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Effects of long term nitrite therapy on functional recovery in experimental ischemia model

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ABSTRACT

Our data have shown that nitrite therapy can rescue the ischemic brain when injected <3 h after cerebral ischemic-reperfusion (I/R) injury and its effects can be prolonged to 4.5 h in combination with memantine. We investigated whether or not long-term nitrite therapy is beneficial in ischemic brains. Sodium nitrite (1–100 μg/kg ip) or saline were administered to rats subjected to focal I/R injury for 7 days beginning 24 h after I/R. Behavioral tests for 5 weeks revealed better functional recovery in the high-dose nitrite group than the control group. Other nitrite groups with relatively low doses showed no functional benefits. Hemispheric atrophy was attenuated by approximately 30% in the high-dose nitrite group. High-dose nitrite therapy also reduced inflammatory cytokine levels and caspase activity in the subacute period, and increased $BrdU^+MAP2^+$ and $BrdU^+laminin^+$ cells, and vascular density in the 5-week ischemic brain. Long-term nitrite therapy, when initiated 24 h after I/R, corrected the subacute hostile environment, induced tissue and vascular regeneration, and improved functional recovery. Early and subsequent long term nitrite therapy may be effective in the management for ischemic stroke patients.

1. Introduction

Totally integrated therapy in ischemic stroke necessitates early restoration of blood flow, acute cytoprotection, regeneration of destructed tissues, and enhancement of biological functions [1]. The potential approach to treat acute stroke might exist with cytoprotective agents. However, no neuroprotective candidates have proven to be clinically efficacious. Nitrites have biological advantages over other agents with respect to the rapid generation of nitric oxide (NO) under hypoxia and acidosis [2,3]. Nitrite infusions have been used for selective NO delivery to ischemic brains with a potential effect in vasodilation, reduction of oxidative stress, and modulation of mitochondrial respiration [4,5].

Despite the overwhelming beneficial role against acute cerebral ischemia–reperfusion injury by nitrites, there is much less information regarding chronic therapy for delayed cytoprotection. For acute stroke, the effect of nitrites is attenuated according to the delay of injection time, and may become toxic at a very late stage [4,5]. It is well-known that overproduction of NO in a hostile environment leads to neurodegeneration by oxidizing proteins, damaging DNA, and inducing the lipoperoxidation of cell membranes. This complicated action raises a new concept of chronic therapy following a harmful stage, designed to avoid potential toxicity, and to induce delayed cytoprotection and regeneration. This concept has been recently tested in a murine model of hind-limb ischemia [6].

Ischemic brain needs vascular and neural regeneration for functional restoration. Neurogenesis is closely linked to angiogenesis, which supports tissue survival, repair, and functional reorganization. A number of factors and agents have been tested to induce angiogenesis and neurogenesis in experimental models, but with no significant clinical benefits. NO is known to mediate proliferation and differentiation of brain cells and vascular endothelial growth factor (VEGF)-induced angiogenesis [7–9]. A recent trial with chronic nitrite therapy in limb ischemia showed that nitrite restored ischemic limb blood flow, and stimulated angiogenesis and vasculogenesis in a NO-dependent manner [6]. Taken together, the chronic treatment of ischemic brains with nitrite might be beneficial for therapeutic regeneration.

When the acute ischemic injury signals are stabilized, the ischemic brain is set for regeneration and brain plasticity becomes the key to stroke recovery. In the present study, we attempted to examine the therapeutic effect of continuous nitrite therapy starting 24 h after cerebral ischemia. We assessed histologic findings involving cell death, and cellular proliferation and differentiation, as well as mortality and behavioral outcome.

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2. Methods and materials

2.1. Model and drug administration

We performed all the experimental procedures with institutional approval in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Sprague–Dawley rats (male, 12 weeks old; Seoul, Genomics, Republic of Korea), weighing 200–220 g, were subjected to focal cerebral ischemia injury. Focal cerebral ischemia injury was generated by intraluminal filamentous occlusion of the middle cerebral artery (MCA) for 90 min, followed by reperfusion, as have been described previously [4,5]. The reperfusion was done by removing the MCA occlusive filament. After confirmation of significant neurologic deficits at 24 h post-ischemia, animals were randomly assigned to either saline or sodium nitrite treatment for 7 days. Treatment with sodium nitrite (Sigma–Aldrich, St. Louis, MO, USA) was done at 3 different doses (1 μg/kg, 10 μg/kg, and 100 μg/kg, dissolved in 0.9% saline) to verify whether or not the efficacy depends on the dose used. This dose was based on prior work in the hind-limb ischemia [6]. Animals received intraperitoneal injection of saline or sodium nitrite twice daily from 24 h after ischemia. The time of delivery was determined to avoid the potential toxicity to increase the oxidative stress in the acute stage. The animals were grouped as follows: normal control ($n = 9$), ischemia control ($n = 15$), ischemia-1 μg/kg ($n = 15$), ischemia-10 μg/kg ($n = 15$), and ischemia-100 μg/kg ($n = 15$).

2.2. Behavior tests

To assess the effect of chronic nitrite treatment on neurologic outcome, we performed behavioral tests with a modified limb placing test (MLPT) and rotarod test with 35 days of follow-up after cerebral ischemia (baseline, 1, 3, 7, 14, 21, 28, and 35 days; $n = 6$ per group). The MLPT assessed the sensorimotor integration of the forelimb and the hindlimb by three compartments [4,5]. Each behavioral test was performed by 2 investigators blinded to the treatment status of the rats.

2.3. Measurement of hemispheric atrophy

At the end of behavioral testing (on 35 day after ischemia), each rat was anesthetized and perfused through the heart with 100 mL cold saline and 100 mL of 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline. After 24 h of fixation in 4% paraformaldehyde, the brains were cryoprotected with 30% sucrose for 24 h and cut using a cryostat (Leica CM 1900) into 30-μm sections. Six sections throughout the striatum were Nissl-stained. The total hemispheric areas of each section were traced and measured with an image analysis system (Image-Pro Plus; Media Cybernatics). The morphometric analyses involved computer-assisted hand delineation of the area of the striatum, cerebral cortex, and ventricle, as well as the whole hemisphere.

2.4. Molecular study

To examine the molecular changes underlying inflammation and cell survival, we conducted RT-PCR for TNF-α, IL-6, IL-1β, Fas, and Fas-L, and Western blotting for poly(ADP-ribose) polymerase (PARP) and caspase-3 7 days after ischemia ($n = 3$ per group). Total RNA was isolated from the homogenates of the ischemic brains, using TRI reagent (Sigma–Aldrich), and semi-quantitative RT-PCR was done using a First Strand cDNA Synthesis kit (Roche, Molecular Biochemicals, Indianapolis, IN, USA) with the following primer sets: TNF-α, 5′-TAC TGA ACT TCG GGG TGA TTG GTC C-30 (sense) and 5′-CAG CCT TGT CCC TTG AAG AGA ACC-3′ (antisense); IL-6, 50-CTT GGG ACT GAT GTT GTT GAC-3′ (sense) and 5′-CTC TGA ATG ACT CTG GCT TTG-3′ (antisense); IL-1β, 5′-GAA GCT GTG GCA GCT ACC TAT GTC T-3′ (sense), 5′-CTC TGC TTG AGA GGT GCT GAT GTA C-3′ (antisense); Fas, 5′-CAA GGG ACT GAT AGC ATC TTT GAG G-3′ (sense) and 5′-TCC AGA TTC AGG GTC ACA GGT TG-3′ (antisense); and Fas-L, 5′-CAG CCC CTG AAT TAC CCA TGT C-3′ (sense) and 5′-CAC TCC AGA GAT CAA AGC AGT TC-3′ (antisense). Transcript levels were quantified by scanning photographs of gels using appropriate imaging software (Molecular Analyst®; Bio-Rad, Hercules, CA, USA). The mRNA expression levels were normalized to GAPDH. The proteins from tissue lysates were separated by SDS–PAGE and transferred onto nitrocellulose membranes. Blots were incubated for 1 h with PARP and caspase-3 antibodies (Cell Signaling Technology). Anti-β-actin antibody (Santa Cruz Biotechnology) was used as a control. Anti-β-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used as a control. Blots were developed using an enhanced chemiluminescence system (Pierce, Rockford, IL, USA), digitally-scanned (GS-700, Bio-Rad), then analyzed (Molecular Analyst®). Relative optical densities were calculated versus measured values of β-actin.

2.5. Measurement of caspase activities

Caspase activities were measured using commercial enzyme-linked immunosorbent assay kits (caspase-3; Promega, Madison, WI, USA; caspase-8; BD Biosciences, San Jose, CA, USA; and caspase-9, Chemicon International) 7 days after cerebral ischemia, as described previously [10]. Lysed brains ($n = 3$ per group) were incubated in a reaction buffer containing 10 mmol/L dithiothreitol at 37 °C for 3 or 4 h. Colorimetric measurements were made using a plate reader (Molecular Devices).

2.6. Analysis of neurogenesis and angiogenesis

To evaluate the regeneration characteristics, BrdU (100 mg/kg) was administered intraperitonealy once daily for 7 consecutive days from the day of nitrite administration. The rats were sacrificed after the completion of 35-day behavioral tests ($n = 6$ per group). Brain sections were processed for immunocytochemistry, as described previously [4,5,11]. The specimens were incubated overnight at 4 °C with primary antibodies, and then incubated for 1 h at room temperature with secondary antibodies. Primary antibodies were as follows: monoclonal anti-BrdU (1:300, Pharmingen); anti-mitogen-activated protein-2 (anti-MAP2, MAP2 and 1:1000; Sigma–Aldrich); anti-glial fibrillary acidic protein (anti-GFAP, 1:1000; Sigma–Aldrich); anti-laminin (1:100; Sigma–Aldrich); and anti-endothelial barrier antigen (EBA, 1:200; Stenberger Monoclonals). FITC-conjugated anti-sheep IgG (1:100; Biodesign) or Cy3-conjugated anti-mouse IgG antibodies (1:300; Jackson Immunoresearch) were used for the secondary antibodies. We selected corresponding coronal sections for comparisons, as determined using a rat atlas [12]. Quantification was done in the peri-infarct areas. Regions of interest (ROIs) are depicted in Fig 3A. $BrdU^+$ cells were counted in five-to-seven 30 μm coronal sections per animal, spaced 210 μm apart, by two investigators blinded for group allocation. The counts were made using a 40× objective by placing an optical grid (field size, 250 μm × 250 μm). The density of BrdU-labeled cells is expressed as cells per cubic millimeter. The $BrdU^+$ cells were characterized by double-labeling with neuronal, glial, or endothelial cell markers. The number of double-labeled $BrdU^+MAP2^+$, $BrdU^+GFAP^+$, $BrdU^+laminin^+$, and $BrdU^+EBA^+$ cells was counted under a confocal microscope. For evaluation of vascular density and endothelial proliferation, every EBA^+ profile was traced and recorded manually into an image analysis system (Image-Pro Plus™). A profile was defined as the net surface area of EBA immunolabeling. Each traced outline was then

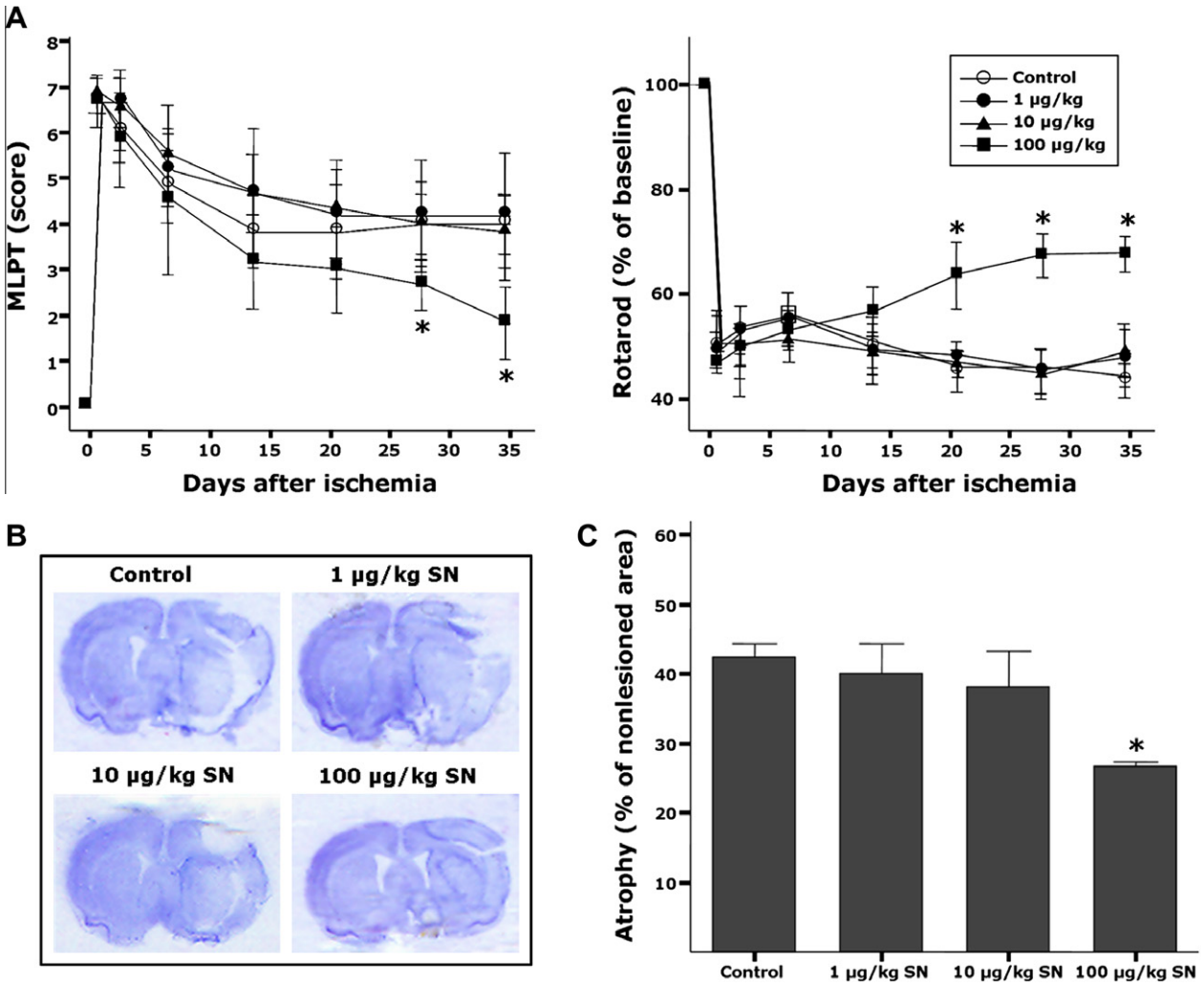

Fig. 1. Effect of chronic nitrite therapy on functional and histologic outcomes. To assess functional recovery, MLPT and rotarod tests (A) were performed serially during 35 days after induction of ischemia. Data are presented as the mean ± SD. The values are compared within the group at the indicated time. $^{*}P < 0.05$ versus saline-treated control group ($n = 6$ [ANOVA]). (B) Representative photomicrographs of Nissl staining 35 days after ischemia show the effect of saline, or nitrite at various dosages on the hemispheric atrophy. (C) Chronic nitrite therapy induces a dose-dependent reduction in hemispheric atrophy as compared with the saline-treated group. Bars represent the mean ± SD. $^{*}P < 0.05$ versus saline-treated control group ($n = 6$ [ANOVA]).

analyzed for its diameter, and the average values were determined. Endothelial cell division was detected by double-staining for BrdU and EBA. The number of total endothelial cells and the numbers of $BrdU^{+}$ endothelial cells in 20 enlarged and thin-walled vessels in the peri-infarct areas were counted from each rat, from which the proportions of $BrdU^{+}$ endothelial cells were calculated.

2.7. Statistical analysis

All data in this study are expressed as the mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA), followed by a Tukey test (when appropriate) or Student's *t*-test if normally distributed. For non-normal distributions we used the Mann–Whitney *U* test. A probability value <0.05 was considered the minimum level of statistical significance.

3. Results

3.1. Functional recovery by chronic nitrite therapy in focal cerebral ischemia

To examine the therapeutic applicability of chronic nitrite treatment in cerebral ischemia, we tested the effects of different dosages (1, 10, and 100 μg/kg of sodium nitrite). Intraperitoneal sodium nitrite administration did not affect the mortality rate over the 35-day examination period when compared with the control group (25.0% in the ischemia control, and 30.8% in the ischemia-1 μg/kg SN, 30.8% in the ischemia-10 μg/kg SN, and 18.2% in the ischemia-100 μg/kg SN groups, $P = 0.885$ [chi-square test]). At baseline, the neurologic status in the MLPT and rotarod test was equal between the nitrite-treated groups and the ischemia-control group. The ischemia-100 μg/kg SN group showed better functional recovery characteristics, with significant differences from day 28 on the MLPT and day 21 on the rotarod test (Fig. 1A; $n = 6$, $P < 0.01$ [Student's *t*-test]). On day 35, the group of rats which had been treated with 100 μg/kg of nitrite scored <2 points on the MLPT and exhibited a 22% better performance on the rotarod test. However, the other nitrite groups (1 and 10 μg/kg) showed no significant difference compared to the ischemia-control group (Fig. 1A).

3.2. Reduction of hemispheric atrophy by chronic nitrite therapy

We determined whether or not the improved functional recovery was associated with the reduction of final infarct volume 35 days after ischemia. We found a clear dose-dependent decrease

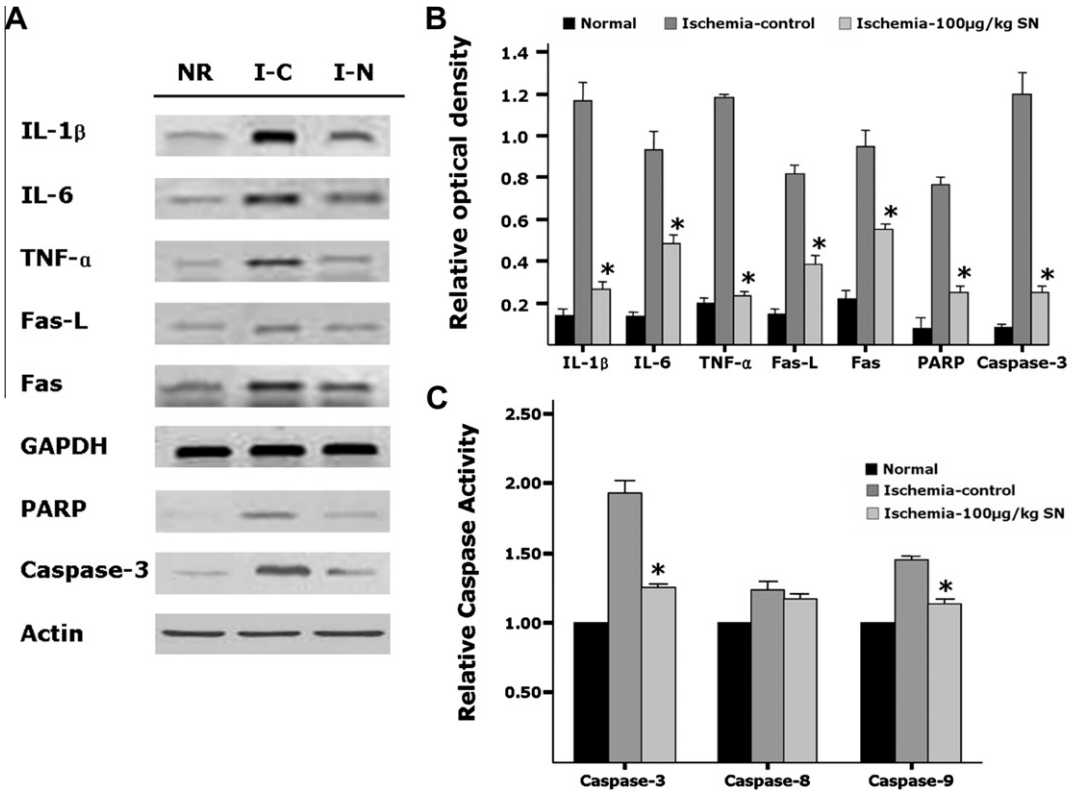

Fig. 2. Effect of chronic nitrite therapy on inflammatory cytokines and caspase activity in acute ischemic brains. Inflammation-related cytokines, PARP, and caspase-3 were analyzed by RT-PCR or Western blotting 7 days after induction of ischemia (A). RT-PCR results for IL-β, IL-6, TNF-α, Fas-L, and Fas were analyzed in terms of the optical density ratio to the glyceraldehyde-3-phosphate dehydrogenase, while the data for PARP and caspase-3 were analyzed by the optical density ratio to the actin (B). In the caspase activity assay (C), the nitrite-treated ischemia group exhibited reduced expression of caspase-3 and -9. $P < 0.05$ versus ischemia control ($n = 3$ [Mann–Whitney U test]).

in infarct volume and hemispheric atrophy with 100 μg/kg of sodium nitrite providing the most substantial effect (Fig. 2B). Hemispheric area analysis showed a profound atrophy of the lesioned-hemisphere in the ischemia-control group. The ischemia-100 μg/kg SN group had less hemispheric atrophy by 40% compared with that observed in the ischemia-control group, while the administration of relatively low doses of nitrite did not cause any statistical change in atrophy (Fig. 2C; $n = 6$ per group, $P < 0.01$ [ANOVA followed by Tukey's b test]).

3.3. Inflammatory cytokines and caspase activities

Inflammatory responses lead to acute and delayed cell death. To verify if 7 days of nitrite therapy prevent this inflammatory response and apoptotic cell death, we performed RT–PCR to measure the expression of mRNA of cytokine-related genes, and Western blotting to measure the expression of PARP and caspase-3 7 days after ischemia. Ischemic brains expressed the mRNAs of IL-1β, IL-6, TNF-α, and Fas-L in abundance, whereas the normal brains expressed the mRNAs at very low levels. Nitrite-treated ischemic rats exhibited lower expressions of these molecules (Fig. 2A). Relative optical density analysis demonstrated significant decreases in IL-1β, IL-6, TNF-α, Fas-L, and Fas mRNA levels in the ischemia-100 μg/kg SN group compared with the ischemia-control group (Fig. 2B; $n = 3$, $P < 0.05$ respectively [Mann–Whitney U-test]). Western blotting revealed the up-regulation of PARP and caspase-3 expression by the induction of cerebral ischemia (Fig. 3A). The relative optical densities of PARP and caspase-3 expression were significantly reduced by 100 μg/kg of nitrite compared with the ischemia-control group (Fig. 3B; $n = 3$, $P < 0.01$ [Mann–Whitney U test]). Caspase-3 and -9 activities in the ischemia-100 μg/kg SN group were significantly lower than those of the ischemia-control group (37% decrease in caspase-3; 26% decrease in caspase-9; $n = 3$, $P < 0.01$ [Mann–Whitney U test]).

3.4. Neurogenesis and angiogenesis

Functional regeneration of ischemic tissues depends on stimulation of neurogenesis and restoration of tissue blood flow [13,14]. Thus, we determined whether or not the chronic intravenous administration of nitrite also affects the neurogenesis, endothelial proliferation, and cerebral microvascular profiles. We processed 35-day brain sections from the ischemia control and ischemia-100 μg/kg SN groups for immunohistochemistry with dual antibodies against BrdU and specific markers for mature neurons (MAP2), astrocytes (GFAP), and endothelial cells (laminin). $BrdU^+$ cells were distributed in the peri-infarct areas similarly in the ischemia control and ischemia-100 μg/kg SN groups, but were increased in the latter group (Fig. 3A). Numerous scattered neurons, glia, and endothelial cells in these areas were also stained with the BrdU antibody (Fig. 3B). In the quantitative analysis, ischemia itself increased $BrdU^+$ cells by 4.8-fold in the peri-infarct areas (Fig. 3C; $n = 6$, $P < 0.05$ [Mann–Whitney U-test]), while chronic nitrite treatment in the ischemic brains increased $BrdU^+$ cells by 7.0-fold in the peri-infarct areas (Fig. 3C; $n = 6$, $P < 0.05$ [Mann–Whitney U-test]). Nitrite treatment produced a greater amount of cells of neuronal (MAP2, MAP2 and 32 ± 9 versus 12 ± 8 cells) and endothelial lineages (laminin, 50 ± 10 versus 30 ± 10 cells), compared to the ischemia-control group (Fig. 3C; $n = 6$, $P < 0.05$ [Mann–Whitney U-test]). To confirm the role of nitrite as an angiogenic factor, sections were also double-immunolabeled for both EBA and BrdU. We found a robust increase

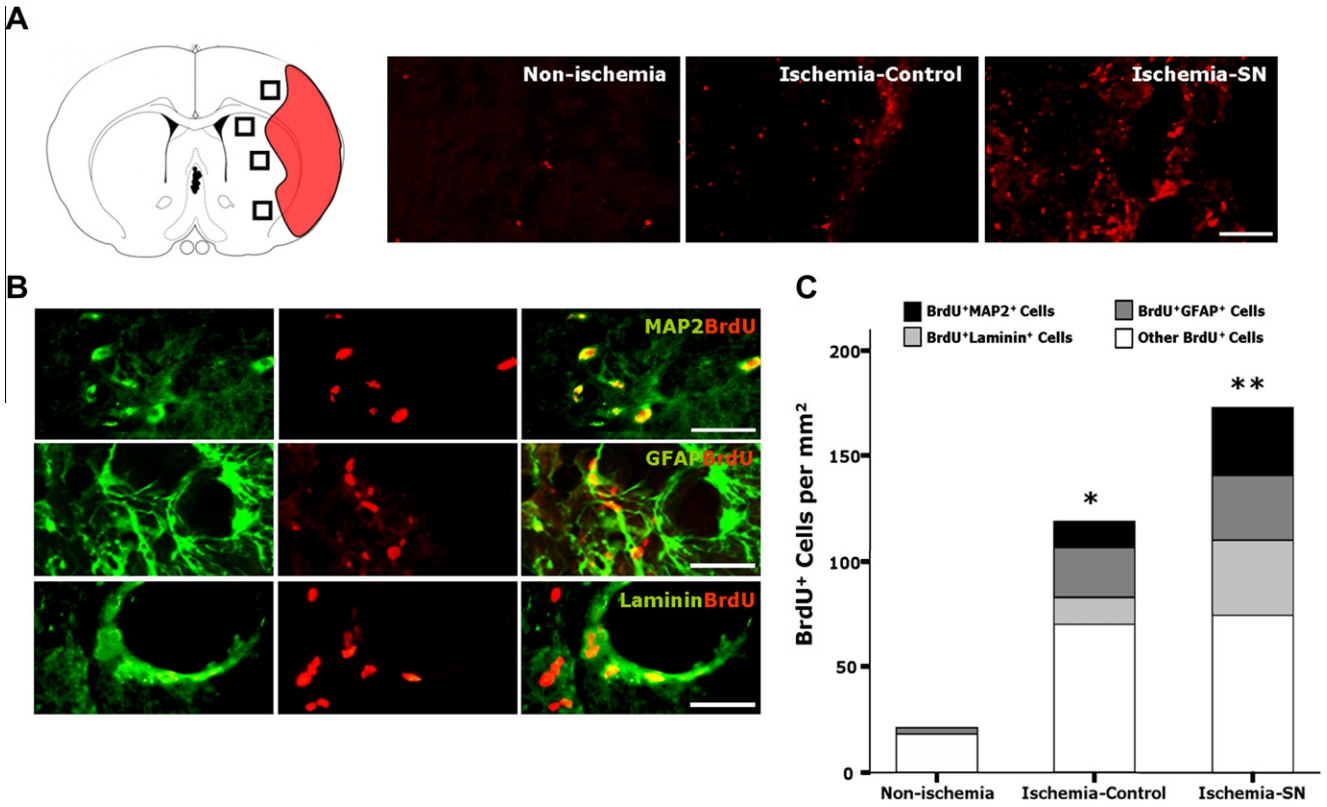

Fig. 3. Effect of chronic nitrite therapy on tissue regeneration. Thirty-five days after induction of ischemia, brain sections were immunostained for BrdU, allowing us to evaluate tissue regeneration in the ischemic brains. $BrdU^+$ cells were quantified in the four regions of interest (ROIs) as depicted in (A). Phenotype (B) of $BrdU^+$ cells were determined by immunostaining for BrdU (red) and marker (green) of mature neurons (MAP2), astrocytes (GFAP), or endothelial cells (laminin). Quantification analysis (C) demonstrated that chronic nitrite therapy produced numerous cells of neuronal and endothelial lineage in the peri-infarct regions. $^*P < 0.05$ compared with non-ischemia group ($n = 6$ [Mann–Whitney U test]). Scale bars, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the cerebral microvessels and amount of BrdU co-localization with EBA staining after nitrite treatment compared to the control group (Fig. 4A). The mean diameter of the microvessels was significantly larger in the ischemia-100 μg/kg SN as compared with the ischemia-control group (Fig. 4B; 3.9 ± 0.6 μm versus 5.6 ± 1.0 μm; $n = 6$, $P < 0.01$ [Mann–Whitney U-test]). When enlarged and thin-walled vessels containing $BrdU^+$ endothelial cells were counted (Fig. 4C), the fraction of $BrdU^+$ endothelial cells in the peri-infarct areas was 4.9 ± 0.8% and 15.7 ± 3.6% in the ischemia control and ischemia-100 μg/kg SN groups, respectively ($n = 6$, $P < 0.01$ [Mann–Whitney U-test]).

4. Discussion

Nitrite administration starting 24 h after ischemia was beneficial in improving functional recovery in the focal cerebral ischemia model. The improved functional outcomes had a strong association with the mitigation of brain atrophy. The chronic nitrite therapy led to a reduction of inflammatory cytokines and cell death signaling molecules in the subacute ischemic brains. Tissue protection was accompanied by increases of new vessels and neurons. These findings suggest that nitrite therapy exerts a wide impact on tissue cytoprotection and regeneration during ischemia.

For chronic therapeutic use of nitrites, the delivery time must be adjusted depending on the anticipated effect and the surrounding condition. Tissue nitrite deficiency is implicated in the progression of ischemic injury. In the limb ischemia model, nitrite uptake from blood to tissue occurred early within 3 days, and persisted up to 7 days, while blood nitrite levels continuously decreased [6]. Chronic nitrite therapy could lead to early tissue accumulation of nitrites in ischemic versus non-ischemic tissues [6]. In cerebral ischemia, tissue nitrites are initially depleted, but increased over hours via activation of neuronal and inducible NOS. At the time of NO increase, exogenous nitrite may increase the burden of NO and free radical toxicity. It is therefore of great importance when nitrite therapy is given in a cerebral ischemia model. In the present study, nitrite supplementation was initiated 24 h after induction of ischemia, and maintained for 7 days. Nitrite therapy during this period did not alter the mean arterial blood pressure and mortality.

For dose–response studies, we examined a range of sodium nitrite doses (1–100 μg/kg) via ip injection twice a day. Of the nitrite doses tested, only the 100 μg/kg dose represented good efficacy, which was consistent with a maximum effect in the heart, liver, and limb [15]. While the dose of nitrites required for protective effects in acute cerebral ischemia was remarkably low, a relatively large dose of nitrites was needed to effectively protect and repair injured brains. Although the mechanism underlying this discrepancy is not clear, the time difference might be associated with the different efficiency of hypoxic NO release and different desired action of NO.

We propose the reduction of delayed cell death and the enhancement of regeneration as potential mechanisms of good functional recovery by continuous nitrite treatment. Apoptosis is a highly regulated process that requires energy for protein synthesis or activation, and thus develops in a delayed fashion in ischemic injuries [16]. One important family of proteins involved in apoptosis is the caspases. Although NO has a complicated influence on apoptosis which depends on cell type, the concentration and

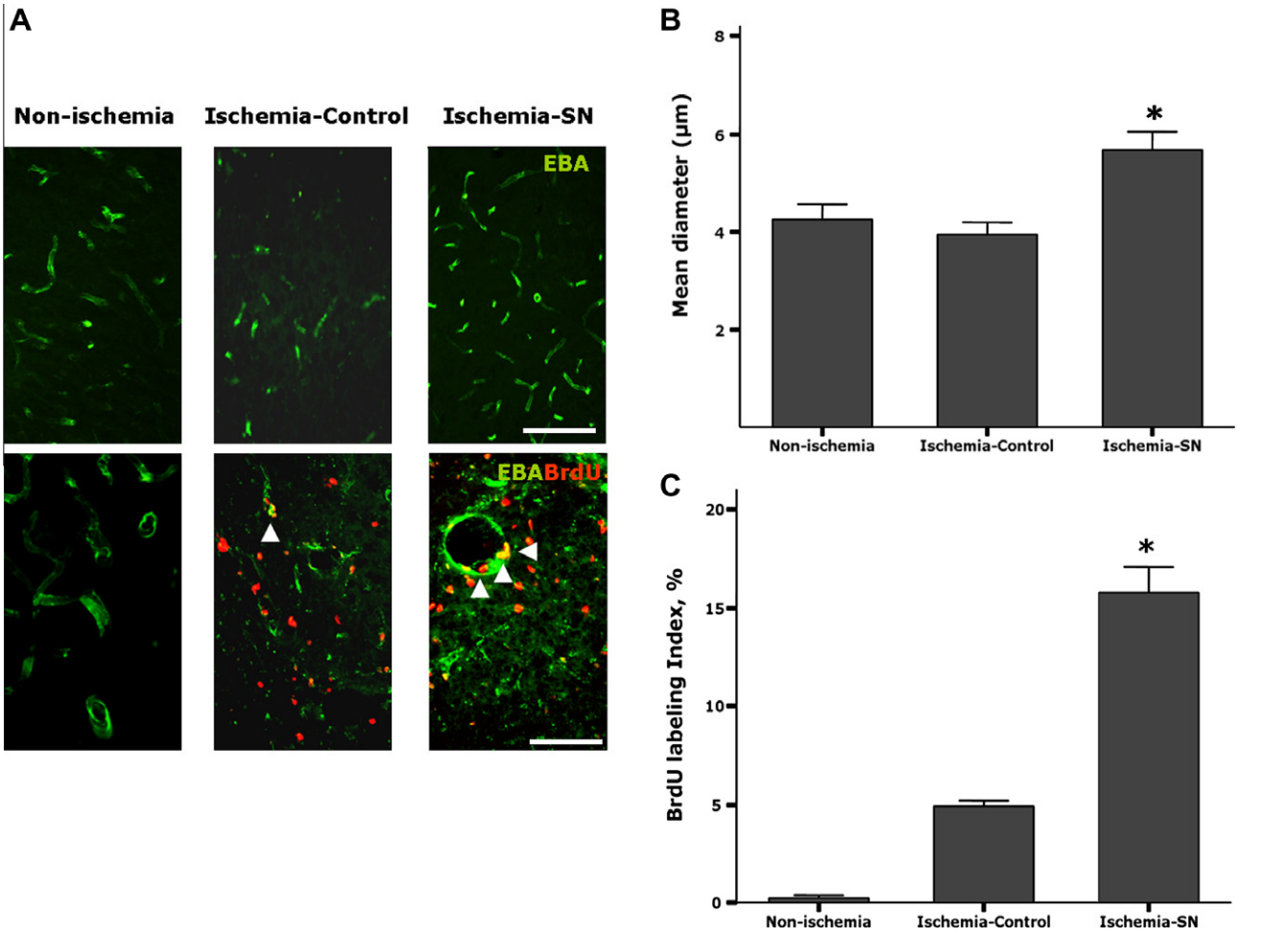

Fig. 4. Capillarization and endothelial proliferation by chronic nitrite therapy. Representative photographs (A) show EBA^+ microvessels and $BrdU^+EBA^+$ proliferating endothelial cells 35 days following ischemia. Arrowheads indicate that some BrdU-positive cells are EBA immunopositive in the peri-infarct regions. The mean diameter (B) was significantly greater in the nitrite-treated rats than in the non-ischemia and ischemia-control groups. In addition, chronic nitrite therapy significantly increased the BrdU labeling index (C). $^*P < 0.05$ compared with the ischemia-control group ($n = 6$ [Mann–Whitney U test]). Scale bars, 100 μm.

presence of other cellular factors, it usually inhibits caspase-3, and possibly other caspases [17], as demonstrated in the present study. Alternatively, NO regulates blood flow to tissues, and indirectly can suppress cell death signals. In addition, NO prevents inflammatory cell infiltration by inhibiting the expression of cell surface adhesion molecules P-selectin, vascular adhesion molecule-1 (VECAM-1), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1) [18,19]. In our study, the inhibition of inflammatory responses might attenuate delayed cell death.

In the present study, we found that prolonged nitrite therapy stimulated neurogenesis and angiogenesis in the ischemic brains, identified by increased BrdU labeling of neuronal and endothelial cells. The neural progenitors, possibly originating from stem cells located in the SVZ, reach the peri-infarct areas by chemokines or other injury signals. NO-induced mitochondrial biogenesis has been reported to be linked to proliferation and differentiation of neural cells [7,20,21]. Angiogenesis is essential to provide oxygen and nutrients to neural regeneration. NO is known to be one of the key biochemical mediators in stimulating angiogenesis in injured tissues [22]. NO is an important signaling intermediate governing tissue factor, and VEGF-dependent angiogenesis [8,23,24]. This cellular genesis did not occur in non-ischemic tissues, indicating that nitrite therapy serves as an ischemia-dependent NO donor. Further studies are warranted to determine key signaling mechanisms of neurogenesis and angiogenesis.

Integrative therapeutic strategies aimed at protection and regeneration following stroke remains scarce. A single treatment of nitrite has previously been reported to alleviate acute injury following ischemic stroke, but here we show that the continuous treatment of nitrite is involved in improving the functional recovery not only by protecting brain tissue, but also by regenerating vessels and neurons. Therefore, nitrite therapy tailored to the disease stage may have future clinical implications.

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