Dietary Nitrates Supplementation Improves Revascularization in Chronic Ischemia

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Background—Revascularization is an adaptive repair mechanism that restores blood flow to undersupplied ischemic tissue. Nitric oxide plays an important role in this process. Whether dietary nitrate, serially reduced to nitrite by commensal bacteria in the oral cavity and subsequently to nitric oxide 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 hind-limb surgery with excision of the left femoral artery was conducted. Blood flow was determined by laser Doppler. Capillary density, myoblast apoptosis, mobilization of CD34+/Flk-1+, migration of bone marrow–derived CD31+/CD45−, plasma S-nitrosothiols, nitrite, and skeletal tissue cGMP levels were assessed. 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 eradication of the nitrate-reducing bacteria in the oral cavity through the use of an antiseptic mouthwash.

Conclusions—Long-term dietary nitrate supplementation may represent a novel nutrition-based strategy to enhance ischemia-induced revascularization. (Circulation. 2012;126:1983-1992.)

Key Words: mouthwashes ■ revascularization ■ nitrates ■ nitric oxide ■ nitrites

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, and cell adhesion molecules.1 Members of the vascular endothelial growth factor family and the stromal cell–derived factor 1 have been described to play a central role in initiating angiogenesis, vasculogenesis, and arteriogenesis.2–6

Editorial see p 1939
Clinical Perspective on p 1992

Nitric oxide (NO) derived from endogenous enzymatic production via endothelial NO synthase is a well-known proangiogenic molecule that regulates both vascular endothelial growth factor signaling and the recruitment of bone marrow–derived endothelial progenitor cells.7–9 Disruption of endothelial NO synthase impairs the release of endothelial progenitor cells into the circulation and their migration to the site of injury,8 thus attenuating vascular regenerative processes.

Inorganic nitrate from dietary sources is converted in vivo to nitrite and then NO and other bioactive nitrogen oxides.10,11 Nitrate is found in considerable amounts in our everyday diet, and leafy green vegetables such as spinach, lettuce, or beetroot have particularly high concentrations.12 In the oral cavity, commensal nitrate–reducing bacteria effectively reduce nitrate to nitrite, which is swallowed with ∼1 L saliva per day and thus continuously enters the circulation.13 The relevance of exogenous supplementation with dietary nitrate for cardiovascular functions has recently been shown. After a 3-day nitrate-enriched diet, diastolic blood pressure was reduced significantly in healthy nonhypertensive volunteers.14 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 at considerably lower levels.

On the basis of the recently described NO-like effects of dietary nitrate, we evaluated whether long-term 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 hind-limb ischemia model.

Methods

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

Animals
Male Naval Medical Research Institute (NMRI) mice 14±3 weeks old with an average body weight of 30±6 g were obtained from the local animal house. C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Os mice were kindly provided by 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 μmol 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. 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% wt/vol 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.

Hind-Limb Ischemia Model
Under complete anesthesia after ketamine (45 mg/kg) and xylazine (10 mg/kg) injections, chronic ischemia was generated in compliance with well-established unilateral hind-limb surgery protocols. In brief, after a 1-cm skin incision was made 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 with 7–0 silk.
sutures (Serag-Wiessner, Nails, Germany). The femoral artery was then excised between the ligations. Wound closures were conducted with single-layer sutures with 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 (Perimed, Stockholm, Sweden) immediately after surgery and for the next 7 days. Blood flow was measured as changes in laser frequency, represented by pixels of different colors. The mean hind-limb perfusion was calculated as the ratio of ischemic to nonischemic side by a blinded observer.

Capillary Density Measurement
Paraffin-embedded serial sections (6 μm) obtained from skeletal muscles of the ischemic and nonischemic (control) hind limb were used for immunostaining. To define capillaries on day 7 after surgery, a primary antibody against CD31 (platelet/endothelial cell adhesion molecule-1; Santa Cruz Biotechnology, Santa Cruz, CA) 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 leukocytes. Tissues were mounted by the use of Vectashield DAPI nuclear counterstain. Only CD31 and CD45 cells were calculated per 1-mm² hind-limb section.

Determination of Myoblast Apoptosis
Seven days after chronic hind-limb ischemia, the hind limbs were removed from mice, and the bones were dissected. The muscle was then placed in 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 Buffer (25 mmol/L) and Dulbecco modified Eagle medium (PromoCell, Heidelberg, Germany). Myoblasts were cultured in HAM’s F-10 medium (PromoCell) supplemented with 20% FCS, 10% equine serum, and 0.2% penicillin/streptomycin on collagen-coated dishes. After incubation and sufficient fibroblast 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, NJ) with the integrin-α7 antibody (Antikoerper-Online GmbH, 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 with a Nikon fluorescence microscope (Nikon Instruments Europe BV Germany, Düsseldorf, Germany), and TUNEL-positive (green) and total (blue) nuclei were counted in 3 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 hind-limb 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 endothelium-regenerating cells into ischemic tissue, NMRI and enhanced green fluorescence protein (EGFP) 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 to 6×10⁸ bone marrow cells were injected into the heart of the wild-type recipients in which bone marrow had been lethally irradiated with 6.5 Gy (2×10 minutes). Four weeks later, wild-type mice were subjected to nitrate supplementation for 7 days and long-term ligation of the femoral artery.
Determination of Bone Marrow–Derived Cells

Seven days after long-term 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 was performed to unmask inflammatory cells. Nuclei were counterstained with DAPI. EGFP/CD31/CD45 cells were calculated per 1-mm² hind-limb section.

Biochemical Analysis

Plasma nitrate, nitrite, and S-nitrosothiol levels were measured with high-performance liquid chromatography (ENO20, Eicom, Dublin, Ireland) and chemiluminescence18 and skeletal tissue cGMP level was determined with the Biotrak™ cGMP competitive enzyme immunoassay system (GE Healthcare, Munich, Germany) following the manufacturer’s protocol. Tissue cGMP levels were expressed per 1 mg wet tissue.19

Statistical Analysis

Results are presented as mean±SEM unless stated otherwise. Data were analyzed by 1-way ANOVA and post hoc Bonferroni multiple-comparison tests and Kruskal-Wallis with Mann–Whitney test with GraphPad Prism 5 and IBM SPSS version 20 software to compare differences between multiple groups. The Student unpaired t test was used to analyze 2 groups. A value of P<0.05 was considered statistically significant.

Dietary Nitrate Improves Perfusion Recovery in Chronic Hind-Limb Ischemia

To determine whether dietary nitrate supplementation augments the recovery of tissue perfusion, we subjected mice to femoral artery ligation, and the effects on perfusion recovery were assayed by laser Doppler perfusion imaging immediately after surgery and the next 7 days (Figure 1A and 1B). Perfusion recovery in the ischemic hind limb was significantly improved in mice treated with nitrate compared with controls (73±4% versus 59±4%; P<0.02; Figure 1B and 1C). Eradication of the nitrate-reducing bacteria in the oral cavity through antiseptic mouthwash twice daily (Figure I in the online-only Data Supplement) did not show the nitrate-mediated positive effect on perfusion (55±3%; P<0.02; Figure 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 μmol/d) over 7 days showed an increase in plasma S-nitrosothiol, nitrite, and nitrate levels compared with controls (S-nitrosothiol, 349±98 versus 9±1.9 nmol/L; nitrite, 5.8±1.7 versus 0.5±0.1 μmol/L; nitrate,
451 ± 75 versus 40 ± 17 μmol/L; Figure 2A and 2B). The nitrite and S-nitrosothiol concentrations were markedly lower in mice that underwent an antibacterial mouthwash (Figure 2B). We also observed lower amounts of 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 (Figure 3A–3D).

Dietary Nitrate Increases Revascularization After Long-Term 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 hind-limb surgery (Figure 4A). The capillary density was higher in the nitrate-treated group compared with controls (123 ± 20 versus 59 ± 15 cells per 1 mm²; P = 0.045; Figure 4A and 4B) and mice receiving nitrate and antibacterial mouthwash (57 ± 7 cells per 1 mm²; P < 0.02; Figure 4A and 4B), as evident by CD31⁺ staining in skeletal muscle tissue.

Dietary Nitrate Attenuates Apoptosis in Myoblasts in Chronic Hind-Limb Ischemia
To evaluate the remaining regenerative capacity of tissue in chronic ischemia, hind limbs were removed from mice on day 7 after surgical ligation of the femoral artery, and myoblasts were isolated and cultured and underwent TUNEL staining for the detection of apoptotic myoblasts (Figure 5A and 5B). The amount of apoptotic nuclei was lower in the nitrate-treated group compared with controls (67 ± 16 versus 197 ± 19 cells per 1 mm²; P < 0.001; Figure 5B and 5C). Blocking of oral nitrate reduction by antibacterial mouthwash showed no beneficial effects of nitrate (169 ± 26 versus 67 ± 16 cells per 1 mm²; P < 0.02; Figure 5B and 5C).

Dietary Nitrate Increases the Mobilization and Migration of Endothelium-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 with the control group (P < 0.001; Figure
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, we investigated the migration of this mobilized population into ischemic tissue using NMRI and EGFP transgenic mice for bone marrow transplantation. The NMRI recipients underwent lethal irradiation and subsequent EGFP+ bone marrow cell transplantation to generate marked hematopoietic chimeras. After 4 weeks of recovery, these animals received nitrate supplementation and control treatment before long-term 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 hind limbs. 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 with animals on a low-nitrate diet (P=0.029; Figure 6D and 6E). No effects on the migration of bone marrow–derived EGFP+CD31+/CD45− cells were seen with nitrate when antiseptic mouthwash was used (P=0.034; Figure 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 result is consistent with recent findings in studies investigating the role of nitrite injections in ischemia, indicating that other nitrite-signaling pathways for regulating angiogenic activity may exist. However, it does not exclude the existence of cGMP-mediated effects because counterregulatory 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 present study, we demonstrate that dietary nitrate supplementation improves revascularization through mechanisms
involving the mobilization and migration of endothelium-regenerating cells. This is accompanied by an attenuation of myoblast apoptosis.

Despite endeavors to promote blood vessel formation by administration of proangiogenic factors, gene therapy, or targeting of microRNAs, clinically applicable strategies have not yet been developed or are still in a preclinical phase.21–24 Although proangiogenic properties of NO have been demonstrated, direct application of NO or classic NO donors in vivo bears a high risk of failure owing to lack of targeted delivery, development of tolerance, cellular toxicity, and risk of hypotension.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.28 A cytoprotective role of nitrite in the setting of myocardial, liver, kidney, and brain ischemia/reperfusion injury has previously been demonstrated.19,20,30 In ischemic angiogenesis, a continuous pharmacological intervention with nitrite injections resulted in an increased vascular density in the hind limb and stimulated endothelial cell proliferation.16 Nitrite has several properties that make it an attractive drug candidate. Its conversion into bioactive nitrogen oxides is slow,31 and the effect is not subjective to tolerance.32 Therefore, one can deliver it even in fairly high doses without the risk of classic NO-related side effects such as hypotension. In addition, first-passage metabolism in the liver is minimal compared with that of classic 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 with a half-life of 6 hours, it continuously generates nitrite and bioactive nitrogen oxides like...
S-nitrosothiols. Recent research in animals and humans has confirmed the 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} increase in 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 and cardiovascular and renal injury in salt-induced hypertension.\textsuperscript{39}

These findings from experimental animal studies and 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 demonstrate here that dietary nitrate supplementation strongly augments perfusion recovery in chronic hind-limb ischemia 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\textsuperscript{+}/Flk-1\textsuperscript{+} cells, and migration of bone marrow–derived CD31\textsuperscript{+}/CD45\textsuperscript{−} cells into ischemic tissue. The associative relation, increased plasma nitrite levels and an increased mobilization of CD34\textsuperscript{+}/Flk-1\textsuperscript{+} cells from the bone marrow, has also been observed in patients with coronary artery disease receiving nutrients that increased NO bioavailability.\textsuperscript{40}

In line with these findings, the results of the present 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 carried out mainly by satellite cells with a contribution of NO.\textsuperscript{41} Quiescent satellite cells become active and proliferate on 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, disruption of the nitrate-NO pathway by long-term eradication of the oral microflora completely abolished the beneficial effects of the dietary nitrate supplementation. This daily intervention effectively suppressed the increase in circulating nitrite and S-nitrosothiol levels that is observed after intake of nitrate-rich food or dietary nitrate supplementation in drinking water.\textsuperscript{15, 38, 42} The fact that mouthwash prevented increases in plasma nitrite and all observed beneficial effects of nitrate provides significant mechanistic insight because it suggests an important role of intermediate nitrite formation in the 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 because recent data show that protein S-nitros(yl)ation is an obligate concomitant process of anaerobic respiration on nitrate in Escherichia coli.\textsuperscript{43}

It is important to note that the exact identity of the final mediator(s) of the nitrate effects is still not settled and that 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 the formation of bioactive nitration products such as nitro fatty acids.\textsuperscript{11, 48, 49} This oxidative chemistry is emerging as an alternative nitrite-mediated signaling mechanism\textsuperscript{33, 50} that 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.

**Conclusions**

We show here that dietary nitrate supplementation increases the regenerative capacity of ischemic tissue and that this effect depends critically on 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.

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**Disclosures**

None.

**References**

Dietary Nitrate Improves Revascularization

1991

Hendgen-Cotta et al


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**CLINICAL PERSPECTIVE**

With the worldwide increase in cardiovascular diseases in recent decades, the need for novel preventive and noninvasive therapeutic strategies has grown tremendously. In this context, there is accumulating evidence that inorganic nitrate from dietary sources is able to influence the hallmarks of cardiovascular functions, including blood pressure regulation. The bioactivation of nitrate from dietary or endogenous sources is carried out mainly by commensal bacteria that express effective nitrate reductase enzymes and are located in the gastrointestinal tract and on body surfaces. Under conditions of low oxygen tensions, nitrate and nitrite are physiologically recycled in blood and tissues to form nitric oxide and other bioactive nitrogen oxides that mediate cytoprotective signaling in the setting of pathological ischemia. The present study provides the first evidence that dietary nitrate supplementation improves revascularization in chronic ischemia. This study identified that dietary nitrate supplementation increases mobilization and migration of regenerative cells, improves the regenerative capacities of chronically ischemic tissue, and decreases apoptosis at the site of ischemia. Eradicating the commensal bacteria in the oral cavity and thus interrupting the bioactivation of the ingested nitrate decreased circulating levels of bioactive nitrogen oxides and reversed all of these beneficial effects. These data underscore the potential therapeutic value of inorganic nitrate and suggest the possible application of a nutritional approach in the prevention and treatment of cardiovascular diseases.
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SUPPLEMENTAL MATERIAL

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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 \( n = 3 \).