Dietary Nitrate Supplementation Improves Revascularization in Chronic Ischemia

Running title: Hendgen-Cotta et al.; Dietary nitrate improves revascularization

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Journal Subject Code: [95] Endothelium/vascular type/nitric oxide
Abstract:

**Background** - Revascularization is an adaptive repair mechanism that restores blood flow to undersupplied ischemic tissue. Nitric oxide (NO) plays an important role in this process. Whether dietary nitrate, serially reduced to nitrite by commensal bacteria in the oral cavity and subsequently to NO and other nitrogen oxides, enhances ischemia-induced remodeling of the vascular network is not known.

**Methods and Results** - Mice were treated with either nitrate (1 g/L sodium nitrate in drinking water) or sodium chloride (control) for 14 days. At day 7, unilateral hindlimb surgery with excision of the left femoral artery was conducted. Blood flow was determined by laser Doppler. Capillary density, myoblast apoptosis, mobilization of CD31+/CD45-, migration of bone-marrow derived CD31+/CD45+, plasma S-nitrosothiols, nitrite, and skeletal tissue cyclic guanosine monophosphate (cGMP) levels were assessed. EGFP (enhanced green fluorescence protein) transgenic mice were used for bone marrow transplantation. Dietary nitrate increased plasma S-nitrosothiols and nitrite, enhanced revascularization, increased mobilization of CD34+/Flk-1+ and migration of bone-marrow derived CD31+/CD45- cells to the site of ischemia, and attenuated apoptosis of potentially regenerative myoblasts in chronically ischemic tissue. The regenerative effects of nitrate treatment were abolished by eradicating nitrate-reducing bacteria in the oral cavity through an antiseptic mouthwash.

**Conclusions** - Chronic dietary nitrate supplementation may represent a novel nutrition-based strategy to enhance ischemia-induced revascularization.

**Key words:** nitric oxide; revascularization; inorganic nitrate; nitrite
Revascularization is a process aimed at maintaining and restoring tissue viability during chronic ischemia. The underlying signaling mechanisms are complex and include cytokines, chemokines, proteinases, as well as cell adhesion molecules. Members of the vascular endothelial growth factor (VEGF) family and the stromal cell derived factor 1 alpha (SDF-1α) have been described to play a central role in initiating angiogenesis, vasculogenesis, and arteriogenesis.

Nitric oxide (NO) derived from endogenous enzymatic production via endothelial NO synthase (eNOS) is a well-known pro-angiogenic molecule that regulates both VEGF signaling and the recruitment of bone marrow-derived endothelial progenitor cells (EPCs). Disruption of eNOS impairs the release of EPCs into the circulation and their migration to the site of injury, thus attenuating vascular regenerative processes.

Inorganic nitrate from dietary sources is converted in vivo to nitrite and then NO, and other bioactive nitrogen oxides. Nitrate is found in considerable amounts in our everyday diet, and leafy green vegetables such as spinach, lettuce, or beetroot have particularly high concentrations. In the oral cavity, commensal nitrate-reducing bacteria effectively reduce nitrate to nitrite which is swallowed with approximately 11 saliva per day and thus continuously enters the circulation. The relevance of exogenous supplementation with dietary nitrate for cardiovascular functions has been recently shown. After a 3-day nitrate-enriched diet, diastolic blood pressure was reduced significantly in healthy non-hypertensive volunteers. A commercially available antibacterial mouthwash administered daily for 1 week to rats supplemented with nitrate via drinking water has been shown to suppress the oral microflora to such an extent that the conversion of nitrate to nitrite in the oral cavity was strongly reduced. Consequently, the blood pressure lowering and gastroprotective effects of nitrate were abolished. In contrast to pharmacological application of the anion nitrite, dietary nitrate is
nontoxic even in higher doses whereas nitrite can cause serious harm already at considerably lower levels.

Based on the recently described NO-like effects of dietary nitrate, we evaluated whether chronic dietary nitrate supplementation matched to the effect of a human diet rich in vegetables would improve ischemia-induced revascularization and tissue regeneration using a mouse hindlimb ischemia model.

Methods

Chemicals

All reagents were obtained from Sigma-Aldrich (Taufkirchen, Germany) unless indicated otherwise.

Animals

Male NMRI (Naval Medical Research Institute) mice 14±3 weeks with an average body weight of 30±6 g were obtained from the local animal house. C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb mice were kindly provided by Prof. Dr. Masaru Okabe (Osaka University, Osaka, Japan). All experiments were approved by the local ethics committee in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe Treaty Series No. 123).

Sodium nitrate supplementation and antiseptic mouthwash

Sodium nitrate was added to the drinking water for 14 days at a concentration of 1 g/l (~150 μmoles according to the measured daily drinking water consumption). This is equivalent to a rich vegetable intake in human. An equal concentration of sodium chloride served as control. In order to suppress the resident microflora in the oral cavity of the animals, subgroups were treated twice daily for 14 days with a commercial antibacterial mouthwash solution (0.2% w/v
hexetidine/water, Pfizer, Berlin, Germany). The control group received a mouthwash with a water solution only. All mice were weighed after sodium nitrate intake for 7 days. Water and food consumption was recorded daily. Animals underwent surgical excision of the left femoral artery at day 7. At day 14 mice were subjected to imaging, blood sampling, and histological analyses.

**Hindlimb ischemia model**

Under complete anesthesia following ketamine (45 mg/kg) and xylazine (10 mg/kg) injections, chronic ischemia was generated in compliance with well-established unilateral hindlimb surgery protocols. In brief, after a 1-cm skin incision at the medial thigh the femoral artery was separated from the femoral vein and nerve. The part proximal to the outlet of the profunda femoris artery and the distal end (outlet of the saphenous artery) were ligated using 7-0 silk sutures (Serag-Wiessner, Naila, Germany). The femoral artery was then excised between the ligations. Wound closures were conducted with single layer sutures using 4-0 prolene threads. Immediately after surgery, animals received a subcutaneous injection of buprenorphine (0.05 mg/kg). Postoperative analgesia was maintained with buprenorphine (0.05 mg/kg) every 12 hours for 2 days and mice were closely monitored for any signs of distress.

**Assessment of perfusion**

Perfusion was assessed by laser Doppler perfusion imaging (LDPI; Perimed, Stockholm, Sweden) immediately after surgery and for the following 7 days. Blood flow was measured as changes in the laser frequency, represented by different color pixels. The mean hindlimb perfusion was calculated as the ratio of ischemic to non-ischemic side by a blinded observer.

**Capillary density measurement**

Paraffin-embedded serial sections (6 μm) obtained from skeletal muscles of the ischemic and
non-ischemic (control) hindlimb were used for immunostaining. To define capillaries on day 7 after surgery, a primary antibody against CD31 (platelet/endothelial cell adhesion molecule-1 [PECAM-1]; Santa Cruz, Santa Cruz, CA, USA) was used to detect endothelial cells, followed by a rabbit anti-goat Alexa 647 secondary antibody. Double-staining with CD45 antibodies was performed to unmask leucocytes. Tissues were mounted by using Vectashield DAPI (4,6-diamidine-2-phenylindole dihydrochloride) nuclear counterstain. Only CD31+ and CD45- cells were calculated per mm² hindlimb section.

**Determination of myoblast apoptosis**

7 days after chronic hindlimb ischemia, the hindlimbs were removed from mice and the bones were dissected. The muscle was then placed in phosphate-buffered saline (PBS) to keep it moist. Subsequently the muscle was minced and the cells were digested with Pronase in PBS (10 mg/ml) with 1 ml Hepes Puffer (25 mM) and Dulbecco's Modified Eagle Medium (PromoCell, Heidelberg, Germany). Myoblasts were cultured in HAM’s F-10 medium (PromoCell, Heidelberg, Germany) supplemented with 20% FCS, 10% equine serum, 0.2% penicillin/streptomycin on collagen-coated dishes. After incubation and sufficient fibroblasts-adhesion myoblasts were carefully extracted. To verify the myoblast population, fluorescence activated cell sorting (FACS) was performed on an FACS sorter (FACS Canto, BD, Franklin Lakes, New Jersey, USA) using the integrin-α7 antibody (Antikörper-Online G, Aachen, Germany) for sorting. Cells exhibiting DNA fragmentation were identified by terminal transferase dUTP nick end labeling (TUNEL) assay according to the manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany). Cells were visualized using a Nikon fluorescence microscope (Nikon Instruments Europe B.V. Germany, Düsseldorf, Germany) and TUNEL-positive (green) as well as total nuclei (blue) were counted in three
separate fields. Apoptotic susceptibility was presented as the percentage of TUNEL-positive nuclei per total counted nuclei.

**Determination of peripheral blood mononuclear cells**

Blood samples were collected 7 days after hindlimb surgery. Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation. To determine the isolated blood cells, we performed FACS analyses using FITC rat anti-mouse CD 34 and APC rat anti-mouse Flk-1 antibodies (BD, Heidelberg, Germany).

**Bone marrow transplantation**

To assess the migration of bone marrow-derived endothelial-regenerating cells into ischemic tissue, NMRI (Naval Medical Research Institute) and EGFP (enhanced green fluorescence protein) transgenic mice were used for bone marrow transplantation. Bone marrow cells were obtained from the tibias and femurs of male EGFP+ mice. A suspension of single cells was created and prepared for transplantation. To generate chimeric mice, 4-6 x 10⁶ bone marrow cells were injected into the heart of the WT recipients whose bone marrow had been lethally irradiated with 6.5 Gy (2 x 10 min). 4 weeks later, WT mice were subjected to nitrate supplementation for 7 days and chronic ligation of the femoral artery.

**Determination of bone marrow-derived cells.**

7 days after chronic ligation of the femoral artery, paraffin-embedded serial sections (6 μm) obtained from skeletal muscles were stained with rat anti-mouse CD31 antibody, followed by a rabbit anti-goat Alexa 647 secondary antibody. Double-staining with CD45 antibodies were performed to unmask inflammatory cells. Nuclei were counterstained with DAPI. EGFP+/CD31+/CD45− cells were calculated per mm² hindlimb section.

**Biochemical Analysis**
Plasma nitrate, nitrite and S-nitrosothiol levels were measured using HPLC (ENO20, Eicom, Dublin, Ireland) and chemiluminescence\textsuperscript{18}, respectively, and skeletal tissue cGMP level was determined using the BiotrakTM cGMP competitive enzyme immunoassay system (GE Healthcare, Munich, Germany) using the manufacturers protocol. Tissue cGMP levels were expressed per mg wet tissue.\textsuperscript{19}

**Statistical Analysis**

The results are presented as mean±SEM unless stated otherwise. Data were analyzed by one-way ANOVA and post-hoc Bonferroni’s multiple comparison tests and Kruskal-Wallis with Mann-Whitney-Test respectively using GraphPad Prism 5 and IBM SPSS Version 20 software to compare differences between multiple groups. Student’s unpaired t-test was used when analyzing two groups. A value of $P<0.05$ was considered to be statistically significant.

**Results**

**Dietary nitrate improves perfusion recovery in chronic hindlimb ischemia**

To determine whether dietary nitrate supplementation augments recovery of tissue perfusion, we subjected mice to femoral artery ligation and the effects on perfusion recovery were assayed by LDPI immediately after surgery and the following 7 days (Figures 1A and 1B). Perfusion recovery in the ischemic hindlimb was significantly improved in mice treated with nitrate compared to controls (73±4% vs. 59±4%, $P<0.02$) (Figures 1B and 1C). Eradication of the nitrate-reducing bacteria in the oral cavity through antiseptic mouthwash twice daily (Supplemental Figure 1) did not show the nitrate-mediated positive effect on perfusion (55±3%; $P<0.02$) (Figures 1B and 1C).

**Dietary nitrate supplementation augments S-nitrosothiol, nitrite and nitrate levels in plasma without affecting feeding behavior**
Intake of nitrate by drinking water (~150 μmoles/day) over 7 days showed an increase in plasma S-nitrosothiol, nitrite and nitrate levels as compared to controls (S-nitrosothiol: 349±98 nM vs. 9±1.9 nM; nitrite: 5.8±1.7 μM vs. 0.5±0.1 μM; nitrate: 451±75 μM vs. 40±17 μM) (Figures 2A and 2B). The nitrite and S-nitrosothiol concentrations were markedly lower in mice which underwent an antibacterial mouthwash (Figure 2B). We also observed lower amounts in plasma nitrate in mouthwash-treated animals. This could theoretically be due to a decrease in water intake (the nitrate source) in mouthwash treated animals. However, antibacterial mouthwash procedures or the different nitrate content of drinking water had no influence on water and food consumption or body weight (Figures 3A-3D).

**Dietary nitrate increases revascularization after chronic ligation of the femoral artery**

To investigate whether the improvement in tissue perfusion was mediated in part by increased revascularization, capillary density was determined 7 days after hindlimb surgery (Figure 4A). In the nitrate-treated group, the capillary density was higher compared to controls (123±20 cells/mm² vs. 59±15 cells/mm²; *P*=0.045) (Figures 4A and 4B) and mice receiving nitrate and antibacterial mouthwash (57±7 cells/mm²; *P*<0.02) (Figures 4A and 4B) as evident by CD31⁺ staining in skeletal muscle tissue.

**Dietary nitrate attenuates apoptosis in myoblasts in chronic hindlimb ischemia**

To evaluate the remaining regenerative capacity of tissue in chronic ischemia, hindlimbs were removed from mice on day 7 after surgical ligation of the femoral artery, myoblasts were isolated, cultured and underwent TUNEL staining for detection of apoptotic myoblasts (Figures 5A and 5B). The amount of apoptotic nuclei was lower in the nitrate-treated group compared to controls (67±16 vs. 197±19 cells/mm²; *P*<0.001) (Figures 5B and 5C). Blocking of oral nitrate reduction by antibacterial mouthwash showed no beneficial effects of nitrate (169±26 vs. 67±16
Dietary nitrate increases the mobilization and migration of endothelial-regenerating cells

Quantitative FACS analysis of CD34+/Flk-1+ cells in peripheral blood revealed that mobilization of this population was dramatically higher in mice receiving nitrate enriched drinking water compared to the control group ($P<0.001$) (Figures 6A-6C). Again, no nitrate-mediated effects were seen in the group that received antibacterial mouthwash ($P<0.01$) (Figure 6C). After demonstrating the mobilizing effect on CD34+/Flk-1+ cells with dietary nitrate supplementation, the migration of this mobilized population into ischemic tissue was investigated using NMRI and EGFP transgenic mice for bone marrow transplantation. The NMRI recipients underwent lethal irradiation and subsequent EGFP+ bone marrow cells transplantation to generate marked hematopoietic chimeras. After 4 weeks of recovery, these animals obtained nitrate supplementation and control treatment respectively before chronic ligation of the femoral artery was performed (Figure 6A). To assess migration of bone marrow–derived cells into the ischemic tissue, we investigated the localization of EGFP+/CD31+/CD45− cells in paraffin sections of the hindlimbs. Blinded analysis of at least 5 visual fields per section revealed that animals treated with nitrate showed higher amounts of EGFP+/CD31+/CD45− cells at the site of active revascularization compared to animals with low nitrate diet ($P=0.029$) (Figures 6D and 6E). No effects on migration of bone-marrow derived EGFP+/CD31+/CD45− cells were seen with nitrate when using antiseptic mouthwash ($P=0.034$) (Figures 6D and 6E).

Dietary nitrate does not alter tissue cGMP levels

Quantification of cGMP levels by competitive enzyme immunoassay in excised skeletal muscle tissue revealed no impact of nitrate supplementation (4±5 fmol/mg tissue) or mouthwash procedure (9±7 fmol/mg tissue). This is consistent with recent findings investigating the role of
nitrite-injections in ischemia,\textsuperscript{16} indicating that other nitrite-signaling pathways for regulating angiogenic activity may exist.\textsuperscript{20} However, it does not exclude the existence of cGMP-mediated effects since counter regulatory mechanisms may disguise NO-mediated changes in this second messenger.

**Discussion**

Chronically ischemic tissue, a feature of peripheral and coronary artery disease, requires a remodeling of the vascular network to reconstitute and sustain its viability. The physiological repair response, however, is often not sufficient and therapeutic angiogenesis remains an unmet medical need. In the current study, we demonstrate that dietary nitrate supplementation improves revascularization through mechanisms involving the mobilization and migration of endothelial-regenerating cells. This is accompanied by an attenuation of myoblasts apoptosis.

Despite endeavors to promote blood vessel formation by administration of proangiogenic factors, gene therapy, or targeting of microRNAs, clinically applicable strategies have not been developed yet or are still in a preclinical phase.\textsuperscript{21-24} Although proangiogenic properties of NO have been demonstrated, direct application of NO or classical NO donors \textit{in vivo} bear a high risk of failure due to lack of targeted delivery, development of tolerance, cellular toxicity and risk of hypotension.\textsuperscript{25-27} Pharmacological treatment with nitrite, an oxidation product of NO, may offer an alternative therapeutic opportunity. We have recently shown that the administration of nitrite under hypoxia regulates cardiac energetics and functions resembling the characteristics described for acute hibernation.\textsuperscript{28} A cytoprotective role of nitrite in the setting of myocardial, liver, kidney, and brain ischemia-reperfusion (I/R) injury has previously been demonstrated.\textsuperscript{19,29,30} In ischemic angiogenesis a continuous pharmacological intervention with nitrite injections resulted in an increased vascular density in the hindlimb as well as stimulated endothelial cell proliferation.\textsuperscript{16}
Nitrite has several properties that make it an attractive drug candidate. Its conversion into bioactive nitrogen oxides is slow,\textsuperscript{31} and the effect is not subjective to tolerance.\textsuperscript{32} Therefore, one can deliver it even in fairly high doses without the risk of classical NO-related side effects such as hypotension. Also, first passage metabolism in the liver is minimal compared to classical organic nitrates. Moreover, its bioactivation is augmented in ischemic areas thereby making the drug “selective” to ischemic areas.

Dietary nitrate is an effective way of delivering nitrite systemically and having a half-life of 6h, it continuously generates nitrite and bioactive nitrogen oxides like S-nitrosothiols. Recent research in animals as well as in humans has indeed confirmed beneficial effects of dietary nitrate\textsuperscript{14,33-38}. Intake of nitrate-rich foods such as leafy vegetables and fruits, abundant in the mediterranean diet, may represent an opportunity for disease prevention and health modulation of human physiological functions. The NO-like effects of dietary nitrate range from lowering of blood pressure\textsuperscript{14,38} to a reduction in experimental myocardial infarct size,\textsuperscript{33} inhibition of platelet aggregation,\textsuperscript{34} increasing exercise tolerance in peripheral arterial disease\textsuperscript{35} and improvement in intrinsic mitochondrial efficiency.\textsuperscript{36} In addition, nitrate supplementation offered cardioprotection against doxorubicin-induced cardiomyopathy,\textsuperscript{37} and prevented oxidative stress, cardiovascular and renal injury in salt-induced hypertension.\textsuperscript{39}

These findings from experimental animal studies as well as human clinical studies support the hypothesis that nitrate of food origin promotes cardiovascular health and thus presents a novel mechanistic explanation for some of the well-known health benefits of a diet rich in vegetables.

We here demonstrate that dietary nitrate supplementation strongly augments perfusion recovery in chronic hindlimb ischemia \textit{in vivo} via a significant increase in capillary density. This
improvement was associated with an increase in circulating nitrite and S-nitrosothiol concentrations, an elevated mobilization of CD34+/Flk-1+ cells and migration of bone marrow-derived CD31+/CD45− cells into ischemic tissue. The associative relation - increased plasma nitrite levels and an increased mobilization of CD34+/Flk-1+ cells from the bone marrow – has also been observed in patients with coronary artery disease receiving nutrients that increased NO bioavailability.40

In line with these findings, the results of the current study further point to a distinct contribution of dietary nitrate supplementation on tissue viability. Dietary nitrate ameliorates the remarkable capacity of adult skeletal muscles to regenerate myofibers after damage. This rapid repair process is mainly carried out by satellite cells (SCs) with contribution of NO.41 Quiescent SCs become active and proliferate upon injury and display the regenerative capacity of the muscle. Committed daughter cells, the myoblasts, continue to proliferate followed by definite differentiation as initialized by a coordinated cellular signaling.

Intriguingly, the disruption of the nitrate-NO pathway by chronic eradication of the oral microflora completely abolished the beneficial effects of the dietary nitrate supplementation. This daily intervention effectively suppressed the increase of circulating nitrite and S-nitrosothiol levels, which are observed after intake of nitrate-rich food or dietary nitrate supplementation in drinking water.15,38,42 The fact that mouthwash prevented increases in plasma nitrite and all observed beneficial effects of nitrate provides significant mechanistic insight since it suggests an important role of intermediate nitrite formation in bioactivation of nitrate. However, one cannot entirely rule out the possibility that oral bacteria could directly catalyze nitrosation reactions from nitrate without intermediate nitrite formation, since recent data show that protein S-nitros(yl)ation is an obligate concomitant of anaerobic respiration on nitrate in Escherichia coli.43
It is important to note that the exact identity of the final mediator(s) of the nitrate effects is still not settled and multiple possible mechanisms exist. Nitrite as a reservoir can be further reduced to NO via numerous pathways in blood and tissue and this process is indeed accelerated under hypoxic conditions.\textsuperscript{44-47} In addition, nitrite forms other reactive nitrogen oxides including S-nitrosothiols and nitrogen oxide, the latter promoting formation of bioactive nitration products including nitro fatty acids.\textsuperscript{11,48,49} This latter oxidative chemistry is emerging as an alternative nitrite-mediated signaling mechanism\textsuperscript{33,50} which could explain some of the NO-like effects of nitrate and nitrite seen under normoxic conditions. The current data suggest that S-nitros(yl)ation might be a crucial signal transduction pathway and that the formation of S-nitrosothiols might account for the regenerative response to the dietary nitrate supplementation in chronic ischemia. This is supported by the marked increase in circulating S-nitrosothiols observed here after nitrate supplementation.

In summary, we here show that dietary nitrate supplementation increases the regenerative capacity of ischemic tissue and that this effect critically depends upon bacteria-dependent bioactivation of nitrate in the oral cavity. These results suggest that dietary nitrate supplementation may offer an attractive nutrition-based strategy to improve ischemia-induced revascularization.

**Funding Sources:** This study was supported in part by grants from the Deutsche Forschungsgemeinschaft (DFG) (Ra969/6-1 to Prof. Dr. Rassaf and Ke405/5-1 to Prof. Dr. Kelm). Dr. Rassaf is a Heisenberg professor funded by the DFG (Ra969/7-1). Dr. Totzeck received a scholarship from the Deutsche Herzstiftung. Dr. Lüdike is a stipend of the German Cardiac Society.

**Conflict of Interest Disclosures:** None.
References:


**Figure Legends:**

**Figure 1.** Effect of Dietary Nitrate Supplementation on Perfusion Recovery. (A) Experimental protocol. (B) Original laser Doppler perfusion images (LDPI) display hindlimb perfusion before and 7 days after excision of the femoral artery. Mice receiving nitrate (middle row) show increased perfusion compared to control animals (left row). Antiseptic mouthwash (MW) did not exhibit the nitrate-induced gain of perfusion (right row). (C) Nitrate-supplemented mice showed increased perfusion recovery in chronic hindlimb ischemia (black bars, *P*<0.02) and this effect was not observed in mice receiving antiseptic MW (grey bars, *P*<0.02). Data are expressed as mean±SEM (n=21-23).

**Figure 2.** Effect of Dietary Nitrate Supplementation on S-Nitrosothiol, Nitrite and Nitrate Levels in Plasma. (A) Assessment of circulating S-nitrosothiol (RSNO), nitrite (NO$_2^-$) and nitrate (NO$_3^-$) levels (B) Circulating S-nitrosothiol, nitrite and nitrate levels increase following nitrate supplementation during a period of 7 days. Mice receiving antiseptic mouthwash (MW) displayed lower S-nitrosothiol and nitrite levels in plasma. Data are expressed as mean±SEM (n=6-8).
Figure 3. Effect of Dietary Nitrate Supplementation on Body Weight and Feeding Behavior. (A) Experimental protocol. Daily assessment of water, food consumption and body weight. (B–D) Dietary nitrate supplementation and daily mouthwash procedure had no effect on water and food intake and body weight development. Data are expressed as mean±SEM (n=4-5).

Figure 4. Effect of Dietary Nitrate on Revascularization. (A) Experimental protocol. (B) Representative original micrographs of hindlimb sections show amounts of endothelial cells per mm². Dietary nitrate-treated mice show higher numbers of CD31⁺/CD45⁻ cells 7 days after hindlimb surgery whereas the mice receiving mouthwash procedure (MW) did not exhibit this effect. Upper row micrographs show CD31⁺/CD45⁻ stained cells (red), middle row show DAPI stained nuclei (blue). The lowest row shows double stained (merged) cells, indicating endothelial cells. (C) Quantitative analysis of capillary density displayed as CD31⁺/CD45⁻ stained cells per mm² hindlimb. Bars show higher capillary density in dietary nitrate supplemented mice at day 7 (black bars, P=0.045). Treatment with an antiseptic mouthwash did not exhibit the nitrate-mediated increase in capillary density (grey bars, P<0.02). Data are expressed as mean ± SEM (n=6).

Figure 5. Effect of Dietary Nitrate on Apoptosis in Myoblasts. (A) Experimental protocol. (B) TUNEL staining of isolated myoblasts demonstrating lower amounts of apoptotic cells in mice receiving dietary nitrate supplementation (middle column) compared to control treatment (left column) or antiseptic mouthwash (MW, right column). Upper row micrographs show TUNEL positive nuclei indicating apoptotic cells (green), middle row micrographs show DAPI positive cells indicating nuclei (blue). The lowest row shows double stained cells (merged) indicating
apoptotic cells. (C) Quantitative analysis of TUNEL stained myoblasts per hindlimb, displayed as ratio between ischemic and non-ischemic hindlimb and expressed as %. Bars show significantly decreased apoptosis level in mice supplemented with dietary nitrate (black bar). Mice without dietary nitrate supplementation (white bar, \(P<0.001\)) or with antiseptic mouthwash (MW) (grey bar, \(P<0.02\)) did not show nitrate mediated regenerative effects. Data are expressed as mean \(\pm\) SEM (n=9-11).

**Figure 6. Impact of Dietary Nitrate on Mobilization and Migration of Endothelial-Regenerating Cells. (A) Experimental protocol. (B)** Exemplary flow cytometric analysis shows the total mononuclear cells in peripheral blood analyzed at day 7 after onset of hindlimb ischemia. The gated population displays isolated blood cells, stained with FITC rat anti-mouse CD 34 and APC rat anti-mouse Flk-1. **(B and C)** The amount of CD34\(^+\)/Flk-1\(^+\) cells was higher in the nitrate-treated group compared to standard drinking water (\(P<0.001\)). Antiseptic mouthwash (MW) did not show this effect and distinctly prevented accumulation of CD34\(^+\)/Flk-1\(^+\) cells despite nitrate supplementation (\(P<0.01, \text{n}=7-10\)). **(D)** Representative original micrographs of hindlimb sections show amounts of EGFP\(^+\)/CD31\(^+\)/CD45\(^-\) cells per mm\(^2\). Mice receiving dietary nitrate showed an increased migration of EGFP\(^+\)/CD31\(^+\)/CD45\(^-\) cells 7 days after hindlimb surgery (middle column) compared to mice getting the mouthwash procedure (right column). **(E)** Bars show significantly increased EGFP\(^+\)/CD31\(^+\)/CD45\(^-\) cells migration in mice supplemented with dietary nitrate at day 7 (black bars, \(P=0.029\)), whereas antiseptic mouthwash displayed markedly attenuated nitrate-mediated increase in EGFP\(^+\)/CD31\(^+\)/CD45\(^-\) cells migration in chronic hindlimb ischemia (grey bars, \(P=0.034, \text{n}=3-4\)). Data are expressed as mean\(\pm\)SEM.
A

-7
Control
Nitrate supplementation
Nitrate supplementation + Mouthwash (MW)

Day 0
Hindlimb surgery
LDPI
+7

B

Day 0

Day 7

C

Ratio ischemic/non ischemic hind-limb

Ischemia
Nitrate
MW

Perfusion x 100 (%)

+ + +
- + +
- - +

** ***

+ + +
- + +
- - +
A Day -7 -3 0

Control Nitrate supplementation Nitrate supplementation + Mouthwash (MW)

Blood sample Blood sample Blood sample

B Control

\[
\begin{align*}
\text{NO}_3^- & : 1500 \\
\text{NO}_2^- & : 1000 \\
\text{RSNO} & : 400
\end{align*}
\]

Nitrate

\[
\begin{align*}
\text{NO}_3^- & : 1500 \\
\text{NO}_2^- & : 1000 \\
\text{RSNO} & : 400
\end{align*}
\]

Nitrate+MW

\[
\begin{align*}
\text{NO}_3^- & : 1500 \\
\text{NO}_2^- & : 1000 \\
\text{RSNO} & : 400
\end{align*}
\]
A

-7

Control
Nitrate supplementation
Nitrate supplementation + Mouthwash (MW)

Day

Daily Body weight
Water consumption
Food consumption

B

Water per day (mL/g body weight)

Ischemia
Nitrate
MW

+ + + 
- + + 
- - +

D

Food per day (g/g body weight)

Ischemia
Nitrate
MW

+ + +
- + +
- - +

Body weight (g)

Ischemia
Nitrate
MW

+ + +
- + +
- - +
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Circulation. published online September 19, 2012;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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SUPPLEMENTAL MATERIAL

Dietary Nitrate Supplementation Improves Revascularization in Chronic Ischemia

Ulrike B. Hendgen-Cotta, PhD1*, Peter Luedike, MD1*, Matthias Totzeck, MD1, Martina Kropp, MSc1, Andreas Schicho, MS1, Pia Stock, MSc1, Christos Rammos, MD1, Michael Niessen, MS1, Christian Heiss, MD1, Jon O. Lundberg, MD, PhD2, Eddie Weitzberg, MD, PhD2, Malte Kelm, MD1, Tienush Rassaf, MD†

Supplemental Figure 1
Figure legend

Supplemental Figure 1. Antibacterial mouthwash procedure eradicated oral bacterial flora. Oral smear tests were performed before and after mouthwash (MW) procedure. Mice received 3 s of oral MW via an hexitidine containing aerosol can. Aerobic (ae) an anaerobic (an) bacteria were cultured on standard agar plates and colony forming units (CFU) were counted. MW procedure led to an eradication of oral bacteria (p < 0.02). Data are expressed as mean ± SEM (n = 3).