as $\text{CD31}^+\text{GFP}^+\text{CD45}^-$. Obviously, after dietary NO_3^- supplementation Endothelial-regenerating cell migration into ischemic tissue was significantly augmented in contrast to that in *MoW* and *control* limbs (ANOVA and Bonferroni post-test, **, $p = 0.004$; **, NO_3^- vs. *control*; **, NO_3^- vs. *MoW*). Data are given as mean $\pm$ SEM ($n = 6$).

1.4.4 Angiogenesis

It is proposed, that migrated Endothelial-regenerating cells participate in angiogenesis. Besides arteriogenesis (shown in 2.4.5), the amplification of capillaries by angiogenesis and neo-vascularization are important mechanisms promoting regeneration. Thus, angiogenesis was also analyzed. However, instead of in the thigh regions, these reactions concentrate on the calf. Consequently, the examination of capillary density was done by immunofluorescence in the lower leg. To detect capillaries the endothelial marker CD31 was used as illustrated in figure 32. Representative series of photographs of every group were shown, picturing the difference in capillary density. Subsequently, the statistical analysis is given in figure 33. Under ischemic conditions without supplementation, the capillary density counted for 59.2 ± 14.7 CD31⁺CD45⁻ cells/mm², and in *MoW* mice 56.8 ± 6.8 CD31⁺CD45⁻ cells/mm² were evaluated. In contrast, in NO_3^- animals the number of capillaries rose significantly to 123.4 ± 19.7 CD31⁺CD45⁻ cells/mm² (ANOVA and Bonferroni post-test, **, $p = 0.009$; *, NO_3^- vs. *control*; *, NO_3^- vs. *MoW*).

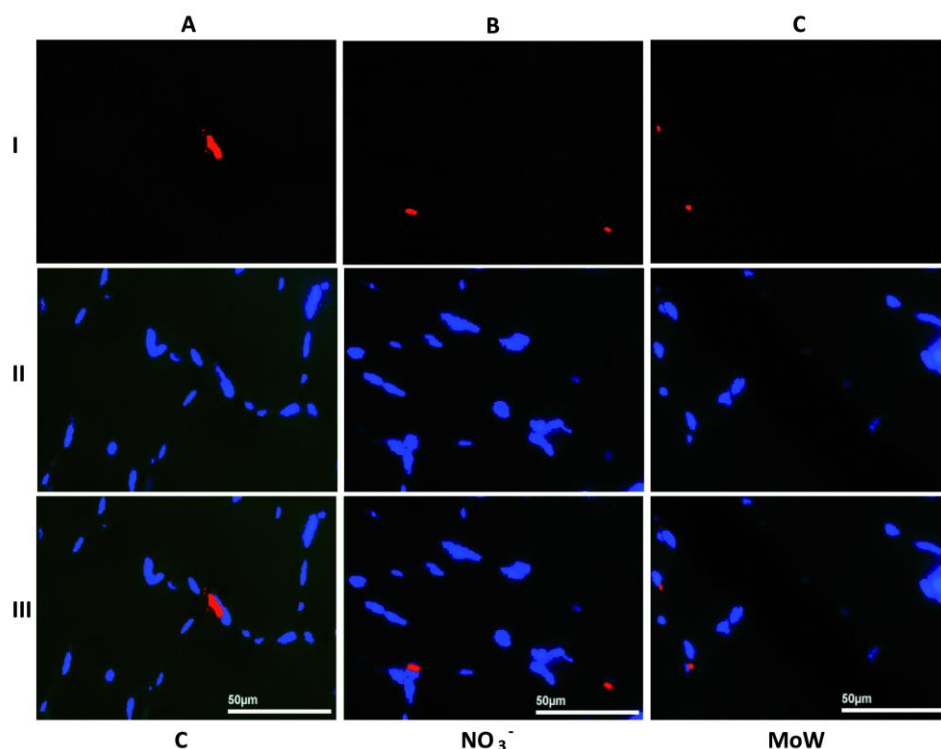


Figure 32. Capillary density in ischemic hind limbs.

A, B, and C Representative images of immunohistochemical CD31 stainings. CD31 is shown red associated to the blue nuclei (arrows). As the pictures are representative for the analyzed groups the difference becomes visible. In the NO_3^- group (**B**) are much more capillaries stained than in the control groups (**A** and **C**). Columns represent the different groups (**A** = *control*; **B** = NO_3^- ; **C** = *MoW*). The rows contain pictures of one staining (**I** = Cy5⁺ (CD31); **II** = UV⁺ (DAP/nuclei); **III** = merged). Magnification x 200.

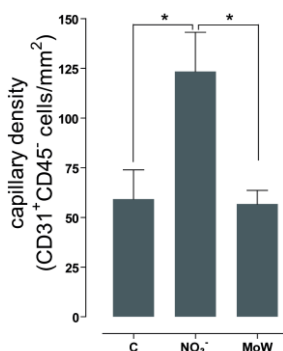
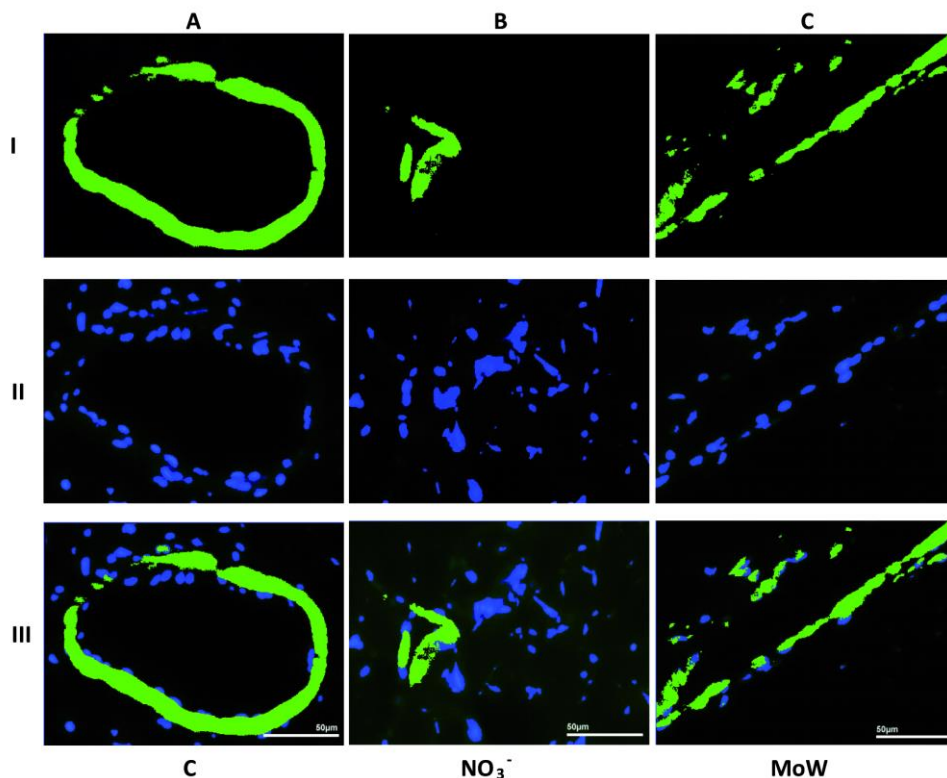


Figure 33. Capillary density in regenerating calfs.

Paraffin slides of the lower leg were stained with immunofluorescent and CD31⁺CD45⁻ vessels were counted. After NO_3^- supplementation, quantity of endothelial cells was significantly amplified (ANOVA and Bonferroni post-test, **, $p = 0.009$; *, NO_3^- vs. *control*; *, NO_3^- vs. *MoW*). Data are given as mean ± SEM ($n = 6$).

1.4.5 Arteriogenesis

After severe ischemia in the extremities, regeneration of the tissue is obtained by restoration of nourishment. Therefore, perfusion has to be recovered, which is achieved by vasculogenesis. Vascularization is comprised of three different mechanisms, which include the growth of arterioles (arteriogenesis), the sprouting of existing capillaries (angiogenesis), and the de-novo development of capillaries (neo-vasculogenesis)^{143, 393, 293}. To investigate whether one or more of these mechanisms are supported by dietary NO_3^- the vascular bed of hind



limbs was analyzed by immunofluorescence.

Figure 34. Representative immunofluorescence photographs of arterioles in the hind limb.

A, **B**, and **C** demonstrate a comparison of three examined collectives. In all groups arterioles could be identified, but no quantitative differences are visible. Arterioles were counted independent from size. Columns represent the three different groups (**A** = *control*; **B** = NO_3^- ; **C** = *MoW*). The rows contain pictures of one staining (**I** = FITC⁺ (SMA- α); **II** = UV⁺ (DAP/nuclei); **III** = merged). Magnification, x 200.

To visualize the Arteriogenesis in addition to the shown angiogenesis (2.4.4), smooth muscle cells were stained with SMA- α in the thighs of the mice. Three examples, including the collective *control*, NO_3^- and *MoW* illustrate the staining and the indifference between the groups (figure 34). Whereas figure 34 visualizes the arterioles qualitative, figure 35 provides quantitative information. After NO_3^- supplementation arteriole density tended to increase compared to controls (*control*: 3.1 ± 0.7 SMA- α^+ vessels/ mm^2 ; NO_3^- : 5.9 ± 1.6 SMA- α^+ vessels/ mm^2 ; *MoW*: 3.8 ± 1.2 SMA- α^+ vessels/ mm^2) but without significance (ANOVA and Bonferroni post-test, ns, $p = 0.25$).

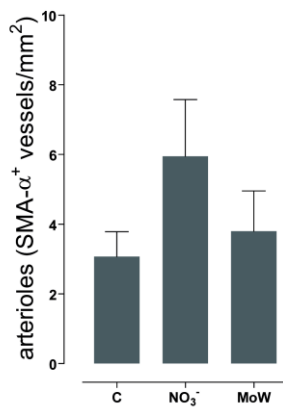


Figure 35. Arteriole density in ischemic hind limbs.

Paraffin slides of the thigh were stained with immunofluorescent and SMA- α^+ vessels counted. No significant change in arteriole density after dietary NO₃⁻ could be detected (ANOVA and Bonferroni post-test, ns, $p = 0.25$). Data are given as mean $\pm$ SEM ($n = 7$).

1.5 Inflammation

1.5.1 inducible NOS

Inflammatory processes are also responsible for a number of stem cell mobilizing pathways³⁴⁵. Furthermore, one sign of inflammation could be the up-regulation of iNOS with subsequent NO-dependent effects^{265, 428}. Due to these mechanisms, immunofluorescent analyses were done to exclude an impact of inflammation on the seen effects, and to ensure that the mobilized stem cells were real Endothelial-regenerating cells and not inflammatory cells (CD45⁺), see also chapter 2.5.3.

A first series of calf slides were stained for iNOS (see figure 36). The randomly chosen photographs illustrate the indifferent low distribution of iNOS in all three groups. Quantification of iNOS expression confirmed the impression of the pictures. There is no difference between *control*, NO₃⁻, and *MoW* mice concerning iNOS expression (shown in figure 37). Levels of iNOS⁺ cells range from 30.8 $\pm$ 6.3 cells/mm² (*control*), to 30.3 $\pm$ 10.1 cells/mm² (NO₃⁻), and 28.9 $\pm$ 7.4 cells/mm² (*MoW*) (ANOVA and Bonferroni post-test, ns, $p = 0.99$).

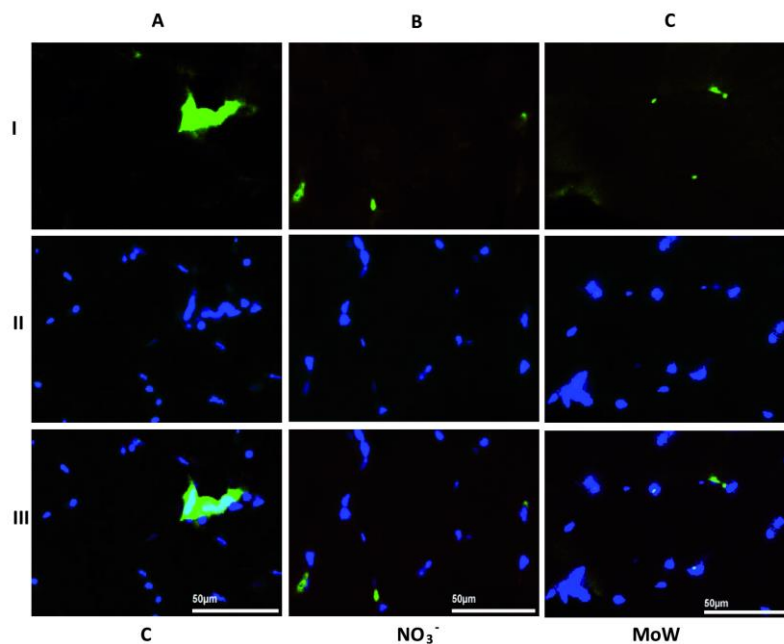


Figure 36. iNOS expression in ischemic muscle tissue.

Representative photographs demonstrate that neither NO₃⁻ supplementation nor MoW treatment changed the iNOS expression in the ischemic hind limbs. **I** The first row shows the green iNOS staining. **II** The second row

presents blue DAPI⁺ nuclei. **III** Finally, the third row unifies the merged pictures. The columns represent the different groups (**A** = *control*; **B** = *NO₃⁻*; **C** = *MoW*). Magnification, x 200.

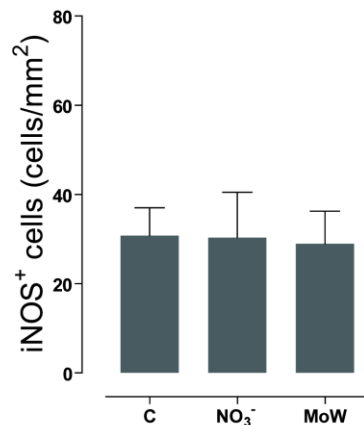


Figure 37. iNOS expression after hind limb surgery.

Leukocyte count in the regenerating muscle measured by iNOS expression is rare seven days after surgery and independent of dietary NO₃⁻ and MoW (ANOVA and Bonferroni post-test, ns, p=0.99). Data are given as mean ± SEM (n=6-10).

1.5.2 Lymphocytes (CD45)

Endothelial-regenerating cells were defined as CD45⁺, therefore CD45⁺ leukocytes could be quantified separately, too. The representative photographs in figure 38 demonstrate the equal low numbers of CD45⁺ leukocytes detected in the examined collectives. An ANOVA with Bonferroni post-test corroborates the impression, that inflammation has no impact (ANOVA and Bonferroni post-test, ns, p=0.2) presented in figure 39 associated with absolute values. In *control* mice 3.7±1.1 cells/mm² were counted, in *NO₃⁻* animals 5.8±1.3 cells/mm² were detected, and in *MoW* mice were 3.3±1 cells/mm² stained.

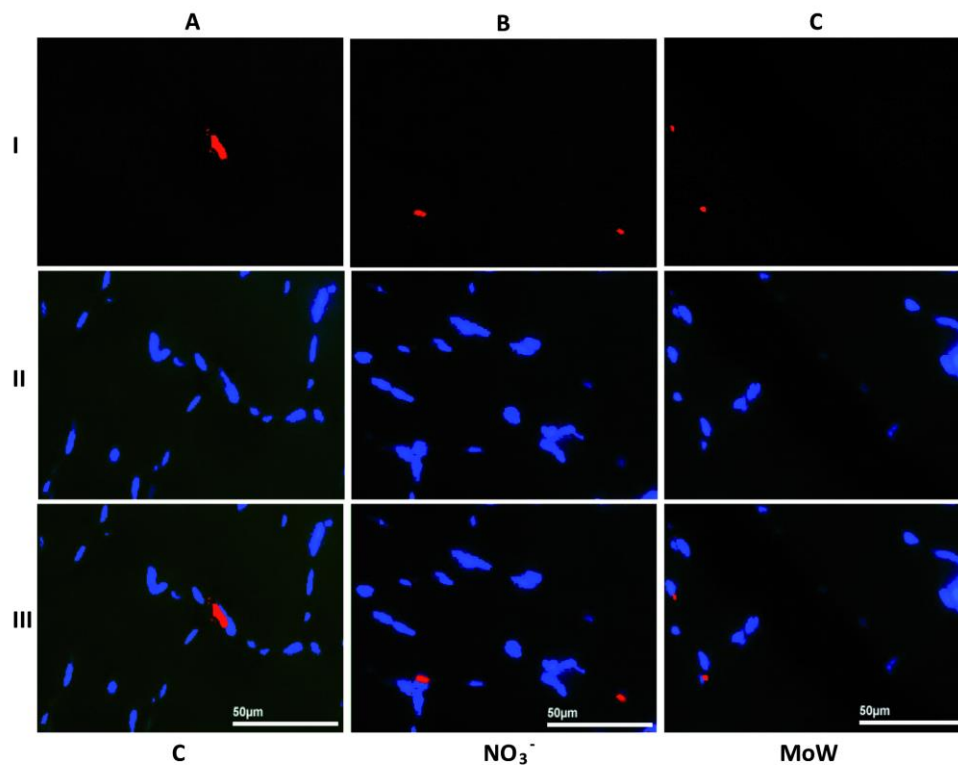


Figure 38. Number of CD45⁺ leukocytes in ischemic hind limbs.

Representative photographs demonstrate that neither NO₃⁻ supplementation nor MoW treatment changed the amount of CD45⁺ leukocytes in the ischemic hind limbs. **I** The first row shows the red CD45 staining. **II** The second row presents blue DAPI⁺ nuclei. **III** Subsequently, the third row encloses the merged pictures. The columns represent the different groups (**A** = *control*; **B** = *NO₃⁻ treated*; **C** = *MoW*). Magnification, x 200.

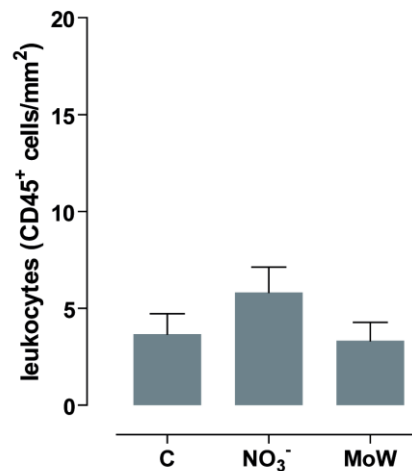


Figure 39. Leukocyte count in murine hind limbs after induction of ischemia.

In all examined samples, independent from supplementation or treatment, the number of CD45⁺ leukocytes lay beyond 15 cells per millimeter (ANOVA and Bonferroni post-test, ns, $p=0.2$). Data are given as mean $\pm$ SEM ($n=10-11$).

1.6 Paracrine Mediators and Angiogenic Chemokines

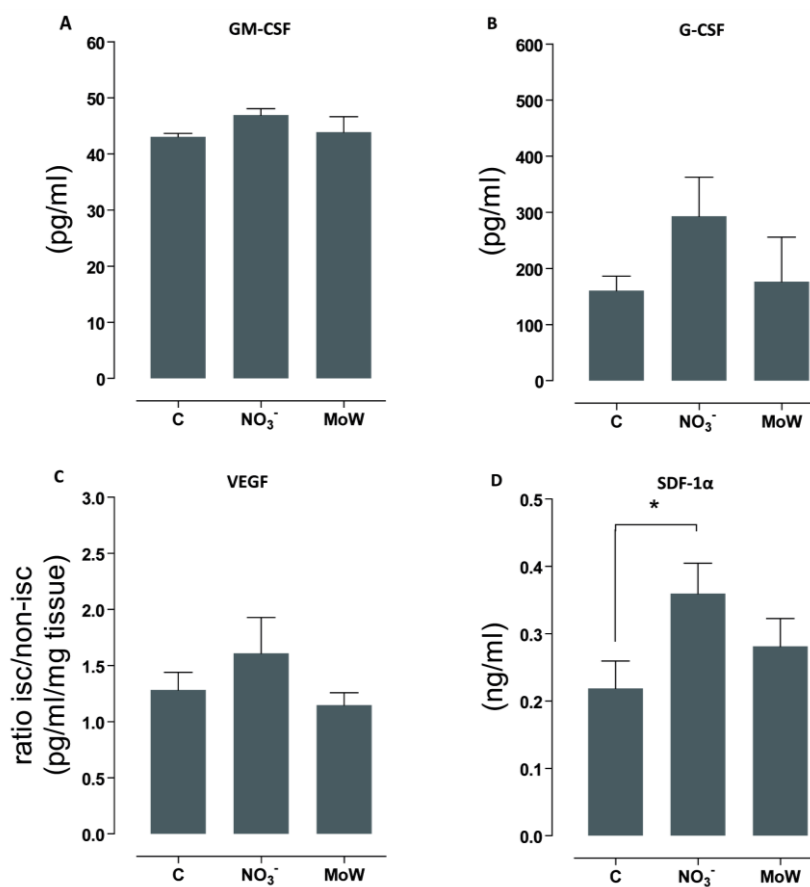
1.6.1 Endothelial-Regenerating Cells and Stem Cell Mobilising Chemokines

Probably, NO does not mobilize Endothelial-regenerating cells directly, but does so through a cytokine cascade. To test the impact of chemoattractors, plasma and tissue samples were tested for their levels. Endothelial-regenerating cells are mobilized from BM¹³⁹. Accordingly, the stem cell factors GM-CSF and G-CSF were analyzed. Furthermore, Endothelial-regenerating cells are known to act pro-angiogen differentiate into EC, because of that, VEGF was tested as well as SDF-1 α . The experimental set-up limited the number of points in time for taking blood samples, so all samples were gained on day 7.

GM-CSF and G-CSF are known stem-cell mobilizing factors¹⁵⁶, but in the actual study neither an impact on GM-CSF nor on G-CSF by NO₃⁻ or MoW could be detected. Figure 40 shows that the levels of GM-CSF were equally distributed (*control*: 43.1 ± 0.6 pg/ml; NO₃⁻: 46.9 ± 1.1 pg/ml; MoW: 43.9 ± 2.7 pg/ml; ANOVA and Bonferroni post-test, ns, $p=0.18$). Similarly, the G-CSF levels remained constant, but with a slight trend to amplification after NO₃⁻ supplementation (*control*: 160.5 ± 25.7 pg/ml; NO₃⁻: 293 ± 69.5 pg/ml; MoW: 176.4 ± 79.3 pg/ml; ANOVA and Bonferroni post-test, ns, $p=0.22$).

Next, pro-angiogen and Endothelial-regenerating cells -mobilizing known factors were examined (presented in figure 40). VEGF is one the most potent angiogenic cytokines^{393, 351} and SDF-1 α is the most prominent factor for Endothelial-regenerating cells release^{365, 327}. However, no significant increase of VEGF (illustrated as ratio from the ischemic and the non-ischemic leg) was measured (*control*: 1.3 ± 0.2 pg/ml; NO₃⁻: 1.6 ± 0.3 pg/ml; MoW: 1.1 ± 0.1 pg/ml; ANOVA and Bonferroni post-test, ns, $p=0.33$).

Solely SDF-1 α was significantly augmented after a 14 d lasting supplementation with dietary NO₃⁻ (*control*: 0.2 $\pm$ 0.04 ng/ml; NO₃⁻: 0.4 $\pm$ 0.05 ng/ml; MoW: 0.3 $\pm$ 0.04 ng/ml; ANOVA and Bonferroni post-test, ns, p=0.09; *, *control* vs. NO₃⁻). Figure 40. Cytokines tested on their impact in hind limb ischemia after dietary NO₃⁻.



A and B Both, the stem cell factors GM-CSF and G-CSF were equally distributed independently from supplementation or treatment (GM-CSF: ANOVA and Bonferroni post-test, ns, p=0.18; G-CSF: ANOVA and Bonferroni post-test, ns, p=0.22). **C** The same counted for the pro-angiogen growth-factor VEGF (ANOVA and Bonferroni post-test, ns, p=0.33). **D** SDF-1 α increased significantly from *control* to NO₃⁻ mice (ANOVA and Bonferroni post-test, ns, p=0.09; *, *control* vs. NO₃⁻). Data are given as mean $\pm$ SEM (n = 3 - 10).

1.6.2 sFasL as paracrine Mediator

Another aim of the study was the evaluation of the paracrine potential of Endothelial-regenerating cells. Besides the examined chemokines in 2.7.1 the Fas/FasL pathway appeared to be a promising candidate, because FasL acts pro-apoptotic as well as proliferative⁴²⁹. To date, it is unknown if Endothelial-regenerating cells express membrane-bound FasL (mFasL). Accordingly, by FACS analysis the Endothelial-regenerating cells were quantified and analyzed for mFasL expression. Indeed, figure 41 revealed that Endothelial-regenerating cells expressed mFasL. Moreover, this expression seemed to be influenced by dietary NO₃⁻. In the *control* group a mean FasL expression of 39.1 $\pm$ 5.6% was detected, which was significantly depleted to 15.4 $\pm$ 3.6% after NO₃⁻ supplementation (ANOVA and Bonferroni post-test, **, p=0.007; **, *control* vs. NO₃⁻) and subsequently increased after extra MoW treatment to 29.9 $\pm$ 5.9%.

Consequently, an ELISA was performed to detect systemic variations in soluble FasL (sFasL) plasma levels mirroring the shift in mFasL expression (see figure 41). Basal levels of sFasL were determined and compared with levels from *control* and NO₃⁻ mice. Basal levels lay by 30.8 $\pm$ 1.9 pg/ml. After surgery a significant increase

of sFasL was observed (ANOVA and Bonferroni post-test, ***, $p=0.0003$, *, *basal* vs. *control*; ***, *basal* vs. NO_3^-), but without significant difference between the *control* and the NO_3^- mice (*control*: 125.5 ± 28.1 pg/ml; NO_3^- : 156.5 ± 32.4 pg/ml).

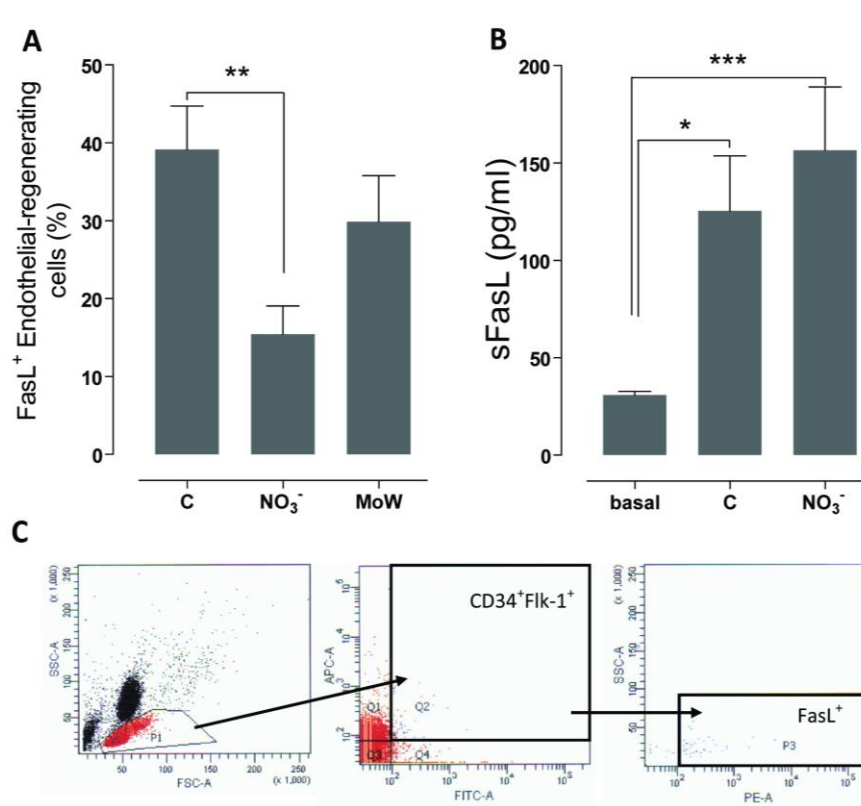


Figure 41. FasL expression on Endothelial-regenerating cell membranes and circulating in plasma.

A Every third Endothelial-regenerating cell of *control* mice expressed FasL on its surface. This expression was significantly modified by NO_3^- supplementation, lowered to about the half. MoW treatment again augmented the expression, but without reaching significance (ANOVA and Bonferroni post-test, **, $p=0.007$; **, *control* vs. NO_3^- ; $n=18-20$). **B** Ischemia augmented sFasL levels, but this increase was not significantly progressed after dietary NO_3^- (ANOVA and Bonferroni post-test, ***, $p=0.0003$, *, *basal* vs. *control*; ***, *basal* vs. NO_3^- ; $n=17-23$). **C** This figure extends the charts of figure 29 B. FasL was determined in the population of double positive MNC, referred to as Endothelial-regenerating cells. Data are given as mean $\pm$ SEM.

1.7 XOR mediated Reduction

In addition to the bacterial NO_3^- reduction, a mammalian NO_3^- reduction is discussed. Recently, a Swedish group postulated the identification of this mammalian NO_3^- reductase as the Xanthine oxidoreductase (XOR)²³. In the actual set-up the cytoprotective effects were abolished by antiseptic MoW, indicating the bacterial reduction as major mechanism. However, in some cases inhibition was not complete, which could be an indicator of a second reductive process. The current work was not focused on this issue, but first started experiments concerning this hypothesis.

In the beginning, like for the *control*, NO_3^- and *MoW* groups, the NO_x metabolism was analyzed for *AP* (*Allopurinol* supplemented) mice. Allopurinol as inhibitor for the XOR was given to the drinking water ad libitum. Figure 42 summarizes the results. Indeed, systemic NO_3^- levels (47.7 ± 6.2 μM), measured in non-ischemic tissue, decreased significantly after AP supplementation (Mann-Whitney test, *, $p=0.03$).

In contrast, NO_2^- levels were unaltered systemically, but locally changed. The local shift was determined by calculating the difference between the ischemic and the non-ischemic tissue (presented as percentage change from non-ischemic tissue). In *control* mice, NO_2^- increased about $45 \pm 18\%$, but nearly no increase was measured after AP supplementation ($7 \pm 15\%$). Instead of this, measured decrease was not significantly (Mann-Whitney test, ns, $p=0.09$) different from NO-heme levels, which differed significantly (Mann-Whitney test, *, $p=0.02$). Altogether, the same pertained for NO-heme as for NO_2^- delta: they were increased in *control* ($7778 \pm 2379\%$) and unaltered in AP mice ($163 \pm 125\%$).

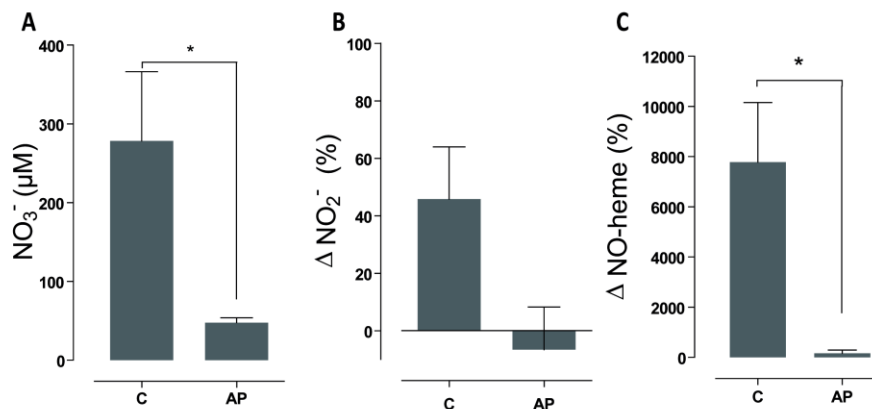


Figure 42. NO_x metabolism determined after XOR inhibition.

A Systemic NO_3^- levels were significantly decreased after XOR inhibition (Mann-Whitney test, *, $p=0.03$). **B** Regarded locally, NO_2^- levels were unchanged by AP inhibition, but diminished in contrast to the control group (Mann-Whitney test, ns, $p=0.09$). **C** NO-heme levels remained constant after AP supplementation, in contrast to the increase in *control* mice (Mann-Whitney test, ns, $p=0.02$). Data are given as mean $\pm$ SEM ($n=4-5$).

In the next step the mRNA expression of XOR and its activity in the muscle were tested, shown in figure 43. Both, expression and activity were diminished by dietary NO_3^- supplementation. Expression was significantly downregulated to 0.34 (NO_3^-) (calculated by REST© by a Pair Wise Fixed Reallocation Randomisation Test and S.E. estimation via a Taylor algorithm, normalized to *control*, *, $p=0.01$, SE range = 0.214-5.41)²⁷⁵.

The markedly active XOR in *control* mice ($14,800 \pm 2255$ $\mu\text{U}/\text{ml}/\text{mg}$) was significantly inhibited by AP (4510 ± 898 $\mu\text{U}/\text{ml}/\text{mg}$) and inclined to decrease after NO_3^- supplementation (9757 ± 1780 $\mu\text{U}/\text{ml}/\text{mg}$) (Kruskal-Wallis test and Dunn's post-test, *, $p=0.046$; *, *control* vs. AP).

Finally, the impact on regeneration was determined with similar assays as for NO_3^- supplementation. The low impact of XOR inhibition on NO_2^- and NO-heme, and the negative effect of NO_3^- supplementation on expression and function of XOR, revised the expectations. Indeed, no measurable effect could be detected in regeneration of perfusion, Endothelial-regenerating cell levels, and in apoptosis (see figure 44). The perfusion ratio remained unaltered in AP ($61 \pm 4\%$) animals compared with *control* mice (t-test, ns, $p=0.79$). Parallel, Endothelial-regenerating cell levels in whole blood were unaffected by AP supplementation ($0.05 \pm 0.01\%$ MNC) (Mann-Whitney test, ns, $p=0.26$). Finally, the apoptotic ratio rested at the levels of *control* mice after XOR inhibition ($187 \pm 45\%$) (t-test, ns, $p=0.79$).

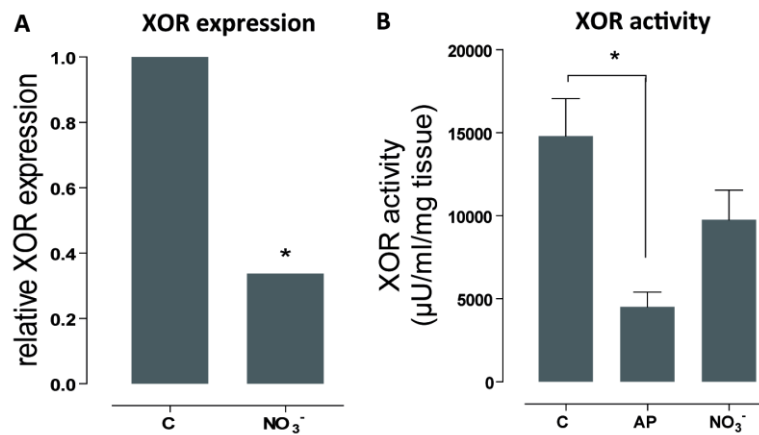


Figure 43. Expression and function of XOR in ischemic muscle.

A mRNA expression analysis by Real-Time PCR revealed a significant downregulation in NO_3^- animals (calculated by REST© by a Pair Wise Fixed Reallocation Randomisation Test and S.E. estimation via a Taylor algorithm, normalized to control, *, $p=0.01$, SE range = 0.214-5.41). **B** Parallel, an enzymatic assay measured a reduction in activity after NO_3^- supplementation. This reduction was confirmed by a significant reduction after AP supplementation, which affirmed the functionality of the assay and the success of AP supplementation (Kruskal-Wallis test and Dunn's post-test, *, $p=0.046$; *, control vs. AP). Data are given as mean $\pm$ SEM ($n = 3 - 5$).

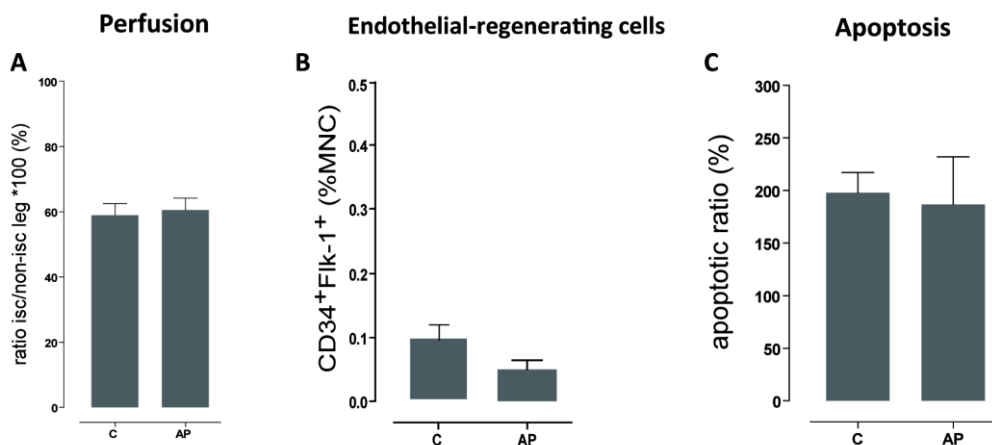


Figure 44. Impact of XOR on regeneration after induction of ischemia.

A Perfusion measured seven days after surgery was not decreased after AP supplementation (t-test, ns, $p=0.79$; $n=10$). **B** Additionally determined Endothelial-regenerating cell levels remained constant (Mann-Whitney test, ns, $p=0.26$; $n=8$). **C** In the end, the apoptotic ratio was unaffected by AP (t-test, ns, $p=0.79$; $n=4$). Data are given as mean $\pm$ SEM.

2. Clinical Studies

In the following, the results of two descriptive, clinical studies will be described. Both were performed in close cooperation with the clinic, combining two goals. The participating clinicians gained deep insights into different imaging tools and possibilities of new molecules as diagnostic or prognostic markers (not presented here) and information concerning the NO metabolism in ischemia was collected. The results were published in two manuscripts including the clinical data^{276, 277}. The following paragraphs focus on the NO metabolism and associated pathways, molecules, and cells.

2.1 Serial Measurements of Whole Blood NO_2^- in an Intensive Care setting²⁷⁶

NO is one of the most important vasodilators in the cardiovascular system²⁸⁰, therefore the question arose whether its' metabolites, NO_2^- and NO_3^- could be used as markers for cardiovascular health. Kleinbongard et al. revealed an association between plasma NO_2^- levels and a number of risk factors for endothelial dysfunction⁵. The current work was designed to extend this work to the collective of seriously ill patients in an intensive care setting and NO_2^- in whole blood. The patient population consists of patients of the intensive care unit (University hospital Aachen, department of cardiology, pneumology, and angiology) and is characterized in table 10. Only patients who got NO-donating drugs or who showed symptoms of refractory shock were excluded.

Blood samples were taken for 10 days every morning. Afterwards, the highest NO_2^- levels were determined. These maximum levels were grouped in low CK-MB level ($<3.7(\text{female})/6.7(\text{male}) \mu\text{g}$) and high CK-MB level ($>3.7(\text{female})/6.7(\text{male}) \mu\text{g}$), representing “no damage” or “myocardial damage”. Separated like this, the mean NO_2^- levels differ significantly with $432 \pm 95 \text{ nM}$ for low CK-MB level and $200 \pm 53 \text{ nM}$ for high CK-MB level (Mann-Whitney test, *, $p=0.039$). Results are presented in figure 45. A cut off value like this has been also looked for in other tested parameters, but only with CK-MB and NO_2^- did an association between parameter and NO_2^- emerge.

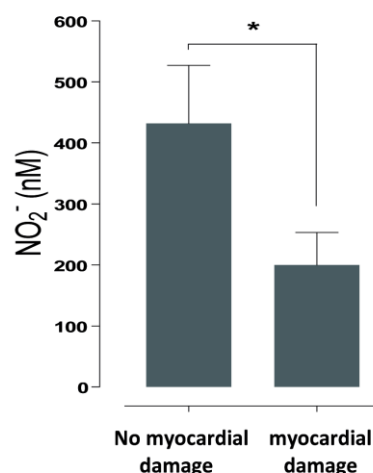


Figure 45. NO_2^- levels of seriously ill patients separated by CK-MB levels.

Patients with low CK-MB levels (no myocardial damage) had significantly higher NO_2^- levels than patients with high CK-MB levels (myocardial damage) (Mann-Whitney test, *, $p=0.039$). Data are given as mean $\pm$ SEM ($n=14+13$).

Table 10. Patient characteristics.

Population enrolled at the intensive care unit in February 2007. (BMI = body mass index)

Characteristics	n=30		
Age - yrs	69±2	Drug therapy – no. (%)	
Sex, male/female – no.	20/10	Inotropes or vasopressors	18 (60)
BMI – kg/m ²	27±1	NO donating drugs	7 (23)
Reason for intensive care – no. (%)		Nutrition – no. (%)	
Cardiogenic shock	11 (37)	Enteral	16 (53)
Hemorrhagic shock	3 (10)	Intravenous	13 (43)
Myocardial infarction	26 (87)	Oral	13 (43)
Respiratory failure	4 (13)	Interventional procedures – no. (%)	
Septic shock	14 (47)	Angioplasty	
Chronic coexisting conditions – no. (%)		Cardiopulmonary resuscitation	8 (27)
Congestive heart failure	19 (63)	Intraaorticballoon counterpulsation	3 (10)
Coronary artery disease	23 (77)	Mechanical ventilation	18 (60)
Chronic kidney disease	9 (30)	Non-invasive ventilation	6 (20)
Chronic obstructive pulmonary disease	8 (27)	Renal replacement therapy	4 (13)
Diabetes mellitus	9 (30)	Outcome parameters – no. (%)	
Hypercholesterolemia	12 (40)	Administration of therapy in refractory shock	6 (20)
Hypertension	17 (57)	Death during hospitalization	10 (33)
Liver disease	3 (10)	Hypoxic brain damage	2 (7)
Smoking	13 (43)		
Renal replacement therapy	4 (13)		

2.2 New techniques in cardiac imaging and diagnostic of the ischemia/reperfusion-injury in the acute myocardial infarction²⁷⁷

The results of the first study gave insights into the importance of NO in myocardial infarction, leading to an expanded series of experiments, embedded in a study concerning new imaging and diagnostic tools of the ischemia/reperfusion-injury.

Study population consisted of STEMI (ST-segment elevation myocardial infarction) patients received PCI (percutaneous coronary intervention) within 12 h after symptom onset. From this moment, the patients were observed for one week, and three bloods, and echocardiographic analyses, and one CMR (cardiovascular magnetic resonance) examination to trace regeneration of myocardial function, inflammation, and NO metabolism (explained in figure 46) were carried out.

Table 11 summarizes the patient characteristics. Only first-infarctions were included and as expected, the group was composed mainly of elderly men with a conglomeration of risk factors, but only rare pre-existing conditions. Most infarctions occurred in the RCA (right coronary artery) and were cured with Stent implantation.

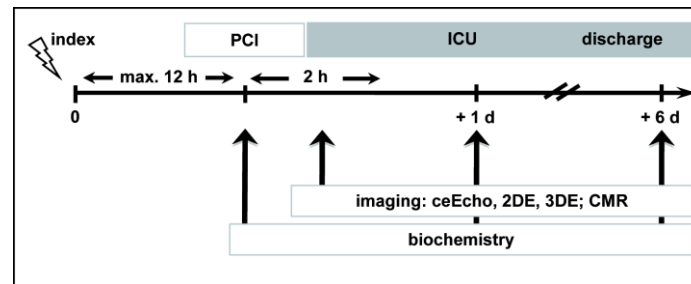


Figure 46. Study set up.

Patients were enrolled in the study if PCI took place within 12 h after symptom had onset. Before PCI the first blood sample was drawn, echocardiographic examination followed within 2 h after PCI. The second series of analyses happened 24 h later and the last examination, including CMR test, was done on day six before discharge. (3DE = three dimensional echocardiography; 2DE = two dimensional echocardiography; ceEcho = contrast-enhanced Echocardiography; CMR = cardiovascular magnetic resonance; PCI = Percutaneous coronary intervention; ICU = intensive care unit).

Table 11. Patient characteristics of the STEMI collective.

Patients were excluded if symptom onset amounted to more than 12 h infarction and the occluded artery was not closed for the first time. Data are given in percentage terms or as mean $\pm$ SEM. (BMI = Body mass index; CAD = coronary artery disease; PCI = Percutaneous coronary intervention; CABG = coronary artery bypass graft surgery; GP = glycoprotein; LAD = left anterior descending artery; LCX = left circumflex artery; RCA = right coronary artery; BMSE = bare metal stent; DES = Drug Eluting Stent; TIMI = Thrombolysis In Myocardial Infarction; CK = creatine kinase; CK-MB = creatine kinase myocardial band).

Characteristics	n=42
Age - yrs	60 $\pm$ 2
Sex, male/female	74/26
BMI - kg/m ²	27.3 $\pm$ 0.8
Coronary risk factors	
Family history of CAD	39
Current or recent smoking	76
Hypertension	50
Hypercholesterolemia	35
Diabetes mellitus	20
Cardiac history	
Prior myocardial infarction	2
Prior heart failure	0
Prior coronary revascularization (PCI or CABG)	0
Peripheral vascular disease	2
Prior stroke	0
Procedural characteristics	
GP1IIa/IIIb inhibitor	80
Culprit lesion	
LAD	37
LCX	15
RCA	48
Proximal LAD	17
Stent implantation	
BMSE	72
DES	26
No stent	4
TIMI flow grade	
Before PCI	1.3 $\pm$ 0.2
After PCI	2.9 $\pm$ 0.03
Time from symptom onset to reperfusion- h	5.1 $\pm$ 0.5
Multivessel disease	46
Enzyme release – peak	
CK - IU/l	1887 $\pm$ 310
CK-MB - μ g/l	210 $\pm$ 35
Additional emergency treatment	
Dobutamine and/or norepinephrine	17
Mechanical ventilation	4
Cardiopulmonary resuscitation	4
Hospitalization time - d	8 $\pm$ 1

2.2.1 NO Metabolism

Similar to the first study, NO metabolites were analyzed. However, NO_3^- and NO_2^- levels of STEMI patients remained constant over the observation period, as shown in figure 47 (Repeated measured ANOVA and Bonferroni post-test; NO_3^- : ns, $p = 0.26$; NO_2^- : ns, $p = 0.69$). NO_3^- levels ranked between $30.04 \pm 3.6 \mu\text{M}$ (0 d), $25 \pm 3.4 \mu\text{M}$ (1 d), and $26.7 \pm 2.7 \mu\text{M}$ (6 d). NO_2^- levels started with $143 \pm 29 \text{ nM}$ (0 d), rose to $1689 \pm 33.9 \text{ nM}$ (1 d), and stayed at $163 \pm 25.8 \text{ nM}$ (6 d).

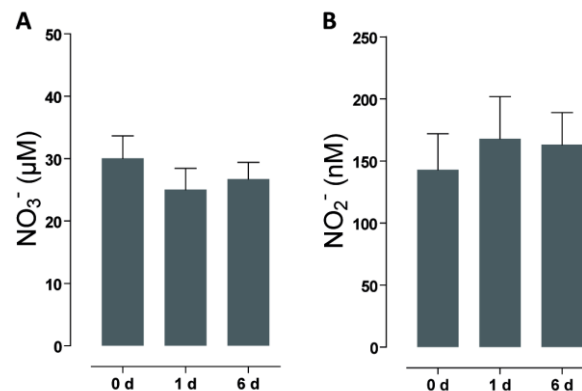


Figure 47. NO_3^- and NO_2^- levels determined in patients suffered from a STEMI.

A NO_3^- levels lacked a difference within the first week after the ischemic event (Repeated measured ANOVA and Bonferroni post-test, ns, $p = 0.26$). **B** In parallel, NO_2^- levels remained constant (Repeated measured ANOVA and Bonferroni post-test, ns, $p = 0.69$). Data are given as mean $\pm$ SEM ($n = 22-25$).

2.2.2 Endothelial-Regenerating Cells and Chemokines

As the presented animal study revealed an association between NO_3^- and Endothelial-regenerating cells, level of $\text{KDR}^+\text{CD34}^+$ cells in whole blood were also determined in the clinical setting (figure 48). Endothelial-regenerating cell levels of STEMI patients remained low from the beginning (0.08 ± 0.02 % MNC) to the end (0.09 ± 0.02 % MNC) of the observation period. After a lapse of 24 h, levels did not exceed 0.07 ± 0.02 % MNC and remained low after six days (0.09 ± 0.02 % MNC) (Friedman test and Dunn's post test, ns, $p = 0.77$).

Subsequently, two cytokines associated with inflammation and Endothelial-regenerating cells were analyzed. Shown in figure 49 A-D are SDF-1 α , a known mobilizing cytokine, and NAP-2, which activates adhesion of neutrophils. SDF-1 α even decreased on admission ($1739 \pm 60.1 \text{ pg/ml}$) and declined further significantly within the first day ($1522 \pm 48.9 \text{ pg/ml}$), but rose to $2077 \pm 90.8 \text{ pg/ml}$ at day six (Repeated measured ANOVA, ***, $p < 0.0001$; **, 0 d vs. 1 d; **, 0 d vs. 6 d; ***, 1 d vs. 6 d). In comparison, the levels of CAD patients were determined ($2077 \pm 90.8 \text{ pg/ml}$), which were significantly augmented in contrast to the minimum SDF-1 α level in STEMI patients (t-test; ***, $p < 0.0001$). Instead, NAP-2 level seemed to be elevated while infarction ($2420 \pm 512.4 \text{ pg/ml}$) and diminished in the following (1 d: $1485 \pm 352.3 \text{ pg/ml}$; 6 d: $946.7 \pm 198.4 \text{ pg/ml}$). The decline to day six was significant (Repeated measured ANOVA, *, $p < 0.012$; *, 0 d vs. 6 d). They corresponded to the levels determined in CAD patients ($1405 \pm 183.5 \text{ pg/ml}$), which were significantly lower than the elevated levels in STEMI patients on day 0 (t test, *, $p = 0.04$).

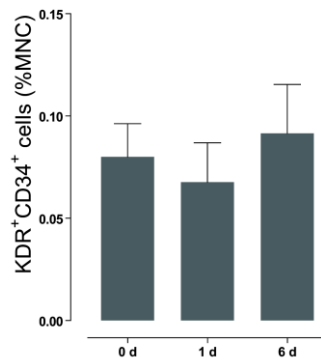


Figure 48. Endothelial-regenerating cells determined by FACS.

Endothelial-regenerating cells were stained as fast as possible after blood taking. At all points in time levels show similar niveau (Friedman test and Dunn's post test, ns, $p=0.77$). Data are given as mean $\pm$ SEM ($n=13$).

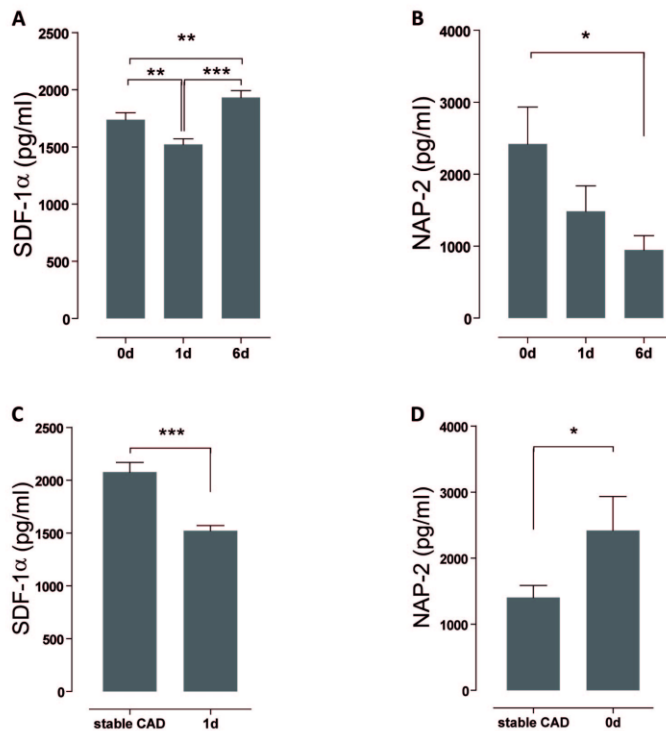


Figure 49. Cytokines in the context of cardiovascular disease.

A and **D** SDF-1 α level decreased significantly in the course of infarction and tended to increase to the level of CAD patients within one week (Repeated measured ANOVA, ***, $p < 0.0001$; **, 0 d vs. 1 d; **, 0 d vs. 6 d; ***, 1 d vs. 6 d; t-test; ***, $p < 0.0001$, CAD vs. 1 d). **B** and **C** NAP-2 level were significantly augmented in STEMI patients, decreasing in six days towards the level measured in CAD patients (Repeated measured ANOVA, *, $p < 0.012$; *, 0 d vs. 6 d; t test, *, $p = 0.04$, CAD vs. 0 d). Data are given as mean $\pm$ SEM ($n = 18-29$).

2.2.3 Inflammation

It is known that PCI leads to the ischemia/reperfusion (I/R) injury¹²⁵. Indeed, the reperfusion of the infarcted heart, leads to a second injury, which impairs the heart aggravating the damage of the infarction. One of the mechanisms, which is involved in this process, is inflammation. Therefore, several participating parameters were determined. Figure 50 presents the most important mediators TNF- α , IL-6, and CRP.

TNF- α levels were elevated in the first sample (7.7 ± 0.6 ng/l). The continued increase over the observation period (8.5 ± 0.7 ng/l (1 d); 9.4 ± 0.8 ng/l (6 d)) reached significance after six days (Repeated measured ANOVA and Bonferroni post-test, *, $p = 0.01$; **, 0 d vs. 6 d).

In contrast, IL-6 reached its peak after 24 h (2.7 ± 0.2 ng/l) and begun to decrease after 6 d (2.2 ± 0.2 ng/l). The variable started with 1.5 ± 0.2 ng/l on admission, rising significantly, tested with a parametrical analysis after transforming the data into their natural logarithm (Repeated measured ANOVA and Bonferroni post-test, ***, $p < 0.0001$; ***, 0 d vs. 1 d; *, 0 d vs. 6 d; *, 1 d vs. 6 d).

Similarly, CRP (transformed to its natural logarithm) rose after 1 d (from 1.4 ± 0.2 mg/l on admission to 2.7 ± 0.2 ng/l after 24 h), but stayed elevated after six days (2.2 ± 0.2 ng/l). Variance analysis revealed significance (Repeated measured ANOVA and Bonferroni post test, ***, $p < 0.0001$; ***, 0 d vs. 1 d; ***, 0 d vs. 6 d).

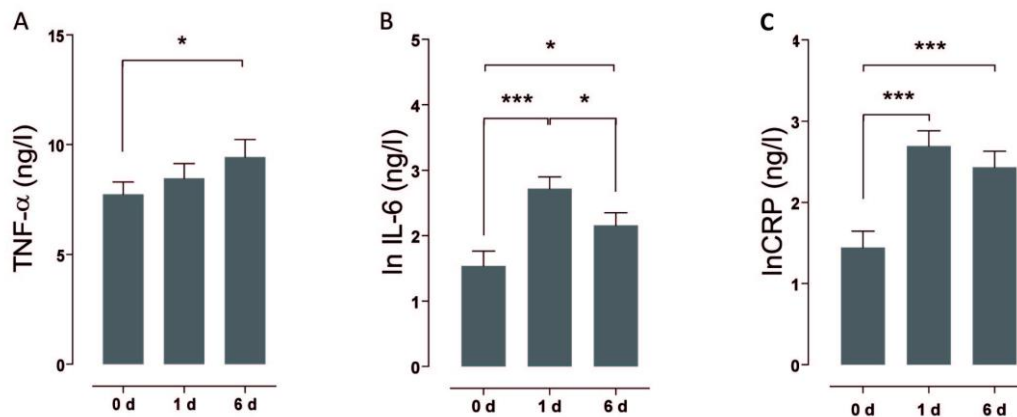


Figure 50. Time course of TNF-α, IL-6, and CRP over six days after myocardial infarction.

A TNF-α elevated within the first hours of infarction, rising continuously during the following week (Repeated measured ANOVA and Bonferroni post-test, *, $p = 0.01$; **, 0 d vs. 6 d) **B** IL-6 increased after 24 h with a fast subsequent decrease (Repeated measured ANOVA and Bonferroni post-test, ***, $p < 0.0001$; ***, 0 d vs. 1 d; *, 0 d vs. 6 d; *, 1 d vs. 6 d) **C** CRP levels were augmented after 24 h, but stayed elevated (Repeated measured ANOVA and Bonferroni post test, ***, $p < 0.0001$; ***, 0 d vs. 1 d; ***, 0 d vs. 6 d). Data are given as mean $\pm$ SEM ($n = 32-37$).

A second series of blood samples was taken from CAD (coronary artery disease) patients before PCI as control (figure 51). Unexpectedly, TNF-α levels of CAD patients were significantly increased (14.8 ± 1 ng/l) in contrast to the levels of STEMI patients on admission (t-test, ***, $p < 0.0001$). However, IL-6 level were significantly diminished in CAD patients (1.2 ± 0.1 ng/l) (t-test, ***, $p < 0.0001$). Similarly, the CRP levels of CAD patients (0.2 ± 0.2 ng/l) were compared to the peak levels in STEMI patients (1 d) elucidating a significant decrease (t-test, ***, $p < 0.0001$).

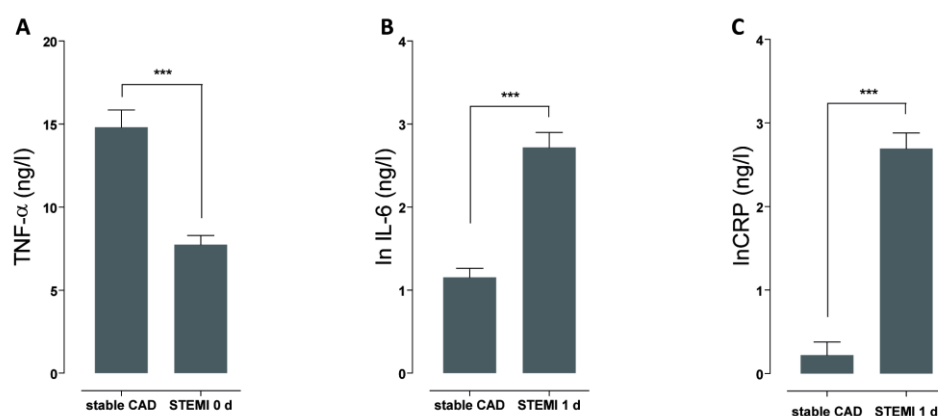


Figure 51. TNF-α, IL-6, and CRP levels of CAD patients in comparison with STEMI patients.

A TNF-α levels were significantly increased in CAD patients (t-test, ***, $p < 0.0001$). **B** and **C** IL-6 and CRP levels were significantly decreased (t-test, ***, $p < 0.0001$). Data are given as mean $\pm$ SEM ($n = 26-32$).

Discussion

NO_3^- is a ubiquitous molecule. People avoid ingesting too much of it in soda. Nevertheless, only a minority really knows why NO_3^- levels are regulated by the administration, and how manifold NO_3^- sources – exogene and endogene are. The current work tries to rescind some of the reservations showing that supplementation with dietary NO_3^- could strengthen regeneration after an acute event of hind limb ischemia and that this supplementation increases the level of NO whose balance is potentially disturbed in ischemia. In science, it was considered, for a long time, that NO_3^- was toxic, carcinogen, and had low reactivity. In the one-way street of NO metabolism it was the blind end without turning area. Moreover, in contrast to NO_2^- , which was used as a marker for NO, NO_3^- could not be used in this context, because of its high suggestibility by nutrition.

Studies from the forties⁴⁶ demonize NO_2^- but also NO_3^- to be toxic causing methemoglobinemia. More recent work discussed the carcinogen potential of NO_2^- and NO_3^- by the reaction of NO_2^- with secondary amines to nitrosamines. However, analyzing these articles, data are inconclusive and questionable; the results concerning methemoglobinemia are true, but exaggeratedly presented and not adapted to the situation^{19,53,57,60,64,70,279}. It is improbable that dietary NO_3^- could influence adult NO_2^- levels in a toxic way, because NO_2^- and NO_3^- are currently produced endogenously. For NO_3^- to be toxic, it has to be reduced to NO_2^- , but up to 70 % of ingested NO_3^- is excreted (on average 67.3 mg/day from 104 mg/day, meaning that only 36.7 mg/day NO_3^- are absorbed and available to be metabolized to NO_2^-). In addition, 1 mg/kg NO_3^- are produced endogenously every day (e. g. 80 mg/day for an 80 kg man). Instead of this, only 1 – 2 g NO_2^- is considered to be acute toxic. Just the adult methemoglobin-reductase will experience problems to detach NO_2^- from haemoglobin, permitting the binding to O_2 , which finally leads to methemoglobinemia. Only in babies, whose methemoglobin-reductase is not as active as the adult one, should the NO_2^- and NO_3^- intake be restricted⁵³. The carcinogen potential of nitrosamines is generally accepted, but epidemiological studies analyzing potential associations between NO_3^- and NO_2^- consumption and cancer are controversial. Michaud et al. found no connotation between NO_3^- or NO_2^- intake and tumor growth⁷⁰. In conclusion NO_3^- was considered to be an inert molecule without benefit, but only risks. Recent work has shed new light on NO_2^- , discovering it as NO reservoir available in ischemia, and there are considerations to broadcast this concept on NO_3^- , which would be refillable by healthy diet! Why would this be useful? NO is one of the most potent vasodilators and signaling molecules in the cardiovascular system, but has a very short half-life and must be produced permanently²⁸⁰. However, in ischemia regular NO synthesis is impaired, because O_2 is a necessary cofactor for eNOS, the NO synthesizing enzyme¹¹⁷. Thus, under these conditions an oxygen independent NO synthesis is needed, which could be attained by reduction of NO_3^- to NO_2^- , and further to NO. Orally ingested NO_3^- is reduced in the oral cavity by bacterial nitrate reductase²⁸¹. Resulting NO_2^- arrives circulation, tissue, and stomach where it is further reduced to NO by acidic and enzymatic reduction. Indeed, patients with atherosclerosis show diminished NO_2^- levels, lacking enough endogenous NO_2^- to fulfil the NO demand²⁸². Therefore, a controlled high intake of NO_3^- could have the potential to intervene in the progress of disease.

To develop guidelines and beneficial strategies, the mechanisms behind a protective effect should be investigated. Vasodilatation initiated by NO occurs by activating the GC, which produces cGMP²⁸⁰, but it is unknown whether the same reaction is responsible for cytoprotection mediated by NO_2^- derived NO in

ischemia. It is known that stem cells and cytokines act protective by pro-angiogenic reactions. One of the most prominent populations is the group of Endothelial-regenerating cells. They leave their stem cell niche in BM, enter circulation and migrate into the ischemic tissue. *In vitro* studies could show that this chemotactic migration is supported by NO⁸⁰. Arrived there, Endothelial-regenerating cells differentiate into EC mediating angiogenesis and neo-vascularization. Whether Endothelial-regenerating cells build up capillaries by itself, or secrete angiogenic factors supporting vascularization, is a matter of debate^{179,180,187,283}. Many studies in the last decade investigated the paracrine potential of these progenitors, showing that Endothelial-regenerating cells transmit hematopoietic and angiogenic effects^{145,284}, depress oxidative Stress¹⁷⁹, and influence inflammatory responses¹⁸¹. The question arose by which pathway Endothelial-regenerating cells in hind limb ischemia operate. The Fas/FasL pathway was discovered as a death-receptor pathway initiating apoptosis²⁸⁵. However, recent work could show that this pathway is not as unidirectional as was originally thought. A membrane-bound and a soluble form of the receptor and the ligand could be distinguished and particularly the sFaL molecule supports proliferative effects.

Clinical studies confirmed that the mentioned processes and factors are impaired in cardiovascular disease. Thus, this specific combination of activators, progenitor cells, and pathways seems to be convenient to aid in cardiovascular disease, offering a promising target for beneficial intervention.

Indeed the current work presents an approach to increase regeneration in ischemia by dietary NO₃⁻ supplementation and evaluates NO_x metabolism, Endothelial-regenerating cells, and angiogenic cytokines in severe ill patients. It could be shown that in ischemic disease, NO_x metabolites are diminished and that a nutritional supplement of NO₃⁻ is able to fill the lacking mediating regenerative effects. Furthermore, the data show that the protective effects of NO₃⁻ are associated with an increase in Endothelial-regenerating cells, which express FasL. The illustration in figure 52 summarizes the proposed mechanism of protection mediated by dietary NO₃⁻.

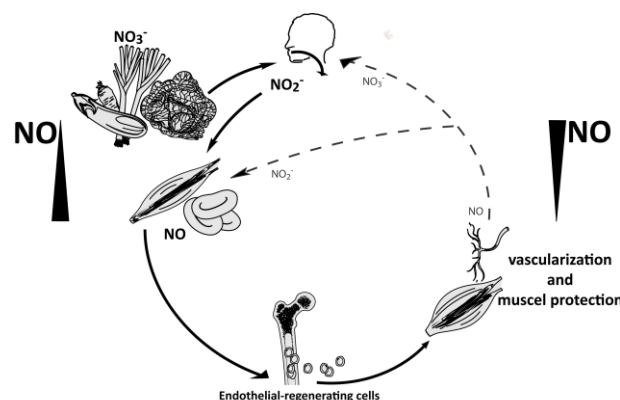


Figure 52. Cytoprotection augmented by dietary NO₃⁻.

In the oral cavity, orally ingested NO₃⁻ is reduced to NO₂⁻. The accrued NO₂⁻ is metabolized further to NO by hemoglobin in erythrocytes and myoglobin in skeletal muscle tissue. Subsequently, Endothelial-regenerating cells are mobilized from BM and migrate into the ischemic tissue mediated by the originated NO. Finally, the Endothelial-regenerating cells support invigoration of vascularization and protection of muscle progenitors. The strengthened capillary network delivers new O₂, which increases endogenous production of NO by eNOS. Moreover, initiating this pathway the synthesized NO reenters the circuit supporting regeneration. The model does not claim to be complete, but only summarizes the stations which were analyzed in the current work.

1. NO Bioavailability is Replenished by Dietary NO_3^-

The first idea to increase endogenous NO level is to medicate with NO, of course. However, NO is a very instable molecule, which is only chemical-bond obtainable. Recent work could show that hemoglobin and myoglobin are able to reduce NO_2^- to NO under hypoxic conditions^{24,32}, and NO_2^- is an ubiquitous molecule in food and water. Nevertheless, it could be problematic to administer NO_2^- because of its toxicity and the suspicion that it contains carcinogen^{53,286}. Considering that, the presented work favored to deliver NO in the form of NO_3^- in drinking water. Indeed, NO_3^- is reduced to NO_2^- too, but the fact that one additional reaction is needed, that about three quarter of ingested NO_3^- are directly excreted, and that NO_3^- usually is a component of “healthy” foods lessen the inappropriate concern about NO_3^- . Another advantage of dietary NO_3^- is the redundancy of pills and injections, avoiding the problem of complications and compliance.

Figure 15 illustrate that, in a mouse model a 14 d-long administration of NO_3^- in drinking water amended endogenous NO_x -levels without influencing constitution. A subpopulation of animals was held separately and daily food and water consumption as well as weight were controlled, confirming autonomy of dietary NO_3^- . Additionally, quantitative and qualitative determination of oral flora affirmed the success of inhibiting bacterial NO_3^- reduction by antiseptic mouthspray, presented in figure 14. Hence, MoW treatment was established as suitable negative control.

NO metabolites were determined in healthy and ischemic animals. It was expected that NO levels in ischemic animals were decreased in comparison to the levels in healthy animals.

Indeed, figure 19 C compares NO_2^- levels of healthy and ischemic mice. Independent from collective the ischemic levels were significantly lower, supporting the hypothesis of a lack of NO in ischemia. Subsequently, clinical studies could show that ischemia also has a strong impact on NO_2^- levels²⁷⁶, which is discussed below (5. NO Balance is disturbed in Ischemic Syndromes).

In relation to NO_2^- plasma concentrations in *control* animals, shown in figure 19 B, it could be proposed, that ischemic levels even decline under basal levels. Additionally, in figure 17 A (NO_3^- levels) and B (NO_2^- levels) all collectives (*basal*, *control*, NO_3^- , *MoW*) are contrasted with each other before induction of ischemia. The bar graphs prove that endogenous NO_3^- and NO_2^- levels were increased by enhanced ingestion of dietary NO_3^- . NO_2^- levels show, that this dietary NO_3^- is reduced to NO_2^- and forwarded into circulation by commensal bacteria in the oral cavity, proven by the abolishing of the process by antiseptic MoW. The effect which could be seen after MoW could be ascribed to the drug, but could not be ascribed to the spraying. Surprisingly, NO_3^- levels were diminished after MoW treatment. Since MoW treated mice could not metabolize and recycle NO_3^- it must circulate unchanged. Thus, missing NO_3^- in plasma, urine and feces were analyzed. Feces levels were negligible and indifferent (data not shown), as confirmed by literature²². The same accounts for NO_2^- levels in urine, which could be disregarded²² and, indeed, levels remained unchanged, independent from supplementation or treatment (figure 18 B). In contrast, urinary NO_3^- levels obviously increased after NO_3^- supplementation and MoW treatment (from about 1500 μM to > 9000 μM). Physiological NO_3^- levels in human urine fluctuate between 250-2000 μM ²² elucidating a huge range. Therefore, urinary levels could not be used as sensitive marker for NO metabolism or bioavailability, but intake could be estimated^{22,287–289}. Indeed, the results shown

in figure 18 A illustrate a significant increase in NO_3^- concentrations after NO_3^- supplementation, but no difference between the two collectives supplemented with NO_3^- . Amplification in NO_3^- and NO_2^- levels after NO_3^- supplementation should also be corroborated after induction of ischemia. Instead of this, the data shows that the extra parameter – ischemia – augmented variance and diminished levels in such a manner that, NO_3^- levels after NO_3^- supplementation and MoW treatment were significantly augmented, compared to the concentrations in *control* mice. However, no difference could be determined between the NO_3^- and MoW groups, shown in figure 18 A. Moreover, no difference in NO_2^- levels could be detected (figure 18 B).

Next, NO_3^- and NO_2^- levels were determined in tissue (figure 20 A and B). Overall, the increasing effect of dietary NO_3^- is blurred, but nevertheless the NO_3^- group had the highest NO_3^- level, declined after MoW. Considering elevated ischemic NO_2^- levels, it became clear, that NO_2^- is accumulated in ischemic tissue in comparison to healthy control tissue (muscle tissue from the non-operated leg). Additionally, this difference is significant in NO-heme data, suggesting a reduction to NO, shown in figure 20 C. Summarized, the data illustrate that dietary NO_3^- is reduced to NO via NO_2^- and arrives at regions of demand. However, the effect is not as clear as it is before the induction of ischemia and in plasma. For this reason, ischemia represents an additional parameter and a loss of NO metabolites from plasma to tissue. Finally, the levels of protein-bound NO were determined and displayed in figure 20 D. Without supplementation, RNO and RSNO levels were separated in two halves amounting to 50 %, and this proportion remained relatively constant after NO_3^- supplementation. The levels seemed to be higher and a bit postponed to more RNOs, but huge variance hampered a statistical analysis and a clear statement.

Furthermore, cGMP levels were determined. In the endothelium, NO activates the sGC which produces cGMP²⁸⁰. Thus, increased cGMP levels suggest increased NO level and mechanistic action via the sGC. However, the cGMP data in figure 21 does not differ between the various groups. Seemingly, under these conditions NO acts via another pathway. These results confirmed a former study, in which a pro-angiogenic effect of NO_2^- was analyzed⁸¹.

2. Dietary NO_3^- Augments Regeneration in Hind limb Ischemia

2.1 Perfusion and Necrosis

Aim of the present work was not only to show, that dietary NO_3^- is suitable to enhance NO bioavailability, but also to prove the beneficial effect of ischemia. Over the last decade, much research was done recognizing a beneficial potential of the NO metabolites NO_2^- and NO_3^- . Duranski et al. and Bryan et al. suggested a benefit of dietary NO_2^- in liver ischemia and I/R injury^{84,85}. Furthermore, in a model of hind limb ischemia i. p. injected NO_2 , attained an increased regeneration in relation with angiogenesis⁸¹. Subsequently, several studies could determine an impact in host defense and protection from gastric ulcer^{17,281,290}. Finally, a blood-pressure lowering effect of dietary NO_3^- was shown²⁸¹. All finished studies proposing a beneficial and cytoprotective effect of NO metabolites laid the foundation for the present work. Here, the approach of dietary supplementation and a model of chronic ischemia were combined. NO_3^- was chosen instead of NO_2^- because of its lower toxicity. PAD and chronic ischemia as model were preferred, as the long-term goal is an early intervention, preventing severe acute events like an infarction. Indeed, figure 22 A illustrates that dietary NO_3^-

amplified regeneration in a significant, physiologic relevant manner. The bar chart shows that through dietary NO_3^- an improvement of regeneration of about 15 % is achieved. The presentation of representative LDPI pictures in figure 22 B before and seven days after surgery clarifies that perfusion remained low in *control* and *MoW* mice, in contrast to the enhanced perfusion after NO_3^- supplementation.

Overall, regeneration appears to be relatively low, but the results have to be regarded along with other studies. Comparison of papers in literature show great differences, dependent on mouse strain. Many studies used the C57BL/6J strain for experiments, which is characterized by a well regeneration^{81,265,291–294}. On the other hand, a series exists in which impaired strains were used like immunodeficient mice¹⁵⁹. In the present work, the NMRI strain was chosen; a mouse with less regeneration than the C57BL/6J strain but healthy in contrast to e.g., nude mice. This, in health and regeneration, intermediate strain seemed to be best transferable for PAD. Besides, the impairment of transgenic or immunodeficient mice, the differences are probably attributable to diverse vessel architectures²⁹⁵. However, no literature is known to analyze the architecture for NMRI mice, but genealogy shows the narrow relationship to CD1 mice, thus a similar vessel architecture could be suggested^{296,297}. This outbred strain shows a mixture of the two extreme types Balb C and C57BL/J6, reflected in regeneration capacity. Balb C mice show many independent vessel trees with no connection to neighboring ones, hampering regeneration. In contrast, C57BL/J6 mice have one big vessel tree, in which many neighboring collaterals are able to compensate for a deficiency. Scheme in figure 53 tries to visualize the principle variation. Additionally, differences in immune system are responsible. C57BL/J6 mice show much more lymphocytes than BalbC mice, which is reflected in better regeneration in C57BL/J6 mice²⁴⁰.

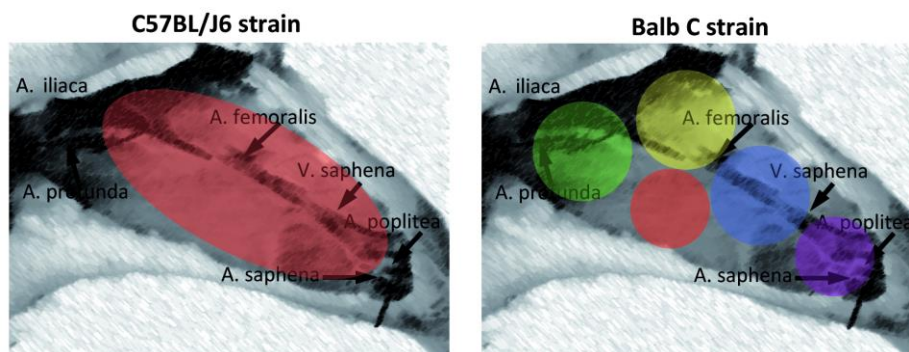


Figure 53. Visualization of vessel architecture in C57BL/J6 and Balb C mice

The operative site of the used NMRI strain is shown, but overlaid circles should demonstrate the different vessel architectures in C57BL/J6 and Balb C mice. Every circle denotes a vessel tree, different colors strengthen the independency of the single trees. In the C57BL/J6 strain one vessel tree subserves a big area of the leg. In contrast, in the Balb C strain many single vessel trees exist, every one responsible for a small tissue area. The scheme is not anatomically correct, but should clarify the principle, transferring the results from the murine m. spinotrapezius to the murine hind limb²⁹⁵.

Furthermore, varieties in surgery lead to different results^{261,294,298}. Under current conditions the chosen strain regenerates to about 60 %, which includes LDPI measurements until seven days after surgery. The 12 week old, male mice were held in groups including BM-transplanted animals. Although maximum regeneration is not obtained after seven days, this point in time was chosen to plan experiments to be as short as possible and as long as needed. Indeed, after seven days a significant improvement in perfusion could be detected. In other set-ups, no difference emerged after seven days¹⁵⁹ or disappeared after longer observation

periods²⁶⁵. In general, the huge interindividual differences have to be considered, which are even bigger in outbred strains like NMRI than in inbred strains like C57BL/J6²⁹².

In addition to perfusion, necrosis was determined as a second outcome parameter. In total, 63.6 % of the mice developed necrosis, although average percentage for the *control* and *MoW* groups was above this (65 % and 77 %), whereas the NO_3^- mice showed lower levels (48 %). Overall, necrosis was common but minor in most cases (necrotic toes but no dead leg), which appeared to be the reason for lacking significance. The mouse strain influences the results, if another mouse strain had been analyzed the results could be different. Taking that into consideration, the results are quite good.

After induction of a severe ischemia in a healthy animal, an improvement of perfusion by 15 % could be achieved by dietary NO_3^- supplementation which more often than not is the decisive factor of necrosis.

Increased perfusion and reduced necrosis leads to the salvage of the leg, which mainly means protection of the skeletal muscle^{299–302}. Although the skeletal muscle is one of the most ischemia resistant tissues, a severe lack of O_2 engenders in necrotic and apoptotic cells, including satellite cells^{244,246,303,304}. These are stem cells, activated by trauma or ischemia, differentiate into myoblasts and hence, are responsible for muscle regeneration^{254,256,305,306}. Mauro et al. discovered the satellite cells in 1961²⁵⁴, but it has only been clear since the 1990's, that really satellite cells are mainly responsible for regeneration, supported by BM-derived stem cells after severe damages^{257,307}. Parallel studies focused on a definite characterization of this heterogeneous population^{255,257,308} and the evidence that satellite cells are real stem cells with the potential to self-renewal^{307–312}.

Due to this, in ischemia it should be a goal to protect the satellite cells. Thus, to get an image of regeneration capacity of the skeletal muscle after dietary NO_3^- supplementation, coming as close as possible to the *in vivo* status, isolation and direct *ex vivo* analysis of satellite cells was established^{313,314}. Necrosis was determined by the presented macroscopic score, thus satellite cells were analyzed for apoptosis. Meanwhile, it is known that satellite cells become necrotic and apoptotic too^{244,246,303,304}. However, it is a matter of debate by which pathway apoptosis is mounted and conducted^{247–249}. Therefore, a TUNEL assay was chosen, detecting all apoptotic cells by fragmented DNA, irrespective of initiating stimulus and underlying pathway.

Concerning the skeletal muscle, the role of myoglobin in dietary NO_3^- mediated regeneration is taken into account. Two recent studies show increased muscle apoptosis in myoglobin over-expressing mice, suggesting a detrimental role for myoglobin, explained by its NO-scavenging function^{315,316}. However, both studies used a transgenic mouse model ($\text{Mb}^{+/+}$) for analysis in comparison to wild-type mice. This strain shows a less dense capillary network³¹⁶, potential trigger for the results. Additionally, transgenic mice are often weaker than their wild-type littermates. Instead of this, a work from our laboratory evaluated a protective role of NO_2^- in I/R injury mediated by a NO_2^- reduction function of myoglobin³². Moreover, this study stated that under hypoxic conditions myoglobin prefers to reduce NO_2^- than to scavenge NO ³². In a parallel study of van Weel et al., it was proposed that VEGF increased myoglobin expression supports regeneration after hind limb ischemia³¹⁷. Indeed, actual results show, that necrosis is reduced by dietary NO_3^- supplementation (figure 23) and further work of our laboratory also show a significant reduction of apoptosis⁴³¹. The analysis demonstrates that necrosis

increases in response to ischemia, evaluated by the increased necrosis in *control* mice. However, in NO_3^- mice necrosis is diminished. Subsequently, this protection is reduced after MoW treatment. Only in the presence of functional myoglobin, which can reduce NO_2^- to NO effectively, does this protective reaction becomes possible. The hypothesis presented included this reaction, which is further increased by a positive feedback, in the case of satellite cell protection. Viable satellite cells account for new muscle fibers, expressing more myoglobin, expanding NO_2^- reduction.

Summarized, investigation of necrosis corroborated the protective effect of dietary NO_3^- on muscle tissue. In addition to the increased perfusion, the results illustrate that the affected tissue is protected.

2.3 Arteriogenesis and Angiogenesis

The mentioned protection of satellite cells and subsequent improved regeneration is probably attributable to two mechanisms. First, bioactivated NO acts as paracrine transmitter directly mediating muscle protection by activation of signal transduction pathways or stimulation of protective acting cells, which will be discussed later. Secondly, the muscle is supported by improved nourishment, accomplished by augmentation of arteriolar and capillary network. Indeed, much works concerning the hind limb model focused on investigation of vascularization under diverse conditions and after several treatments^{81,177,224,300,302,317–321}.

Vascularization includes three different mechanisms of vessel synthesis. In the thigh, origin of the conductance arteries of the leg, occlusion and ischemia increases shear stress inducing arteriogenesis^{293,318}. This process is triggered by shear stress, which leads to an outgrowth of small arterioles to arteries^{143,318}.

In ischemia, as in hind limb model, loss of one major artery is followed by increased shear stress in neighbouring arterioles. To pick up increased blood flow these arterioles grow out to arteries. Thus, it is undisputed that in hind limb model, arteriogenesis is a major mechanism restoring perfusion^{234,293}, nevertheless, results are controversial. Therefore, it is unclear if increasing arteriogenesis is a promising target to improve regeneration. Apart from many studies, which ignore arteriogenesis^{81,261,265,302,317}, there are works showing no difference in arteriogenesis dependent on treatment^{315,321,322}. On the other hand, in many studies positive effects on arteriogenesis could be initiated^{177,224,323}. To explain these differences in results, one has to distinguish between the effects of treatment^{317,322,324}, and the detrimental effects of disease^{234,318} or knock-out genes^{177,315,323}. Studies using knock-out models are always difficult to interpret and to transfer to humans, because effects of a knock-out are often pleiotrop and do not imitate human diseases. Hidden compensatory effects additionally influence results. Such studies give important insights into concerning mechanisms, but can not simply be translated into treatment recommendations. Studies with immune impaired or diabetic animals could also be problematic. Immunodeficient animals do not show physiological reactions, which has to be considered for interpretation³²⁵. Diabetes promotes the development of PAD, as a result many diabetic patients suffer from PAD, but not all PAD patients have diabetes. Both are forms of PAD, but with different origins leading to different treatment strategies. Interpreting results in literature against this background of arteriogenesis seems to be influenced by inflammation²³⁸ and induced by shear stress, which activates eNOS^{177,318}. Furthermore, significant differences could be detected by analyzing different strains and different ages of mice^{295,326}. Finally, it is not the absolute number of arterioles that seems to be the optimal parameter

for analysis rather the size^{168,324,327} and seems not to be a promising target for therapy in otherwise healthy mice^{317,322}. The present work could not show differences in arteriole density (figure 34 and 35). Dietary NO₃⁻ delivers NO independently from eNOS, probably initiating different pathways, which is why no eNOS dependent effects could be detected. Also, eNOS does not seem appropriate for treatment, because this enzyme does not work without O₂, as in ischemia. Moreover, after treatment, no substantial inflammation pre-exists after seven days (figure 36-39), which is needed for considerable arteriogenesis²³⁸. Additionally, the LDPI results suggest a CD1-like vessel architecture, with minor arterioles able to grow-out to arteries²⁹⁵.

In the calf, other mechanisms of vascularization dominate. Here, the capillary network is solidified by angiogenic processes²⁹³. In the adult organism, new capillaries are formed by the sprouting of existing capillary networks (angiogenesis) or de-novo vessel formation (neo-vasculogenesis)¹⁴³. These most analyzed parameters in hind limb ischemia seem to be promising targets for increasing regeneration as they are associated with tissue preservation and reduced necrosis and susceptible by dietary supplementation^{223,261,265,317,320,328,329}. Moreover, many studies suggest an association between NO and angiogenesis^{81,226,234,330-332}. Thus, in the present work it was hypothesized that dietary NO₃⁻ increases angiogenesis, which could be confirmed. Figures 32 and 33 demonstrate a profound increase in CD31 positive endothelial cells. After NO₃⁻ supplementation the amount of EC detected was twice as high as that found in *control* legs were and this angiogenic effect was abolished by antiseptic MoW. To exclude the counting of false positive inflammatory cells, staining was combined with the leukocyte marker CD45 (EC were defined as CD45⁻CD31⁺), and the photographs in figure 30 and the bar graph in figure 31 present the analysis of CD45⁻ cells.

Kevil et al. suggest an impact on ICAM expression in endothelial cells by NO, positively influencing its polarity³³¹, but most analyses stopped at the level of NO, and do not investigate by which mechanisms NO promotes angiogenesis. Murohara et al. wrote that the detailed processes “remained enigmatic”²²⁶, Kumar et al. and Katayama et al. only converse on the “NO-pathway” and the numerous positive effects on EC^{81,330}. The present work tried to close this gap to some degree. Apart from treatment with known angiogenic factors, e. g. VEGF, therapy with stem cells is one of the major research issues in this field. The 1997 discovered EPC show potential to differentiate into EC and to incorporate into sites of ischemia¹³⁸, and research focused on this population. Based on recent work, in this work the population is called Endothelial-regenerating cells¹⁴⁴. In 2005 our laboratory could demonstrate a direct proliferative and mobilizing effect of NO on EPC¹³⁶, and in 2008 a NO-dependent pathway was suggested in the pro-angiogenic, EPC-mobilizing function of pleiotrophin¹³⁷. Recently, the chemotactic response from Endothelial-regenerating cells on chemokines was combined with a chemokinetic response on NO⁸⁰. Taken together, in the current study it was hypothesized that dietary NO₃⁻ supports angiogenesis by indirect mobilisation of Endothelial-regenerating cells.

3. Endothelial-Regenerating Cells are Mobilized by Dietary NO₃⁻

3.1 Endothelial-Regenerating Cells in Plasma and Tissue

Already in 1998, it was postulated that angiogenesis is an eNOS dependent process; impaired in eNOS^{-/-} mice, and blocked by NOS-inhibitors in WT mice^{226,227}. It was concluded that defective eNOS, referred to endothelial dysfunction, is responsible for disturbed angiogenesis in CAD. However, it is a debatable point whether

promoting eNOS (with its substrate L-arginine e. g.) would be an effective treatment, because of the essential cofactor O_2 , which is lacking in ischemia. Aicher et al. analyzed the reduced angiogenesis in eNOS^{-/-} mice from another point of view, discovering that it is due to depressed Endothelial-regenerating cells mobilization¹⁷⁷. Moreover, in 2005 a first relation to CAD patients was found. Increased plasma levels of the endogenous NOS inhibitor ADMA correlated with decreased EPC mobilization, function, and differentiation³³³. Additionally, in diabetes mellitus, which is a known risk factor for CAD, EPC are reduced in a NO-dependent manner^{229,334}. Therefore, attempts were done to strengthen the EPC-NO axis, but independently of eNOS. Asaki et al. treated mice with infrared light daily for five days, which increased angiogenesis and regeneration in hind limb ischemia significantly⁷⁹. However, such therapy seems to be very time-consuming and therefore difficult to transfer to patients. Thum et al. increased NO levels medicinally with organic nitrates in rats and humans²³³. Even treatment with benfotiamine (vitamine B3 derivate) obtained valid results²³⁴. Other approaches include hyperoxia and treatment with angiogenic cytokines (e. g. SDF-1 α)³³⁵. However, all medicinal treatments need high compliance by patients. Thus, the positive impact of moderate intake of red wine on EPC and NO was investigated *in vitro*, and *in vivo* in an animal model and a clinical study (healthy volunteers)^{72,225}. A second study, focusing on nutrition, tried to increase Endothelial-regenerating cells level with Stem-Kine (Aidan Products, Chandler AZ)³³⁶. However, neither alcohol (although in moderate dimensions) nor an expensive food supplement seem to be ideal for sustained support, different from dietary NO_3^- .

To determine the impact of dietary NO_3^- , in the present work CD34⁺KDR⁺ and DilacLDL/UEA positive cells, isolated from MNCs were analyzed as described by Asahara et al.¹³⁸ and redefined as Endothelial-regenerating cells (and CFU-EC) by Prater et al.¹⁴⁴. In figure 25 and 26 representative photographs of the culture and the characterizing staining with Dil-acLDL/UEA are exposed. Following characterization quantification was done with FACS, gating all mononuclear cells (figure 27 B). Bar graph in figure 27 A elucidates a fourfold increase in Endothelial-regenerating cells in blood after NO_3^- supplementation. Subsequently, it should be investigated, whether Endothelial-regenerating cells migration is also improved by dietary NO_3^- . Therefore, BM-transplantation was established and controlled by blood smear (figure 29). Leukocytes of transgene GFP⁺ mice are fluorescence green. Blood smear analysis proves similar fluorescence by GFP⁺-BM transplanted mice. Next, transplanted GFP⁺ cells even could be discovered and characterized as Endothelial-regenerating cells after differentiation in tissue, by its GFP-fluorescence in combination with the endothelial marker CD31 and the exclusion of leukocytes by a negative CD45 staining. Figure 30 and 31 illustrate that dietary NO_3^- supplementation increased the number of Endothelial-regenerating cells in tissue threefold. The mobilization of Endothelial-regenerating cells is increased and the migration into ischemic areas is also increased after NO_3^- supplementation. In conclusion, it could be revealed that dietary NO_3^- increases Endothelial-regenerating cells, plasma and tissue levels, indicating amplified number and function of the cells. Through corroborating literature^{79,80,136,137,226} it could be assumed that NO_3^- is reduced to NO, increasing its bioavailable levels, subsequently mediating mobilization and chemokinesis on Endothelial-regenerating cells. Moreover, Heiss et al. showed that chemokinetic function of NO possibly initiates a positive feedback loop in Endothelial-regenerating cells, which are able to independently produce NO⁸⁰.

Further effects including, increased survival, diminished apoptosis, enhanced dispersal and adhesion direct referable to the NO_3^- -NO- Endothelial-regenerating cell axis could be supposed but still need to be investigated. One cell can not fulfil all these functions by replacing damaged cells. Nonetheless, many of such cytoprotective effects were seen after improving Endothelial-regenerating cells function or number, which was why the paracrine capacity of Endothelial-regenerating cells was analyzed^{171,179,180,337,338}. In ischemia we have different sides of hypoxia, the necrotic core and the border zone. The border zone includes necrotic and apoptotic cells, unsalvageably damaged, but also injured cells, which could be preserved. As a consequence, such tissue need a) a mechanism that destroys the necrotic cells and remove its residues, b) a process able to recognize damaged cells and initiate apoptosis (if it is not initiated by endocellular pathways), and c) a mechanism which supports injured cells to survive and the tissue to regenerate. The following paragraphs will examine this question, discussing paracrine suggestibility and potential of Endothelial-regenerating cells.

3.2 Inflammation

Briefly, inflammation has two important functions, first to recognize and destroy pathogens and secondly to remove remnants of pathogens or dead cells³³⁹. Thus, the occurrence of inflammatory processes was presumed, but two analyses should give an insight in the extent of these processes in hind limb ischemia. To determine inflammation directly in the affected tissue, and to get a quantifiable parameter, immunofluorescent analyses were chosen. iNOS and CD45 positive cells were stained in the lower ischemic leg.

The inducible NOS originally cloned in macrophages, is expressed in various cell types including endothelial cells, smooth muscle cells, and cardiac myocytes^{340,341}. Different from the constitutive expressed eNOS, iNOS expression is induced by special stimuli, initiating inflammation, respectively^{342,343}. Thus, the number of iNOS expressing cells in the ischemic leg should give an insight into the extent of inflammation seven days after induction of ischemia. Additionally, a big iNOS expression should have an impact on the NO level in the tissue, influencing outcome and interpretation of NO_x analyses.

The actual work shows that iNOS expression is low seven days after surgery (figure 36). In healthy limbs iNOS expression is rare but detectable, and multiplied in acute inflammation³⁴⁴. In comparison, cell counts of about thirty cells per millimeter (figure 37) indicate a persisting inflammation but also in abeyance. Additionally, levels remained constant independently from supplementation or treatment, confirming that dietary NO_3^- has no influence on inflammation and no impact on enzymatic iNOS-derived NO. Furthermore, to exclude false positive results in capillary density and Endothelial-regenerating cell migration analyses, slides were also stained with CD45. This control staining was interpreted solely, to use the possibility to determine the number of leukocytes in the injured tissue, by extending iNOS analysis. Despite transplantation and surgery, leukocyte levels were low (after 7 d), presented in figure 38 and 39, corroborating iNOS analysis. Besides, these results are important for interpretation of the results for capillary density (CD31 staining in figure 32 and 33). Levels of CD31 positive cells differ a lot in literature^{222,224} and were rather low in the current work. It could be assumed, that CD31 expression differs based on the inflammatory status of the tissue. The second name of CD31 – PECAM (platelet endothelial cell adhesion molecule) – identifies the marker as adhesion molecule, whom

expression is increased in inflammation to enable adhesion of leukocytes³⁴⁵. Following that, the presented rather low levels of CD31 positive EC refers to a low inflammatory state.

3.3 Angiogenic Cytokines

After analyzing inflammatory processes, responsible for detritus removing, mechanisms for regeneration and survival remained enigmatic. Protective and proliferative effects could be mediated by cytokines, mobilising immune and progenitor cells. Besides, these cytokines could be secreted by Endothelial-regenerating cells itself¹⁷⁹. This implies that increased cytokine levels portend to augmented mobilization of Endothelial-regenerating cells and amplified secretion of cytokines by this cell population.

GM-CSF and G-CSF are stem-cell mobilizing factors regulating hematopoiesis and are thus potential Endothelial-regenerating cell mobilizing molecules³⁴⁶. Indeed, a clinical study showed a protective effect of GM-CSF treatment in AMI³⁴⁷. However, in the present study no increase of GM-CSF and only a trend to increased levels after NO₃⁻ supplementation in G-CSF levels were determined (figure 40 A und B). As the outcome in the present study was determined one week after induction of ischemia, these analyses were done seven days after surgery. Probably, the impact of GM-CSF and G-CSF finds expression at an earlier date of ischemia³⁴⁶ and that is the reason why no association could be detected here and in other studies³²⁷.

VEGF, discovered in the eighties of the last century, is a growth factor mediating angiogenesis^{348–351}. In addition, VEGF attracts Endothelial-regenerating cells to sides of ischemia^{80,146}. After transplantation adenoviruses encoding VEGF-D angiogenesis is improved in diabetic rabbits and increased VEGF-D expression is detectable six days after transplantation³⁵². In the hind limbs of ischemic mice injected with myoblasts expressing VEGF, increased levels of the growth factor were determined three days after surgery³⁵³. In addition, this study focused on the distribution of VEGF in tissue, suggesting an impact of allocation on success of angiogenesis³⁵³. In samples of infarct patients, VEGF could only be detected in muscle biopsies but not in plasma³⁵⁴. Subsequently, VEGF levels were determined in muscle tissue from ischemic hind limbs. Figure 40 C illustrates the measurement. The rather low levels lack a significant increase after NO₃⁻ supplementation, showing only a trend to it. Possibly, VEGF is acting following the stem cell markers GM-CSF and G-CSF, but good data is not available to support this hypothesis. Another explanation could be that, the compartment in which VEGF was measured was sub-optimal for determination differences in VEGF concentration³⁵³.

Finally, SDF-1 α levels were analyzed in plasma. This chemokine induces mobilization of BM-derived progenitor cells³⁵⁵, by reversing its gradient^{356,357}. Normally, SDF-1 α is produced by BM stromal cells in high concentration^{358,359}, retaining BM progenitor cells in their BM niche³⁶⁰. In ischemia this gradient is disrupted and concentration rises in the target tissue attracting BM cells into it^{356,357}. Moreover, animal and clinical studies proposed a protective effect of SDF-1 α in ischemia^{173,361–364}. The important role of SDF-1 α in Endothelial-regenerating cell mobilization became obvious^{365,366}. Indeed, results (figure 40 D) elucidate a significant increase in SDF-1 α plasma levels after dietary NO₃⁻ supplementation. Thus, SDF-1 α results corroborated and amplified the trend seen for G-CSF and VEGF to significance. After MoW treatment, SDF-1 α tended only to decrease back to basal levels, probably, because of taking blood samples. SDF-1 α is a

chemotactic factor, which attracts Endothelial-regenerating cells to sides of ischemia via a positive gradient with a suggested maximum concentration in the ischemic tissue.

However, in the clinical study a drop of SDF-1 α level in STEMI patients, 24 h after infarction was detected (figure 49). How does that match to the data in the animal study? The results of the STEMI patients gave an insight in the complexity of SDF-1 α regulation. As mentioned above, in a healthy state, SDF-1 α adheres Endothelial-regenerating cells in their niche by maintaining a positive gradient with high concentrations in the BM³⁶⁰. Under pathological conditions like ischemia, this gradient is reversed to mobilize Endothelial-regenerating cells to the side of ischemia, with the need of increased plasma levels in comparison to BM, but with maximum in tissue. Apparently, this gradient is build up in CAD patients, and maintained after induction of acute ischemia, and perpetuated beyond the acute phase. It is proposed, that within this period, a narrow timeframe exists, in which the gradient is shifted extremely to the side of ischemia, and this shift leads to decreased plasma levels 24 h after infarction in STEMI patients, compared to admission, discharge (6 d), and seven days after surgery in the hind limb model. In parallel, the animal study compared collectives, not at different points of time, illustrating an impact of dietary NO₃⁻ on SDF-1 α levels. In summary, all these cytokines support Endothelial-regenerating cells and thus angiogenesis, but there remains the question of which process or factor supports the muscle? The muscle needs mechanisms and pathways able to detect apoptotic cells and to promote survival. Recent studies show that the Fas/FasL pathway could perform both^{250, 367–370}. Of course, one pathway communicating with a cell in both directions appears to be ideal to accomplish this demand.

3.4 Endothelial-Regenerating Cells Express FasL

Apoptosis in contrast to necrosis occurs physiologically. During the so called programmed cell death, mutated, senescent or damaged cells were eliminated without producing toxic cell detritus, initiating inflammation³⁷¹. In principle, two different ways to start apoptosis exist. In the intrinsic or mitochondrial pathway the cell itself commits suicide, in contrast to the extrinsic way, which is started by other cells via death receptors³⁷². The Fas/FasL system belongs to the TNF superfamily and therefore to the family of death receptor pathways^{285,369,373,374}. FasL is mostly expressed on T-cells. One of the more important functions of FasL is to eliminate autoreactive T-cells^{369,370}. Otherwise, the ubiquitous expression of Fas presumes multifaceted functions of this pathway^{369,370,375}. Beyond the classical function of inducing apoptosis, non-apoptotic mechanisms regulated by Fas/FasL were discovered³⁷⁶. There are results describing an impact in angiogenesis and inflammation, a supportive role in lympho- and erythropoiesis, and an upregulation of VEGF and MCP-1 in fibroblasts^{250,377–379}. Tumor growth and invasiveness are increased by Fas/FasL^{380–382}. The pathway could act regenerative in the nervous system³⁸³, but increases keratinocyte proliferation in psoriasis³⁸⁴. Endothelial overexpression of FasL reduces the I/R injury³⁸⁵ and the study by Date et al. emphasizes the differential role of membrane-bound (mFasL) and soluble FasL (sFasL) in I/R injury²⁵¹. Besides the research focusing on different functions of membrane-bound and soluble forms of Fas and FasL, other works concentrated on the affected cell. They demonstrated that, the decision of death or survival is contingent on the status of the cell^{386,387}. This result supports the hypothesis that, in ischemia the Fas/FasL pathway could be the pathway of choice to manage apoptosis and regeneration in variably severe damaged cells.

It is known, that FasL is expressed on immunomodulatory cells, but it could be shown that it is also expressed on CD34⁺ cells³⁸⁸ and on EC³⁶⁷. Therefore, the expression of FasL on Endothelial-regenerating cells, which originated from hematopoietic CD34⁺ stem cells, and are able to differentiate into EC, seems probable. Indeed, current work could show for the first time that Endothelial-regenerating cells express FasL. This expression enables the progenitor cells to communicate with Fas-expressing muscle cells and myoblasts²⁴⁹. In detail, the results of the presented work demonstrates that about 40 % Endothelial-regenerating cells of *control* mice express FasL on their surface (figure 41 A). This expression significantly declined after NO₃⁻ supplementation, which could originate from downregulation or shedding. As literature suggests, a protective role for shed sFasL^{251, 367,389} plasma level of sFasL in ischemic mice was analyzed. Figure 41 B shows a significant rise in sFasL in ischemic mice, but only a trend to a further increase after NO₃⁻ supplementation. Although significance is missing between the ischemic groups, the increased plasma levels support the concept of FasL as important mediator of death and survival in ischemia, proposing an association. Accordingly, it is assumed that shedding of FasL is increased after NO₃⁻ supplementation, lowering FasL⁺ Endothelial-regenerating cells count in FACS measurement. Certainly, the low number of Endothelial-regenerating cells in total has to be involved in the interpretation of results. Consequently, three questions remained unresolved. Are Endothelial-regenerating cells able to secrete sufficient amounts of sFasL to be measurable in plasma? Do both subpopulations secrete Endothelial-regenerating cells in the circulation and Endothelial-regenerating cells in the tissue sFasL, or only one of them? Finally, could sFasL have synthesized from such a rare cell population an impact on regeneration?

Endothelial-regenerating cell levels in plasma were 0.1 %, and 3 – 4 cells/mm² could be detected in skeletal muscle (*control* mice) (figure 27 and 30). Many scientists doubt that these low numbers of cells are able to deliver enough cells for successful capillarisation¹⁸⁷, focusing on paracrine functions^{145,284,324,337}. Acting as paracrine, one cell could influence the fate of a multitude of target cells. Subsequently, the current study hypothesizes a great importance on paracrine functions of Endothelial-regenerating cells, which must not preclude the direct support of neo-vasculogenesis by differentiation. Furthermore, it could be suggested that the reduced number of FasL⁺ Endothelial-regenerating cells after NO₃⁻ supplementation is related to the increased plasma level, indicating a secretion of FasL (in contrast to a decreased expression). Whether only one or both of the analyzed Endothelial-regenerating cells populations (in plasma and tissue) secrete FasL is secondary in this context, but should be analyzed in detail in future.

Nevertheless, it has to be mentioned that the presented hypothesis is only one possible way for Endothelial-regenerating cells to act paracrine. Endothelial-regenerating cells secrete various protective and angiogenic cytokines and molecules. A detailed cytokine array revealed a higher secretion of 108 from 174 tested cytokines in EPC, in contrast to human umbilical vein endothelial cells (HUVEC) presenting a great beneficial potential¹⁷⁹. Cited work concentrated on the protection of endothelial cells against oxidative stress. The authors could show that *in vitro* HUVEC treated with EPC-derived conditioned medium are more resistant to oxidative stress than without treatment¹⁷⁹. Two other works were focused on angiogenesis in ischemia. They proposed that the increased secretion and expression of the angiogenic (growth) factors VEGF, HGF, G-CSF, FGF, IGF, Ang-1, and SDF-1 promised a beneficial effect mediated via these humoral proteins^{145,337}.

In conclusion, it could be shown that dietary NO_3^- improves regeneration in the ischemic hind limb by increasing Endothelial-regenerating cells in plasma and tissue. Furthermore, it could be recommended that these cells do not only differentiate into EC build up capillaries, but also mediate regeneration in a paracrine manner. The supplied evidence that Endothelial-regenerating cells express FasL and that this expression is regulated by dietary NO_3^- , could suggest that sFasL is one of the paracrine mediators secreted by Endothelial-regenerating cells.

4. The Impact of XOR on Reduction of NO_3^- in Ischemia

The fact that, in oral flora NO_3^- reducing bacteria exist has been known for a long time^{390, 391}, and thought to be the only option for mammals to reduce NO_3^- . However, recent work supposed a NO_3^- reducing function in the Xanthine oxidoreductase (XOR) representing first a mammalian NO_3^- reductase, described in 2008²³. The current work focused on dietary NO_3^- and its reduction in the oral cavity by bacteria. Therefore, antibacterial MoW was used as negative control, but single results in this group appeared to presume an “incomplete” NO_3^- reduction. One example is the SDF-1 α concentration in figure 40. The significant worsening of the apoptotic rate in myoblasts, does not achieve *control* levels, despite the significant increase compared with NO_3^- mice. Similarly, SDF-1 α concentrations were lowered after MoW, in contrast to the concentrations after NO_3^- supplementation, but failed to reach *control* levels. Several reasons could explain this phenomenon. Above all, antibacterial MoW kills most but not every single bacterium, which is why it is possible that remnants influenced results. This was shown in literature and confirmed here (figure 14)^{281,391}. It is conceivable that not all analyzed parameters are supported by NO_3^- or NO only, but also by other trigger factors. Thus, repression of NO_3^- reduction does not inhibit the complete reaction. An example for redundant pathways is mobilization of Endothelial-regenerating cells, which is regulated by a plethora of factors^{283,392,393}. Finally, the possibility of a second, mammalian NO_3^- reductase exists!

To get the first insights referring the last, hypothetical option, regeneration was analyzed after inhibiting XOR. A new collective of *allopurinol (AP) supplemented* mice was introduced, in which allopurinol depressed the reducing domain of the enzyme^{394, 395}. Just as in the main experiments, NO metabolism was analyzed, determining NO_3^- , NO_2^- , and NO-heme tissue levels. The NO_3^- level (figure 42 A) decreased significantly after AP supplementation. Based on the proposed inhibition of NO_3^- reduction the NO_3^- concentrations had to be higher than in *control* mice. However, in common with the *MoW* group it is presumed that un-reduced NO_3^- is excreted in great quantities and not detectable in plasma or tissue. Figure 42 B shows the difference in NO_2^- levels between the ischemic and the non-ischemic leg. The left bar illustrates that, in *control* mice the NO_2^- levels are much higher in the ischemic than in the non-ischemic leg. In the *AP* group no increase of NO_2^- in the ischemic tissue was detected, possibly indicating a lack of NO_3^- reduction. The same applies for NO-heme level. In the *control* group, NO-heme concentration rose from about non-detectable (non-ischemic) to multiplies (ischemic), but remained constant in *AP* mice. The decreased NO_3^- tissue levels in *AP* animals as in *MoW* mice, and the lack of reduction-products of NO_3^- (NO_2^- and NO-heme) indicate that NO_3^- reduction could be inhibited by AP supplementation, and that XOR reduces NO_3^- in mammalian tissue. Additionally, the complex results of NO metabolites after NO_3^- supplementation and MoW treatment could be partly explained by this second

reductive mechanism (e. g. the lack of significance in decrease in NO_2^- and NO-heme levels in tissue after MoW treatment in figure 20 B and C).

The second experimental XOR-series tried to determine, whether there is a relation between dietary NO_3^- supplementation and XOR function. Thus, we measured mRNA expression of XOR and activity in the ischemic tissue (figure 43). Both, expression and activity were repressed by dietary NO_3^- . It is recommended that, after dietary NO_3^- supplementation there is no need for XOR mediated NO_3^- reduction. The oral flora is activated in such a great manner, that the reductive function of XOR is dispensable. Otherwise in *control* mice, the demand of NO_2^- and NO is not covered by oral NO_3^- reduction, and XOR encourages the delivery of NO.

The results describing regeneration and impact of Endothelial-regenerating cells after NO_3^- supplementation were unambiguous, which is why analysis after AP supplementation would be exciting. It was decided to repeat the three key experiments to show the outcome (LDPI measurement), the impact on Endothelial-regenerating cells by that indirectly on vascularization (Endothelial-regenerating cell quantification by FACS), and dimension of muscle regeneration (apoptosis in myoblasts). Not one of these analyses yielded a significant result. Indeed, data of *control* and *AP* mice were similar. In conclusion, it could be supposed that XOR is able to reduce NO_3^- , but this capacity is such low in contrast to the bacterial one, that it loses importance in severe ischemia. Perhaps, in local ischemic events, e. g. after small vessel lesions by trauma, XOR intervenes positively. Future research should work out this in detail.

5. NO Balance is Disturbed in Ischemic Syndromes

5.1 Impact of Inflammation and Endothelial-Regenerating Cells in AMI

At a cardiologic intensive care unit most patients suffer from the most severe cardio-vascular diseases to cardiogenic shock. In the majority of these patients, the disease began with endothelial dysfunction and atherosclerosis. This impairment of endothelial function is associated with diminished NO bioavailability, although it is unclear, whether decreased bioavailability is the cause or result of the syndrome^{112–114,117,396–399}. Reduced NO bioavailability refers to disturbed eNOS function^{119, 120}, resulting from declined expression^{78,117,400,401} and destroyed enzymatic activity. Oxidative lipids such as oxLDL can uncouple eNOS, depleting its functionality, increasing synthesis and availability of arginase II, and eliminating the substrate for NO production^{117, 402–404}. In turn, these processes potentate the negative effects with the production of reactive oxygen species and the conversion of NO to peroxynitrite by uncoupled eNOS^{117, 402–404}. In addition, the inflammatory response also induced by oxidized lipids, progresses endothelial dysfunction leading to atherosclerosis¹¹⁷.

Atherosclerosis aggravates over decades, with a worsening condition of the arteries. The first build up lesions initiate an inflammatory process in which the fatty streak originates. Lipids, cartilage, thrombi, and several inflammatory cells migrate to the lesion and deposit there affecting the intima of the artery. These atheromas narrow the lumen of the artery until no remodeling can compensate limited perfusion. If an ischemia (in peripheral arteries) becomes chronic, the patient becomes a PAD. Acutely, the nucleus of the atheroma could break up, circulate as thrombus, and occlude an artery. The patient suffers from a myocardial

infarction, a stroke or similar acute event¹¹⁸. In case of a myocardial infarction, perfusion could be restored by blood thinners or by percutaneous intracoronary intervention (PCI). In the latter, perfusion is fast and as complete as possible reconstituted, but a second injury impairs regeneration. The ischemia-reperfusion-injury (I/R-injury) is characterized by the O₂-paradox, the Ca²⁺-paradox, the pH-paradox, increased inflammation, and mitochondrial re-energization. Despite the need of O₂, Ca²⁺, and restoration of pH, the sudden reaction triggers the injury by producing a transient overflow of these factors¹²⁵.

Many studies focused on reduction of this injury, but on the other hand an attempt can be made to prevent or at least to retard the initial acute event. In this regard, NO bioavailability comes to the fore. In an animal model of hypercholesteremia Mathew et al. show that NO bioavailability is decreased. They measured an impaired response to L-NMMA and a diminished eNOS expression in the coronary arteries⁴⁰⁵. The same group could present an association between oxidative stress and NO bioavailability. Basal NO release remained constant between patients with endothelial dysfunction and controls but, in patients a local increase in oxidative stress markers, which influenced NO bioavailability was determined⁴⁰⁶. In addition, a previous study of our group, transferred the results to humans, showing a positive correlation between decreasing NO₂⁻ levels, as marker for NO bioavailability, and decreasing flow-mediated-dilation (FMD), as marker for endothelial function, after inhibiting eNOS activity⁴⁰⁷. Furthermore, a study from 2006 determined a negative correlation between increasing risk factors for endothelial dysfunction and decreasing NO₂⁻ levels in combination with decreasing FMD²⁸². Finally, the presented animal study could also show that NO_x levels are diminished in ischemia and that supplementation with dietary NO₃⁻ could increase regeneration in the hind limb model. Thus, an association between raising NO_x level and regeneration could be assumed.

The present work extends the results mentioned above to severe cardiovascular ill patients. In the first study patients from the intensive care unit were analyzed. The collective was separated into patients who suffered no or small myocardial infarctions and patients who suffered big infarcts by CK-MB level. Figure 45 elucidates a relation between CK-MB and NO₂⁻ levels. Patients with CK-MB concentrations > 3.7 (female)/6.7 (male) µg had significantly less NO₂⁻ circulating than patients with CK-MB levels < 3.7 (female)/6.7 (male) µg. In addition, this study confirmed that NO₂⁻ level also could be reliably determined in whole blood²⁷⁶. Thus, it is suggested that not only NO derived from endothelial NOS is involved in endothelial dysfunction but, also erythrocyte NOS originated NO, was discovered in our laboratory⁵. The second clinical study presented here focused on NO balance in I/R injury. Therefore, STEMI patients were enrolled in the study and blood was examined before and after PCI. The aim was to investigate whether NO₂⁻ levels further decline during STEMI and whether PCI had an additional impact on NO availability²⁷⁷. During the course of the study both NO₃⁻ and NO₂⁻ levels remained constant. This suggests that an impact on I/R injury is precluded, but not on STEMI. Parallel, the data showed that no regeneration of NO homeostasis occurred within one week after infarction, illustrated in figure 47. Moreover, the NO_x levels were obviously overall low. The data collected in 2006²⁸² ascertained NO₂⁻ concentrations of 351±13 nM for healthy persons and 171±29 nM for persons with four risk factors for endothelial dysfunction. Current data show NO₂⁻ levels of 143±29 nM, suggesting a further decrease of NO₂⁻ in acute cardiovascular events. Besides, the relation between NO₂⁻ level and inflammation had to be investigated, because inflammation is a hallmark of I/R injury¹²⁵. It could be shown that suppression of inflammation

increases eNOS function, augmenting protein level and enzyme activity⁴⁰⁸. Furthermore, the eNOS promoter possesses a binding site for the inflammatory mediator IL-6, and with increased TNF- α level mRNA stability of eNOS is decreased⁴⁰⁹. Thus, it could be assumed that there is an association between NO₂⁻ and inflammatory factors. Therefore, the present work included the measurement of TNF- α , IL-6 and CRP from STEMI patients and CAD controls. Furthermore, the analysis of the neutrophil-activating peptide-2 (NAP-2) was added. This inflammatory, platelet-derived chemokine is known to influence angiogenesis and atherosclerosis^{410–412}, but its function in STEMI remains open.

Overall, TNF- α concentrations were increased, but in comparison to CAD patients the levels decrease after STEMI. These results sustain many studies lacking an increase in TNF- α levels and a deleterious effect of the molecule especially in animal models of AMI^{413–416}. However, other studies let suppose a high increase of TNF- α in AMI^{347,417–419}. Three cited reviews describe the problem of conflicting data concerning TNF- α ^{420–422}. In summary, the effects of TNF- α seemed to be pleiotrop. Additionally, TNF- α acts in a temporal and spatial manner. Together, these characteristics could lead to confusing results in individual, differential set-ups. Concerning this, more specific planned animal or *in vitro* studies are needed to explain the possible relation between NO and TNF- α . The present data reveal a chronic, systemic inflammation in CAD mediated by TNF- α , but not by IL-6 and CRP. Despite this, in STEMI this chronic reaction seemed to be replaced by an acute reaction supported by IL-6 and CRP. These two inflammatory mediators were low in CAD patients, but significantly increased in STEMI patients. Time course elucidated the well-matched interplay. Both, IL-6 and CRP rose until day 1 after infarction, but different to IL-6, which decreases subsequently towards admission level, CRP stayed elevated at least one week. These results amend the image of inflammation caused by I/R-injury. Supporting the data, the current study could show that NAP-2 was increased in STEMI in contrast to CAD patients. Immediately after admission the levels peaked, falling down to CAD level in the course of one week. Gained data refined the knowledge of inflammation in I/R injury and expanded it to the new fellow player NAP-2. Presumably, IL-6 and CRP do not acutely influence NO bioavailability. Despite this, chronic alteration of TNF- α and NO₂⁻ in parallel suggested a relation, which should be intensively analyzed in future studies.

During inflammation different leukocytes were mobilized from BM to support the inflammatory process, but also to enhance regeneration. Within this heterogeneous group Asahara et al. discovered a progenitor cell (EPC), which is mobilized under ischemic conditions, involved in vascularization, and differentiates into EC^{138,138,139,139}. With this in mind, EPC seemed to be predestined to mediate regeneration in cardiovascular disease. However, clinical studies demonstrated that with increasing risk factors and endothelial dysfunction EPC number decreases^{160, 165}. Moreover, Werner et al. detected an inverse correlation between increasing EPC number and a lowered risk of death caused by cardiovascular events²⁰². On the other hand, examinations of EPC in unstable angina or AMI detected an obvious elevation of EPC level in correlation with systemic inflammation^{199, 423} (but independent from PCI⁵⁷), reflecting regeneration capacity¹⁶⁶. Our laboratory postulated a relation between renamed EPC (Endothelial-regenerating cells) and NO levels in CAD⁸⁰. Analyzed population should represent Endothelial-regenerating cells, an angiogenic population involved in vasculogenesis and angiogenesis¹⁴⁴.

In the present study we found low Endothelial-regenerating cell levels over the whole observation period, confirming the suggestion that Endothelial-regenerating cells in patients with cardiovascular disease had decreased in number and function¹⁹⁷. However, it is suggested that mobilization occurred, but was not detectable. A study from 2005 shows such an increase from low basal concentrations only has a limited regenerative capacity compared to SA (stable angina) patients and elderly, healthy controls¹⁶⁶.

Endothelial-regenerating cells were attracted to the ischemic tissue by chemokines, where one of the most prominent is SDF-1 α ^{327, 357}, and *in vitro* studies confirmed the migratory effect on stem cells⁴²⁴. SDF-1 α and its receptor CXCR4 are constitutively expressed in several tissues like BM and skeletal/myocardial muscle tissue⁴²². But despite intensive research, data concerning the mobilizing mechanisms is controversial. Both, animal models and clinical studies suggest a mobilization of stem cells by elevation of plasma levels^{64,356,363}. In contrast, another theory proposes that SDF-1 α decreases in BM and increases in the tissue of demand more than in plasma^{173,357,361,362}. Current data supports a second hypothesis, shown in figure 49. In plasma, STEMI patients had decreased SDF-1 α levels compared to CAD patients. Moreover, the presented study revealed a drop of SDF-1 α in a temporally narrowly defined manner, 24 h after infarction. Data presumes that SDF-1 α levels are chronically increased in CAD, because of the current hypoxic stimulus. In the acute event, gradient shifts in favour of the infarct area, diminishing plasma concentrations.

In conclusion, clinical studies presented here show that NO bioavailability is decreased in myocardial infarction. Besides, a relation between this deficiency and inflammation could be suggested. Moreover, the decreased NO bioavailability is associated with impaired Endothelial-regenerating cell mobilization. In future, therapeutic approaches should try to fulfil the NO reservoir to support the complex mechanism of regeneration in ischemia at its origin.

6. Conclusions and Outlook

Aim of the study was to analyze the beneficial potential of dietary NO₃⁻ in severe ischemia, carried by two pillars. First, the importance of NO homeostasis was shown in elucidating NO disturbance in patients suffering from AMI. Second, an animal study evaluated feasibility and efficiency of dietary NO₃⁻ as a nutritional supplement. Additionally, current work gave an insight in the mechanisms by which NO₃⁻ is reduced and acts.

The pleiotropic signaling molecule NO has important functions in diverse organs from immune defense to act as neurotransmitter to the regulation of a plethora of functions in the cardiovascular system^{17,280,425,426}. Subsequently, disturbances in NO homeostasis could lead to an impaired regeneration or a severe progress of disease¹¹⁷, and supplementation aimed to recover homeostasis should be promising. Even though NO is not the only and original cause of disease, NO imbalance is one of the first events in the progress of disease, e. g. in atherosclerosis caused by hyperlipidemia amongst other things. The lipids like oxLDL, uncouple eNOS and increase synthesis of arginase II, destroying functionality of the NO-synthesizing enzyme and its substrate for NO production^{117,402,404}. Therefore, besides a change of life style, regulation of NO metabolism seems to be a promising target for therapy.

However, NO is a gaseous molecule with a short half life. Thus, deviates have to be found, “storing” NO in another chemical form. Its physiological oxidation products, currently endogenous produced by ourselves, were presumed to be ideal after discovering its reactivity^{24,32}. Moreover, the reduction back to NO preferably occurs under ischemic conditions like atherosclerosis, PAD, or AMI^{24,32}. Nevertheless, there are voices and guidelines which caution against toxicity and cancerogen potential of NO₂⁻ and NO₃⁻, albeit with contradictory results^{53,57,279}. Thus, NO₃⁻, which is less toxic and more stable than NO₂⁻, seems to be the most promising candidate. Besides tolerance, the compliance is an important quality of a drug, which is another advantage of NO₃⁻. It is ubiquitous in diet and daily uptake could be easily increased by adapting composition of food.

The present study underscores the dimension of NO imbalance beyond endothelial dysfunction. It is shown that in severe cardiovascular disease NO homeostasis is severely perturbed. Additionally, it is proposed that this imbalance stands in relation with increased inflammation, decreased Endothelial-regenerating cell mobilization, and SDF-1 α concentration. Subsequently, the animal study supplied evidence that supplementation with dietary NO₃⁻ is feasible and able to modify NO metabolism *in vivo*. Moreover, dietary NO₃⁻ significantly improves outcome after severe hind limb ischemia. Perfusion was increased by parallel decreased necrosis, achieved by improved vascularization. The scheme presented in figure 54 summarizes the mechanism of protection by dietary NO₃⁻.

In detail, not only angiogenesis but also vasculogenesis are supported by dietary NO₃⁻, mediated by increased Endothelial-regenerating cell mobilization. Furthermore, it could be assumed that Endothelial-regenerating cells not only migrate into the ischemic tissue and differentiate into EC, but act paracrine protective to skeletal muscle tissue. For the first time it was determined that Endothelial-regenerating cells express FasL and that this expression is influenced by dietary NO₃⁻. As growing evidence, implicit paracrine functions in Endothelial-regenerating cells¹⁷⁹ delivers new support to this hypothesis, suggesting paracrine effects mediated by the Fas/FasL pathway. Recent data propose a proliferative role of FasL, in addition to its function in apoptosis³⁷⁶. Particularly, the shed sFasL is believed to act as an anti-apoptotic^{251,367}. Thus, the decreased FasL expression on Endothelial-regenerating cells after dietary NO₃⁻ supplementation in combination with increased sFasL plasma levels, indicate a paracrine effect on the muscle tissue mediated by sFasL shed by Endothelial-regenerating cells. Subsequently, it is shown here that dietary NO₃⁻ has a protective impact on muscle tissue and thus myoblasts. It is presumed that the decreased necrosis of the tissue and diminished apoptosis⁴³¹ is associated with improved vitality, augmenting regenerative capacity of the muscle. Thus, the current study present dietary NO₃⁻ as a feasible and potent method to combat against ischemia and gave insights into ensuing mechanisms. Further studies should focus on Endothelial-regenerating cells and its functions. Combinations of *in vitro* and *in vivo* studies could analyze the role of FasL expression. Its modulation by NO₃⁻ and its impact on the skeletal muscle, i. e. myoblasts, myocytes, and surrounding cells (e. g. fibroblasts and EC), would elucidate underlying mechanisms and additional beneficial options.

Finally, the completed analyses concerning the produced NO, pave the way for further studies. For the development of therapeutic strategies underlying mechanisms have to be investigated in detail. The current results suggest that XOR mediated NO₃⁻ reduction does not have a great impact on severe ischemia and that endogenous NO₃⁻ production and reduction is not sufficient to neutralize NO imbalance and cover the demand.

Future work should try to close the mechanistic gap between NO_3^- and Endothelial-regenerating cells, analyzing whether how and via which pathways NO acts. In this context analysis of the impact of eNOS, XOR and endogenous NO should be intensified.

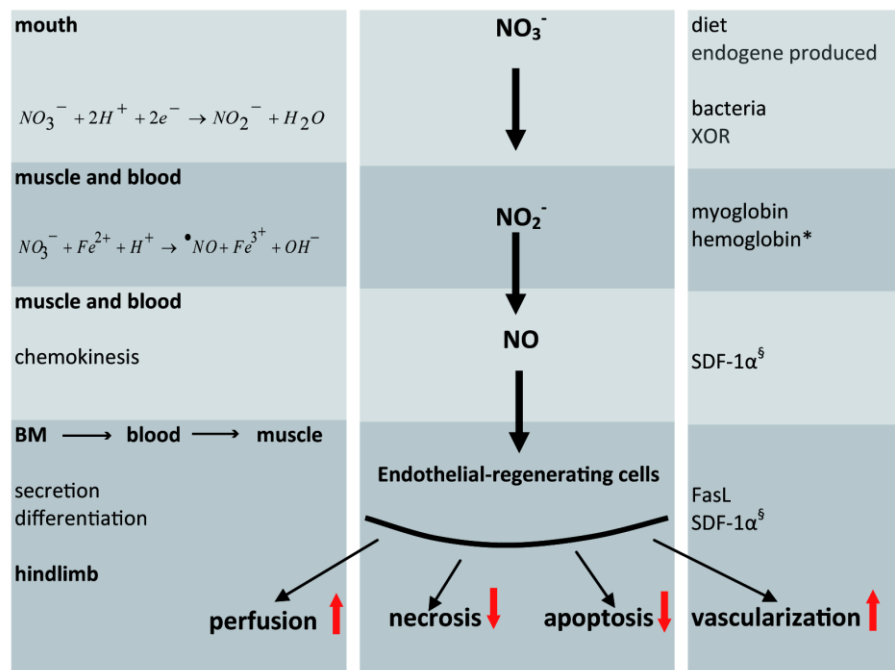


Figure 54. Chemical reactions and involved factors in bioactivation of dietary NO_3^- .

Orally ingested NO_3^- is reduced to NO_2^- in the oral cavity by bacteria. Additionally, endogenous produced NO_3^- is concentrated in saliva and reduced, but not to the same extent as dietary NO_3^- . Reduction could also be performed by XOR in tissue, but is not the major mechanism. Incurred NO_2^- is further reduced to NO by hemoglobin in blood and myoglobin in muscle tissue. Subsequently, NO mobilizes Endothelial-regenerating cells from BM to circulation and tissue, supported by SDF-1α. Finally, the Endothelial-regenerating cells support invigoration of vascularization and protection of muscle tissue. Protective functions of Endothelial-regenerating cells are presumably mediated by FasL. The presented model raises no claim to completeness, but only summarizes the stations analyzed in the presented work. * NO_2^- is also reduced by XOR, protons, ascorbate, and polyphenols. [§]SDF-1α is one of several chemokines influencing mobilization of Endothelial-regenerating cells and secreted of them.

Clinical studies which focus on the relation between NO and Endothelial-regenerating cells in severe ischemia could expand on the results obtained here in a sensible way. Possibly, long-term clinical studies could be commenced on CAD or PAD patients. By increasing their daily NO_3^- intake and observing the hopefully slower progress of disease and prevention of severe consequences (e. g. necrosis, amputation, or AMI), the results gathered here could be transferred to a clinic set-up.

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Kind of contribution	Method	Extent of contribution	Name
Design of the work		Animal study Clinical studies	Tienush Rassaf, Ulrike Hendgen-Cotta, Matthias Totzeck, Peter Lüdi, Martina Kropp Tienush Rassaf, Malte Kelm, Wolfgang Lepper, Eva Kehmeier
Data acquiring	Care and Treatment of the animals	2007, feb - 2010, jan 2010, jan-2010, sep	Martina Kropp Peter Lüdi, Christos Rammos, Andreas Schicho, Dominik Semmler
	Microbiological evaluation of oral flora	Sample preparation (figure 14) Culture and evaluation	Martina Kropp Animal Facility of the University Hospital Düsseldorf
	Hind limb model surgery	2007, feb - 2010, jan 2010, jan - 2010, sep	Martina Kropp Dominik Semmler
	Bone-marrow transplantation	100%	Martina Kropp
	Laser-Doppler-Perfusion-Imaging (LDPI)	2007, feb - 2010, jan Contribution in establishment During surgery and finalization; 2010, jan - 2010, sep	Martina Kropp Michael Niessen Andreas Schicho
	Evaluation of Necrosis Score	2007, feb - 2010, jan 2010, jan - 2010, sep	Martina Kropp Andreas Schicho
	NO ₂ /NO ₃ ⁻ determination via High Pressure Liquid Chromatography combined with diazo coupling	Sample preparation (figure 16-20) Measurement	Martina Kropp Simone Zander, Lisa Frommke
	NO ₂ /NO ₃ ⁻ determination via chemiluminescence Detection (CLD)	100%	Martina Kropp
	RXNO/RNNO determination via chemiluminescence Detection (CLD)	Sample preparation (figure 20) Measurement (figure 20)	Martina Kropp Dominik Semmler, Matthias Totzeck
	NO-Heme determination via chemiluminescence Detection (CLD)	Sample preparation (figure 20) Measurement (figure 20)	Martina Kropp Matthias Totzeck
	cGMP determination via EIA	100%	Martina Kropp
	Isolation of Endothel-Regenerating Cells	90% Figure 28	Martina Kropp Dandan Lou
	Characterization of cultured Endothel-Regenerating Cells by immunofluorescence	Design and supervision Figure 28	Martina Kropp Dandan Lou
	Quantification and characterization of Endothel-Regenerating Cells by flowcytometry (FACS)	100%	Martina Kropp
	Immunofluorescent determination of angiogenesis, neoangiogenesis, arteriogenesis, and iNOS expression Cutting of the slides Staining	20%, design and supervision 80% 100%	Martina Kropp Andreas Schicho Martina Kropp

	Microscopy	100%	Martina Kropp
	Viability analysis of Endothelial-Regenerating Cells by Tetrazolium Salt (WST-8) Assay	100%	Martina Kropp
	Quantification of sFasL by Enzyme-linked-immunosorbent Assay (ELISA)	100%	Martina Kropp
	Isolation of Satellite Cells	50%	Martina Kropp
		50%	Andreas Schicho
	Culture of Satellite Cells	100%	Martina Kropp
	Characterization of Satellite Cells by culture and FACS	100%	Martina Kropp
	Apoptosis analysis of Satellite Cells by Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)	Design, establishment and supervision	Martina Kropp
	Determination of angiogenic and inflammatory cytokines by ELISA		
	Quantification of XOR activity by an enzymatic assay	100%	Martina Kropp
	XOR expression analysis by Real Time PCR	Sample preparation (figure 45 A) Measurement and calculation of results (figure 45 A)	Martina Kropp Ulrike Hendgen-Cotta, Pia Stock
	Clinical Chemistry		Central laboratory University Hospital Aachen
Analysis and interpretation of data			Tienush Rassaf, Ulrike Hendgen-Cotta, Matthias Totzeck, Peter Lüdi, Martina Kropp
Statistical analysis			Tienush Rassaf, Ulrike Hendgen-Cotta, Matthias Totzeck, Peter Lüdi, Martina Kropp
Handling of funding and supervision			Tienush Rassaf
Other	Constructive discussion of study design, results and data interpretation		Tienush Rassaf, Ulrike Hendgen-Cotta, Peter Lüdi, Matthias Totzeck

Curriculum Vitae

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Career

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02/2012 – 09 / 2012	RWTH Aachen, Division of Ophthalmology, University Hospital Aachen (dir.: Prof. Dr. P. Walter) Research assistant
07/2009 – 12/2011	HHU Düsseldorf, Division of Cardiology, Pulmonary Diseases and Vascular Medicine, University Hospital Düsseldorf (dir.: Prof. Dr. M. Kelm) Research assistant (Phd thesis)
02/2007 - 06/2009	RWTH Aachen, Division of Cardiology, Pulmonary Diseases and Vascular Medicine, University Hospital Aachen (dir.: Prof. Dr. M. Kelm) Research assistant (Phd thesis)
01/2007	RWTH Aachen, Division of Pathology (dir.: Prof. Dr. R. Knüchel-Clarke) Practical cell culture course PD Dr. Sabine Neuß-Stein
08/2006	RWTH Aachen, Institute for Biology II (dir.: Prof. Dr. H. Wagner) Diploma thesis in biology
11/2000 - 05/2006	RWTH Aachen, Institute for Biology II (dir.: Prof. Dr. H. Wagner) Behavioural experiments with barn owls Supervision of the practical course „Tierphysiologisches Grundpraktikum“ Supervision of the practical course „Morphologie & Anatomie der Tiere“ Programming of „GUIs“ with „Igor“
10/1998 - 08/2006	RWTH Aachen Study of Biology

Education

1987 – 1996	Lauenburgische Gelehrtenschule Ratzeburg
Graduation	Allgemeine Hochschulreife (equivalent to A-level)

Teaching Experience

since 2009	scientific supervision of Mr. Andreas Schicho (practical part completed)
since 2008	scientific supervision of Mr. Michael Niessen (practical part completed)
2008-2009	scientific supervision & examination of Mrs. M. sc. Dandan Lou (completed)
2000-2006	supervision of several practical courses (see career)

Advanced Education

2010	Perimed Stockholm Practicum including theory & practical use of equipment for LDPI & LDPM
2010	HHU Düsseldorf, Tierversuchsanstalt Fachkundenachweis gemäß §9 des geltenden Tierschutzgesetzes
2010	HHU Düsseldorf, Bildungszentrum Workshop „Projectmanagement for scientific research“
10/2010-04/2012	HHU Düsseldorf, Büro der Gleichstellungsbeauftragten Selma-Meyer-Wiss Mentoring program

Publications

Ulrike B. Hendgen-Cotta, PhD, Peter Lüdike, MD, Matthias Totzeck, MD, **Martina Kropp**, MSc, Andreas Schicho, MS, Pia Stock, MSc, Christos Rammos, MD, Michael Niessen, MS, Christian Heiss, MD, Jon O. Lundberg, MD, PhD, Eddie Weitzberg, MD, PhD, Malte Kelm, MD, FESC, Tienush Rassaf, MD, FESC. Dietary Nitrate Supplementation Improves Revascularization in Chronic Ischemia. *Circulation*. 126(16). 1983-92 (2012)

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Declaration

Hiermit versichere ich, Martina Kropp, dass ich die vorliegende Dissertation eigenhändig verfasst und ausschließlich die angegebenen Quellen und Hilfsmittel verwendet habe.

Genf am 01.05.2013

Martina Kropp